Molecular Mechanisms of Opioid Receptor-dependent Signaling and Behavior

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ABSTRACT

Opioid receptors have been targeted for the treatment of pain and related disorders for thousands of years and remain the most widely used analgesics in the clinic. Mu (μ), kappa (κ), and delta (δ) opioid receptors represent the originally classified receptor subtypes, with opioid receptor like-1 (ORL1) being the least characterized. All four receptors are G-protein coupled and activate inhibitory G proteins. These receptors form homo- and heterodimeric complexes and signal to kinase cascades and scaffold a variety of proteins.

The authors discuss classic mechanisms and developments in understanding opioid tolerance and opioid receptor signaling and highlight advances in opioid molecular pharmacology, behavioral pharmacology, and human genetics. The authors put into context how opioid receptor signaling leads to the modulation of behavior with the potential for therapeutic intervention. Finally, the authors conclude there is a continued need for more translational work on opioid receptors *in vivo*.

OPIOIDS are the most widely used and effective analgesics for the treatment of pain and related disorders. Opiates have been used for thousands of years for the treatment of pain, and in the last century we have made huge strides in the development of opioids derived from naturally occurring opiates within the fields of receptor pharmacology and medicinal chemistry. In addition, opioids are used frequently in the treatment of numerous other disorders, including diarrhea, cough, postoperative pain, and cancer (table 1).

Opioid systems are critical in the modulation of pain behavior and antinociception. Opioid peptides and their receptors are expressed throughout the nociceptive neural circuitry and critical regions of the central nervous system included in reward and emotion-related brain structures. To date, four different opioid receptor systems mu (μ), delta (δ), kappa (κ), opioid receptor like-1 (ORL1) and their genes have been characterized at the cellular, molecular, and pharmacologic levels.[1]

The most commonly used opioids for pain management act on μ opioid receptor (MOR) systems (fig. 1). Although μ opioids continue to be some of the most effective analgesics, they are also mood enhancers and cause activation of central dopamine reward pathways that modulate euphoria. These unwanted side effects have driven researchers at basic and clinical levels to actively pursue other opioid receptors as putative drug targets for pain relief (table 1).

The opioid receptor subtypes were identified pharmacologically and genetically more than 2 decades ago.[1] From that point on, numerous studies have implicated all four opioid receptors in an array of behavioral effects, including analgesia, reward, depression, anxiety, and addiction. In addition, all four receptor subtypes have been characterized at cellular levels with respect to the downstream signal transduction pathways they activate. However, there are fewer studies that have directly linked opioid signal transduction to behavioral events. One of the "holy grails" in opioid pharmacology research has been to identify pathway-specific opiate receptor agonists that could activate antinociceptive signaling without causing μ agonist-mediated euphorigenic responses or κ agonist-mediated dysphoria.[2,3] Understanding the diversity of signaling at opioid receptors and how second messenger activation leads to modulation of pain and reward could reveal novel opioid receptor drug candidates.

In this review, we highlight the current status of *in vitro* molecular pharmacology at opioid receptors and discuss many of the recent advances that connect these molecular stud-

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Received from the Departments of Anesthesiology and Anatomy/Neurobiology, Washington University Pain Center, Washington University School of Medicine, St. Louis, Missouri. Submitted for publication May 1, 2011. Accepted for publication September 13, 2011. Supported by the National Institute on Drug Abuse, National Institutes of Health, Bethesda, Maryland, grant MRB DA025182 (to Dr. Bruchas). Figures 1-3 were prepared by Annemarie B. Johnson, C.M.I., Medical Illustrator, Wake Forest University School of Medicine Creative Communications, Wake Forest University Medical Center, Winston-Salem, North Carolina.

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Table 1. Organ System Effects of Morphine and Its Surrogates

Organ Systems	Effects	Additional Information
Central nervous system	↑ Analgesia	—
	↑ Euphoria	Leading to risk of addiction and abuse
	↑ Sedation	—
	↓ Rate of respiration	—
	↓ Cough reflex	Codeine used for treatment of pathologic cough
	↑ Miosis—constriction of the pupils	
	↑ Truncal rigidity	Most apparent when using fentanyl, sufentanil, alfentanil
	↑ Nausea and vomiting	
Peripheral	Gastrointestinal system	—
	↑ Constipation	
	↓ Gastric motility	
	↓ Digestion in the small intestine	
	↓ Peristaltic waves in the colon	
	↑ Constriction of biliary smooth muscle	
	↑ Esophageal reflux	
	Other smooth muscle	
	↑ Depression of renal function	
	↓ Uterine tone	
	↑ Urinary retention	
	Skin	
	↑ Itching and sweating	
	↑ Flushing of the face, neck, and thorax	
	Cardiovascular system	
	↓ Blood pressure and heart rate if cardiovascular system is stressed	
	Immune system	
	↓ Formation of rosettes by human lymphocytes	
	↓ Cytotoxic activity of natural killer cells	
	Other	
	Behavioral restlessness	

The actions summarized in this table are observed for all clinically available opioid agonists.

ies with opioid behavioral pharmacology. We discuss the advances in opioid receptor pharmacology and highlight the connections between signaling at opioid receptors, tolerance to opioids, and behavioral responses. The review's primary aim is to discuss recent efforts in understanding how opioid receptors mediate a diverse array of molecular or cellular responses while also modulating behaviors such as analgesia, reward, depression, and anxiety. We summarize the modern advances in opioid receptor signaling to mitogen-activated protein kinases (MAPK) and receptor protein–protein interaction networks and propose that there is a strong potential for selective ligand intervention at opioid receptors to treat a variety of central and peripheral nervous system disorders by using biased ligands and pathway-selective pharmacology. Moreover, we highlight how a greater connection between these advances at the molecular levels and behavioral pharmacology is imperative to fully understand the field of opioid pharmacology.

Opioid Tolerance in the Clinic

Before a detailed understanding of the molecular and cellular actions of opioid receptors is developed, it is important to consider their general effects and those observed in daily clinical settings. Different potencies of opiate drug formulations have been effective in the treatment of a variety of acute, chronic, and cancer-related pain disorders. The clinical utility of opioids continues to be limited by a compromise between efficacy and side effects. The most common side effects of opiates can be divided into peripheral effects (constipation, urinary retention, hives, bronchospasm) and central effects (nausea, sedation, respiratory depression, hypotension, miosis, cough suppression), all of which seriously affect the agents' clinical utility and patients' quality of life[4,5] (table 1). There have been many attempts to develop better opioid drugs, but these have been largely unsuccessful because of our incomplete understanding about the development of tolerance to the analgesic effects.[6]

Opioid tolerance is defined typically in the clinic as the need to increase a dose to maintain the analgesic effects. However, this increase in dose can exacerbate the perpetual problem of the side effects mentioned. This continual cycle of insufficient analgesia and side effects is among the greatest challenges of using

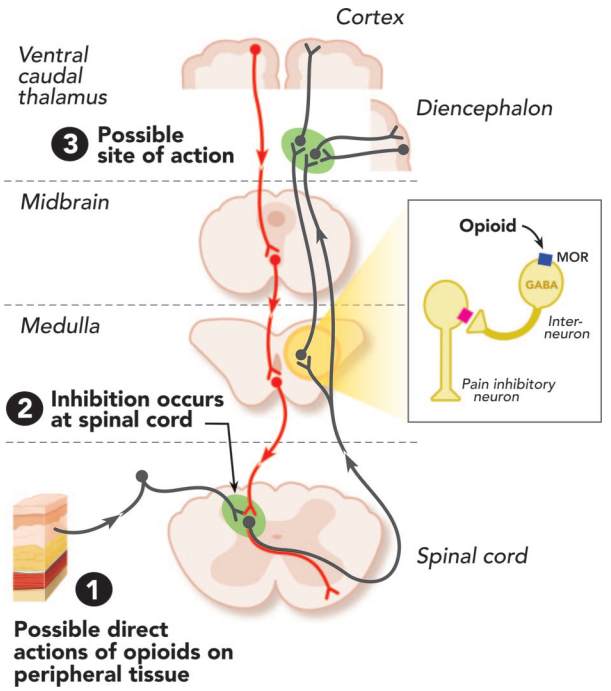

Fig. 1. Sites of action of opioid analgesics. The *gray pathway* shows the sites of action on the pain transmission pathway from periphery to central nervous system. The *red pathway* shows the actions on pain-modulating neurons in the midbrain and medulla. GABA = γ-aminobutyric acid; MOR = μ opioid receptor.

opioids in the clinic. Because of these limitations, opioid tolerance can ultimately lead to low patient compliance and treatment discontinuation. These clinical problems highlight the continued need for a better understanding of the molecular and pharmacologic mechanisms of opioid receptor tolerance, regulation, and signal transduction.

Classic Opioid Receptors Signaling

Opioid receptors are expressed in pain-modulating descending pathways, which include the medulla, locus coeruleus, and periaqueductal gray area. They are also expressed in limbic, midbrain, and cortical structures (fig. 1). The activation of opioid receptors at these locations directly inhibits neurons, which in turn inhibit spinal cord pain transmission.[4,5] The process by which these receptors engage in disinhibition is understood mostly with respect to analgesia; however, research is still active in this area because investigators continue to unravel novel modulatory mechanisms in these opioid circuits.

All four opioid receptors are seven-transmembrane spanning proteins that couple to inhibitory G proteins. After being activated by an agonist, such as the endogenous μ-opioid peptide endorphin, or exogenous agonists, such as morphine and fentanyl, the Gα and Gβγ subunits dissociate from one another and subsequently act on various intracellular effector pathways.[7,8] Early work in opioid receptor pharmacology demonstrated that guanine nucleotides such as guanosine triphosphate (GTP) modulate agonist binding to opioid receptors in membrane preparations from brain tissue. It was later determined that GTPase activity is stimulated by opioid agonists and endogenous opioid peptides.[9] Agonist stimulation of opioid receptors was also shown to inhibit cyclic adenosine monophosphate (cAMP) production in a manner similar to that of other types of G protein-coupled receptors (GPCR).[10] When pertussis toxin was used to selectively adenosine diphosphate (ADP)-ribosylate the G protein, the inhibitory function of opioid receptors on cAMP signaling was found to be $G\alpha_i$ dependent.[11,12] Today it is widely accepted that all four opioid receptor types couple to pertussis-toxin–sensitive G proteins, including $G\alpha_i$, to cause inhibition of cAMP formation.

The classic and perhaps most important aspect of opioid receptor signal transduction relates to opioids' ability to modulate calcium and potassium ion channels (fig. 2). After $G\alpha_i$ dissociation from Gβγ, the Gα protein subunit moves on to directly interact with the G-protein gated inwardly rectifying potassium channel, Kir3. Channel deactivation happens after GTP to guanosine diphosphate hydrolysis and Gβγ removal from interaction with the channel.[13–15] This process causes cellular hyperpolarization and inhibits tonic neural activity. In several reports, the inhibitory effects of opioids on neural excitability were shown to be mediated by interactions of opioid receptors with G protein-regulated inwardly rectifying potassium channel (Kir3).[16,17]

When activated, all four opioid receptors cause a reduction in Ca^{+2} currents that are sensitive to P/Q-type, N-type, and L-type channel blockers.[18] Opioid receptor-induced inhibition of calcium conductance is mediated by binding of the dissociated Gβγ subunit directly to the channel. This binding event is thought to reduce voltage activation of channel pore opening.[19,20] Numerous reports have shown that opioid receptors interact with and modulate Ca^{+2} channels; this has led to the examination of specific Ca^{+2} channel subunits that may be involved in opioid receptor modulation. For instance, it was reported that MOR stimulation results in G protein-dependent inhibition of α_{1A} and α_{1B} subunits.[21]

It is also clear that the acute administration of opioid agonists reduces Ca^{+2} content in synaptic vesicles and synaptosomes, with compensatory up-regulation of vesicular Ca^{+2} content during the development of opiate tolerance.[22,23] In addition, because the activation of μ, δ, and κ opioid receptors inhibits adenylyl cyclase activity, the cAMP-dependent Ca^{+2} influx is also reduced.

The evidence for opioid receptors positively coupling to potassium channels while negatively modulating calcium channels has been reported in numerous model systems and cell types. For many years this was thought to be the primary action of opioid receptors in the nervous system. This coupling of opioid receptors to potassium and calcium channels has been demonstrated in a wide range of systems, from neurons in the hippocampus, locus coeruleus, and ventral

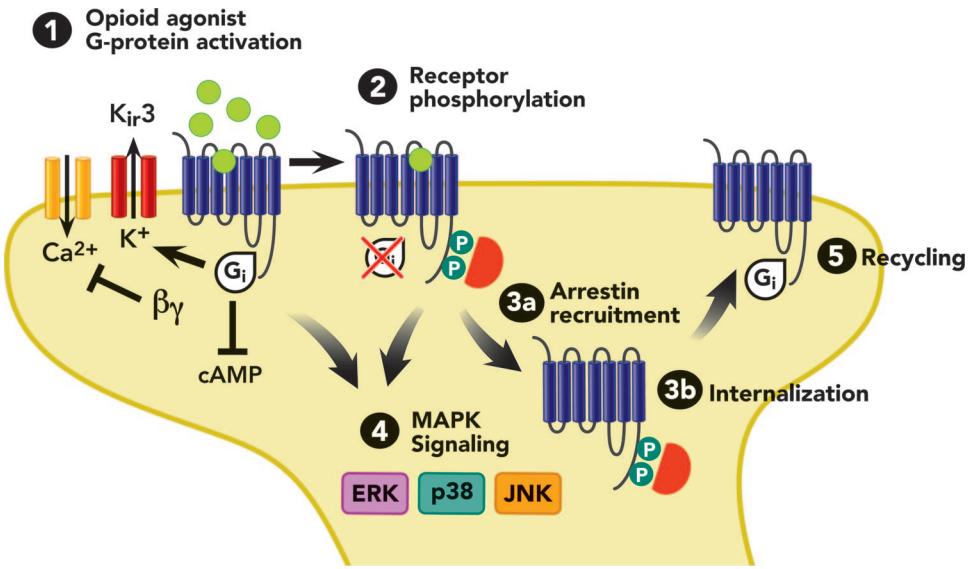

Fig. 2. Summary of opioid receptor signaling. Figure depicts opioid receptor signal transduction and trafficking. In general, all four opioid receptor subtypes (mu [μ], delta [δ], kappa [κ], and opioid receptor like-1 [ORL1]) share these common pathways. New research indicates that selective ligands at each opioid receptor can direct opioid receptors to favor one or more of these signaling events (biased agonism or ligand-directed signaling). *Arrows* refer to activation steps; *T lines* refer to blockade or inhibition of function. $\beta\gamma$ = G protein β-γ subunit; cAMP = cyclic adenosine monophosphate; ERK = extracellular signal-regulated kinase; JNK = c-jun N-terminal kinase; MAPK = mitogen-activated protein kinases; P = phosphorylation.

tegmental area to the dorsal root ganglia, supporting the notion that these channels are highly conserved opioid receptor substrates and represent one of the most important targets for opioid receptor modulation. Newer findings, which we highlight later in this review (see Opioid Receptor Regulation), suggest that although opioid receptors have potent effects on ion channel modulation, they also have slower yet robust effects on other signal transduction pathways.

Molecular Mechanisms of Opioid Tolerance

To date, the molecular and cellular mechanisms mediating the development of tolerance to morphine remain a matter of controversy. Traditionally, it was thought that the down-regulation of opioid receptors after chronic agonist exposure induces tolerance, as reported in *in vitro* studies.[24,25] However, recent *in vivo* studies show that down-regulation does not occur consistently with each and every agonist and may not completely explain tolerance. In light of these findings, it has been suggested that MOR proteins are in fact not down-regulated but instead may be desensitized and uncoupled from downstream signaling pathways.[26] It has been observed that after chronic morphine exposure, levels of the second messenger cAMP are increased. However, this elevation in cAMP may not be attributable to opioid receptor uncoupling from inhibitory G proteins but instead could reflect cellular adaptive changes, including the up-regulation of adenylyl cyclase, protein kinase A, and cAMP response element-binding protein.[27] It is this ineffective regulation of cAMP by morphine that some believe induces tolerance.

It has also been proposed that the regulation of opioid receptors by endocytosis reduces the development of tolerance and therefore serves a protective role.[28,29] After endocytosis, the cellular response is desensitized to the μ agonist, but the receptors can be recycled to the cell surface in an active state, resensitizing the receptor to the agonist. Morphine-activated opioid receptors signal for long periods of time, thereby enhancing the production of cAMP, which is thought to result in tolerance. *In vivo* studies have shown that facilitation of MOR endocytosis in response to morphine prevents the development of morphine tolerance.[28] In addition, it has been shown *in vivo* that the lack of β-arrestin 2 prevents the desensitization of MOR after chronic morphine treatment, and these mice also failed to develop antinociceptive tolerance.[30]

Recent studies have identified how ligand-directed responses, more commonly known as biased agonism, are crucial in understanding the complexity of opioid-induced tolerance. The work of Bohn and colleagues showed how β-arrestin 1 and β-arrestin 2 differentially mediate the regulation of MOR. β-arrestins are required for internalization, but only β-arrestin 2 can rescue morphine-induced MOR internalization, whereas both β-arrestin 1 and β-arrestin 2 can rescue [d-Ala2, N-Me-Phe4, Gly-ol5]enkephalin (DAMGO)-induced MOR internalization.[31] These findings suggest that MOR regulation is dependent on the agonist and may be critical in understanding the mechanism involved in the development of tolerance. Melief *et al.* further showed how acute analgesic tolerance to morphine is blocked by c-jun N-terminal kinase (JNK) inhibition but not G protein-receptor kinase 3 (GRK-3) knockout. In contrast, using a second class of μ agonists (fentanyl, methadone, and oxy-

codone), acute analgesic tolerance was blocked in GRK-3 knockout but not JNK inhibition.[32] Ligand-biased responses are well documented *in vitro* but less so *in vivo*; however, a recent study addressed biased agonism at δ opioid receptors (DOR) *in vivo* showing that DOR agonists with similar binding and analgesic properties but different internalization potencies lead to the development of differential tolerance at DOR.[33] These findings highlight the important implications of ligand-selective responses in GPCR biology[33] and indicate the need for additional work to examine the role and consequences of biased signaling in behavioral models.

Opioid Receptor Regulation

Agonist-induced receptor phosphorylation is believed to be one of the many critical molecular components of opioid tolerance. This process is well established in the GPCR literature and typically occurs after chronic agonist exposure or sustained release of endogenous opioid peptides. Sustained opioid treatment produces tolerance to the acute effects of the drug and can potentially lead to physical and psychological dependence. As a result of this problem, opioid-receptor trafficking, desensitization, and phosphorylation have been extensively examined (for a detailed review see Bailey and Connor[34]). Here we highlight the key findings in this area as they connect potential signaling to tolerance mechanisms.

μ Opioid Receptors (MOR)

One common thread between the opioid receptor subtypes is the interesting observation that receptor trafficking and regulation vary depending upon the agonist. For example, morphine is unable to promote receptor internalization, in contrast to DAMGO, which causes robust internalization.[32,35,36] It is thought that morphine tolerance, a major problem in the clinic, is perhaps mediated by these differences in receptor regulatory activity. Several groups are actively working to discern the various mechanisms for the differences in ligand-dependent MOR regulation, but controversy remains, with some groups hypothesizing that MOR internalization does not actually uncouple the receptor from signal transduction pathways but instead induces recycling of uncoupled receptors to the plasma membrane. Alternatively, the morphine-bound receptor, although not internalized, may still signal at the cell membrane, and because signaling is never attenuated, the cellular machinery adapts to produce tolerance. A recent study has shown that morphine acts as a "collateral agonist" to promote receptor G-protein uncoupling ("jamming") and JNK activation (see MAPK Signaling at Opioid Receptors), whereas fentanyl and DAMGO internalize and desensitize normally.[35] It is plausible that many processes work together to produce receptor regulation and opioid tolerance, and additional study is warranted to continue to decipher these discrepancies.

μ Opioid receptors contain more than 15 serine, threonine, and tyrosine residues that are accessible to protein kinases, which phosphorylate the receptor. All three intracellular loops and the carboxyl terminal tail contain these sites.[37] ^{32}P incorporation experiments have been critical to our understanding of MOR receptor phosphorylation, and combined with our knowledge of site-directed mutagenesis, we now have a clear understanding of the key residues involved in MOR phosphorylation. Rat MORs are phosphorylated at Ser375 in the carboxy terminus,[38,39] and treatment with both morphine and DAMGO causes robust phosphorylation of this residue. However, some reports have suggested that morphine and DAMGO induce different degrees of phosphorylation of Ser375,[39] suggesting that Ser375 may not be the only amino acid residue phosphorylated and responsible for MOR regulation. The highly conserved GPCR "DRY motif" in the second cytoplasmic loop of the μ opioid receptor has been implicated in regulation of agonist efficacy. Phosphorylation of Tyr166 reduced the efficacy of DAMGO-mediated G-protein activation.[40] It has been shown recently that agonist-selective differences in MOR regulation are in fact determined not only by net incorporation of phosphates into the receptor population as a whole but also by individual receptors achieving a critical number of phosphorylated residues (multiphosphorylation) in a specific region of the C-tail.[41] In addition, the same group identified that multiphosphorylation specifically involves the ^{375}STANT^{379} motif required for the efficient endocytosis of MOR. These ligand-mediated differences highlight the ligand-dependent nature of opioid receptor function and require additional further study *in viv*o.

κ Opioid Receptors (KOR)

κ Opioid receptor trafficking shares some common features with MOR regulation because KOR is readily phosphorylated, desensitized, and internalized. KOR is phosphorylated, desensitized, and internalized by the agonists U50,488 and dynorphin 1–17 but not by other agonists, such as etorphine or levorphanol.[42,43] Both dynorphin A and B have been shown to initiate significant receptor internalization in human KORs and three structurally distinct KOR ligands: Salvinorin A (salvA), TRK820, and 3FLB were shown to induce KOR internalization with varying rank orders of potency.[44] There have been conflicting data regarding agonist-induced KOR internalization, which seem to be dependent on the cell line, receptor species, or model system used. In Chinese hamster ovary cells expressing KOR, the selective KOR agonists U50,488 and U69,593 did not cause robust receptor internalization[45]; however, in mouse pituitary tumor (AtT20) cells and human embryonic kidney (HEK293) cells, U50,488 initiated strong internalization of KOR-green fluorescent protein (GFP) proteins.[46–48] Despite this, several groups have found consistency in the ability of KOR to become phosphorylated, internalized, and desensitized by its endogenous opioid peptide dynorphin.

δ Opioid Receptors (DOR)

In contrast to MOR and KOR, DOR were thought to exist primarily (more than 90%) at intracellular sites[49–51] until recently, when mice expressing fluorescently tagged DOR revealed that there was strong membrane localization of DORs *in vivo*.[52] The reasons for this discrepancy between the numerous studies showing intracellular DOR labeling and membrane labeling remain unclear and continue to be matters of controversy. It is plausible that previous studies using DOR antibodies were flawed because of antibody specificity issues, despite the numerous controls conducted. Although DORs tagged with GFP are a powerful *in vivo* tool, they require careful interpretation given that GFP is a large protein that may interfere with the typical DOR trafficking machinery. Additional investigation is required in both cases, and it is plausible that both concepts are indeed true; the concentrations of DOR expressed on the cell surface may well be higher than originally hypothesized, yet a large intracellular pool of DOR protein remains. Nevertheless, DOR seems to be a dynamic opioid receptor that can readily traffic in response to agonists. Some reports have shown that chronic morphine treatment promotes movement of DORs to the cell surface in the dorsal horn of the rat spinal cord.[49] This effect was dependent on MOR receptor activity because blocking or deleting MOR genetically (MOR knockout) prevents the effect.

Like MOR and KOR, desensitization of DOR is controlled *via* phosphorylation, after recruitment of arrestins and sequestration of arrestin-bound receptors.[53,54] Phosphorylation of DOR has been shown with both small-molecule organic ligands and peptide treatments. Once again, c-terminal phosphorylation was shown to be critical for opioid receptor regulation. In DOR, the Ser363 residue is the key phosphorylation site.[55,56] This phosphorylation event was shown to be mediated by GPCR kinase 2 (GRK-2).[56,57] Other studies have demonstrated that other amino acid residues are involved in DOR regulation. For example, Thr353 was found to be important for [D-Ala2, D-Leu5]-Enkephalin (DADLE)-mediated down-regulation of DOR, and Leu245 and 246 act as lysosomal targeting motifs that partake in determining agonist-bound DOR localization.[58,59] Furthermore, ligand-specific variability in agonist-dependent DOR phosphorylation has been observed with potential differences between SCN80- and [D-Pen2,5]Enkephalin (DPDPE)-bound conformations recruiting kinases with various efficacies and potencies.[60]

Opioid Receptor Like-1 (ORL1)

Opioid receptor like-1 (also called nociceptin or orphanin FQ) receptors are the newest members of the opioid receptor family, and few groups have examined their regulatory properties. Agonist-induced internalization of ORL1 is rapid and concentration- dependent.[61] Both the endogenous agonist nociceptin and small-molecule selective ORL1 agonist Ro646198 promote rapid internalization of ORL1. Agonist challenge also reduces the ability of ORL1 to couple to inhibition of forskolin-stimulated cAMP production, suggesting that ORL1 undergoes desensitization mechanisms similar to those of the other three opioid receptor subtypes. ORL1 internalization appears to be more rapid than that of the other opioid receptors, with some groups reporting internalization after only 2 min of agonist exposure in Chinese hamster ovary cells.[61] However, this appears to be dependent on ligand type and cell line expression because ORL1 internalization in human neuroblastoma cells was slower and occurred closer to a 30-min time point.[62] ORL1 receptors recently were demonstrated to cointernalize with N-type Cav2.2 channels after a 30-min agonist treatment.[63] The internalization of the entire signaling complex is not unusual in GPCRs; however, the effect in the case of ORL1 is particularly pronounced and is believed to play a major role in how ORL1 selectively removes N-type calcium channels from the plasma membrane to inhibit calcium influx.

Opioid receptor like-1 receptor regulation is increasingly studied, but our understanding remains in the infant stages compared with that of the other three opioid receptor subtypes. To date, few site-directed mutagenesis studies have been conducted, and receptor regulation in primary neurons, dorsal root ganglion, or dorsal horn neurons remains unknown. As we move forward in understanding opioid receptor signaling and identify novel opioid receptor targets, ORL1 receptors become likely candidates for the future of opioid pharmacology.

Opioid Receptors and Arrestin Recruitment

Phosphorylation by GRK-2 or -3 of μ, δ, and κ opioid receptors leads to arrestin 2 or 3 recruitment. Arrestin molecules are key proteins that bind phosphorylated GPCRs to regulate their desensitization, sequestration, and sorting and ultimately assist in determining receptor fate. Opioid receptors are regulated by arrestin 2 and arrestin 3 binding (also called β-arrestin 1 and β-arrestin 2, respectively), and this interaction depends on the model system and agonist treatment procedure. Mice lacking arrestin 3 have been shown to have a reduced tolerance to μ opioids such as morphine, suggesting that MOR regulation requires arrestin 3.[30,64]

With the use of surface plasmon resonance methods, glutathione s-transferase pull-down assays, and classic immunoprecipitation methods, the C-terminal tails of DOR, MOR, KOR have been shown to be crucial for arrestin 2 or 3 binding. C-terminal carboxyl mutant opioid receptors have been studied widely, and these serine mutant receptors show decreased agonist-induced receptor internalization and arrestin recruitment. Dominant positive arrestins (such as Arrestin-2-R169E or Arrestin-3-R170E) that bind the nonphosphorylated receptors can rescue internalization of serine mutated MOR/DOR/KOR,[48,65] further implicating arrestin dependence in opioid-receptor trafficking. Most studies implicating arrestin have been conducted in heterologous expression systems using overexpressed arrestins and opioid receptor subtypes. These conditions are atypical and do not represent

the likely physiologic state of opioid receptors and arrestins *in vivo*, so these data should be interpreted with caution, and additional studies using *in vivo* approaches are needed to increase our understanding of arrestin–opioid interactions.

MAPK Signaling at Opioid Receptors

In the discussion above, we highlighted that sustained agonist treatment causes GRK phosphorylation at the carboxyl-terminal domain of opioid receptors activating arrestin-dependent receptor desensitization and internalization (fig. 2). During the last several years, GPCR research has discovered that the phosphorylated arrestin-bound GPCR complex is not simply inactive, but that it recruits alternate signal transduction cascades, including MAPKs.[66] The merging of our previous knowledge regarding opioid receptor phosphorylation, arrestin, and cellular mechanisms of tolerance with an understanding of opioid receptor signaling to MAPKs is becoming more appreciated (table 2).

Mitogen-activated protein kinase pathways are diverse signaling cassettes that govern cellular responses, including cell proliferation, differentiation, apoptosis, transcription factor regulation, channel phosphorylation, and protein scaffolding.[93] The MAPK family is composed of 12–15 gene products with the most well-described forms including extracellular signal-regulated kinases 1 and 2 (ERK 1 and 2), JNK1–3, and p38 (α, β, γ, δ) stress kinase. The MAPKs are distinct in that they have the capacity to respond to a variety of stimuli and transmit a diverse array of intra- and extracellular signals.[94] MAPK signaling is regulated by the kinetics of activation, nearby phosphatase activity, and the cellular domain the MAPKs occupy.[93] Initially, ERK MAPKs were shown to require receptor tyrosine kinase transactivation, through epidermal growth factor or brain derived neurotrophic factor (also called TrkB receptors).[95] Later reports directly linked GPCRs to activation of MAPK signaling pathways, and now most, if not all, GPCRs have been found to couple to this pathway.

ERK 1 and 2 Signaling at Opioid Receptors. The most frequently examined opioid-induced MAPK cascade is ERK 1 and 2. Coscia and colleagues have been crucial in developing our understanding of the relationship between opioid receptors and ERK 1 and 2 signaling. In one of the initial studies, MOR and KOR stimulation was demonstrated to initiate ERK 1 and 2 phosphorylation in astrocyte cultures and transfected cell lines.[96] The kinetics of ERK 1 and 2 phosphorylation by MOR and KOR systems vary, yet both receptors can activate ERK 1 and 2 within 5–10 min. MOR-mediated ERK 1 and 2 phosphorylation requires protein kinase C (PKCε) activity, and MOR-dependent ERK 1 and 2 signaling requires GRK-3 and arrestin in primary neurons, glial cells, and heterologous expression systems.[67,68,97] The downstream substrates of MOR-mediated ERK 1 and 2 have been defined in some cases and remain unknown in others. In embryonic stem cells, MOR-dependent ERK 1 and 2 signaling positively modulates and directs neural progenitor cell fate decisions.[98,99] However, in astrocytes chronic morphine can negatively regulate ERK 1 and 2 signaling by tyrosine kinase pathways to ultimately inhibit neurite outgrowth and synapse formation.[69] Most studies use MAPK–ERK (MEK) inhibitors (the proximal upstream kinase) to determine substrates of ERK 1 and 2 signaling in GPCRs; however, few reports have shown direct interaction between μ-opioid–induced ERK and a final substrate. (The *in vivo* implications of MOR-dependent ERK signaling are explored in Opioid Signaling and Behavior.) Several groups are investigating the potential for ligand-specific ERK agonists at opioid receptors.

δ Opioid receptors have also been shown to activate ERK 1 and 2 through G$\beta\gamma$ and Ras signaling cascades[96] and do not necessarily require receptor internalization or receptor phosphorylation for signaling.[100,101] DOR-mediated ERK signaling recently was found to require integrin signal transduction through transactivation of epidermal growth factor receptor pathways. DOR-mediated epidermal growth factor receptor activation also initiated phospholipase C signaling to stimulate ERK 1 and 2 phosphorylation.[83] DOR-dependent ERK 1 and 2 signaling requires additional investigation because, coupled with DOR's critical role in pain and mood regulation, ERK signaling through DOR may reveal a novel mechanism for DOR regulation of neural activity.

KOR-dependent ERK 1 and 2 phosphorylation occurs in a multiphase manner, with an early period of activity between 5–15 min after agonist exposure and a late phase after 2 h of agonist treatment. Similar to other GPCRs,[102] the biphasic ERK 1 and 2 activation for KOR contains an arrestin-dependent late phase[73] and an arrestin-independent early phase. This group identified G$\beta\gamma$ as a crucial mediator in the early-phase ERK 1 and 2 activation by KOR, and showed that arrestin 3 is required for late-phase ERK 1 and 2. KORs activate ERK 1 and 2 through PI3-kinase, PKCζ, and intracellular calcium.[72] However, like MOR and DOR, the substrate for KOR-mediated ERK 1 and 2 has not been identified, although a recent study suggests that KOR-induced ERK 1 and 2 also directs stem cell fate toward neural progenitor development. ORL1 receptor-dependent ERK 1 and 2 activation has not been extensively examined, although one group has shown that ORL1 receptor activation does initiate ERK 1 and 2 phosphorylation.[89] The signaling pathways for ORL1-mediated ERK 1 and 2 phosphorylation in neuronal cell types and *in vivo* need additional investigation.

JNK. The JNK pathway is activated by environmental triggers, including stress, inflammation, cytokine activation, and neuropathic pain.[103,104] Classically, JNK activity can result in transactivation of c-jun, a component of the activator protein 1 transcription factor complex, and JNK phosphorylation is caused by cytokines, including tumor necrosis factor and interleukin-1β.[105] JNK activation has also been implicated in numerous other signaling cascades. JNK typically is activated by Ras-related GTP binding proteins in the ρ

Table 2. A Summary of Current Opioid Receptor-dependent Signaling

Receptor	Cascade/Signaling Pathway	Model	Reference
μ Opioid	↑ ERK 1 and 2 (GRK-3 and arrestin dependent)	*In vivo* (murine)	Macey *et al.* 2006[67]
	↑ ERK 1 and 2 (arrestin dependent)	Astrocytes	Miyatake *et al.* 2009[68]
	↓ ERK 1 and 2 (chronic activation)	Astrocytes	Ikeda *et al.* 2010[69]
	↑ JNK 2 (PKC dependent)	*In vivo* and HEK293	Melief *et al.* 2010[35] Tan *et al.* 2009[70]
	↑ Stat3 phosphorylation	*In vivo* (murine) and CMT-93	Goldsmith *et al.* 2011[71]
κ Opioid	↑ ERK1 and 2	Astrocytes	Belcheva *et al.* 2005[72]
		In vivo	Bruchas *et al.* 2006[48] McLennan *et al.* 2008[73] Bruchas *et al.* 2008[74] Potter *et al.* 2011[75]
	↑ p38 MAPK (dependent on GRK-3 and arrestin)	Striatal neurons	Bruchas *et al.* 2006[48]
		Astrocytes	Bruchas *et al.* 2007[76]
		In vivo	Xu *et al.* 2007[77] Bruchas *et al.* 2011[78]
	↑ JNK 1	*In vivo*	Melief *et al.* 2010[35] Melief *et al.* 2011[32]
	↑ JAK2/STAT3 and IRF2 signaling cascade	PBMC	Finley *et al.* 2011[79]
δ Opioid	↑ ERK 1 and 2	HEK293	Eisinger *et al.* 2009[80] Eisinger *et al.* 2004[81] Audet *et al.* 2005[82]
	↑ ERK 1 and 2 (integrin stimulated, EGFR mediated)	HEK293	Eisinger *et al.* 2008[83]
	↑ ERK 1 and 2 (integrin stimulated, Trk1 mediated)	NG108-15	Eisinger *et al.* 2008[83]
	↑ PI3K/AKT/ ↓ GSK-3β	DOR-transfected CHO cells Rat NAc NG108-15 NG108-15	Olianas *et al.* 2011[84] Heis *et al.* 2009[85]
	↑ PI3K/ ↓ GSK-3β (SRC and AMPK dependent)	DOR-transfected CHO cells	Olianas *et al.* 2011[86]
	↑ PI3K (SRC and IGF-1 dependent)	DOR-transfected CHO cells	Olianas *et al.* 2011[87]
	↑ JNK (AKT dependent Pi3K mediated)	T cells	Shahabi *et al.* 2006[88]
ORL1	↑ ERK 1 and 2	Neuro-2a cells	Harrison *et al.* 2010[24]
		Rats NAc	Chen *et al.* 2008[89]
		In vivo (porcine)	Ross *et al.* 2005[90]
	↑ p38 MAPK	NG108-15	Zhang *et al.* 1999[91]
	↑ JNK	COS7 and NG108-15	Chan *et al.* 2000[92]
		In vivo	Ross *et al.* 2005[90]

↑ = activation; ↓ = deactivation; AKT = serine threonine protein kinase; AMPK = 5′ adenosine monophosphate-activated protein kinase; CHO = Chinese hamster ovary cells; CMT-93 = mouse rectum carcinoma cells; COS7 = monkey kidney fibroblast cell line; DOR = δ opioid receptors; EGFR = epidermal growth factor receptor; ERK1 and 2 = extracellular signal-regulated kinases 1 and 2; GRK-3 = G protein-receptor kinase 3; GSK = glycogen synthase kinase 3; HEK293 = human embryonic kidney cells; IGF-1 = insulin-like growth factor 1; IRF2 = interferon regulatory factor 2; JAK2 = Janus kinase 2; JNK 1 and 2 = c-jun N-terminal kinase; MAPK = p38 mitogen-activated protein kinase; NG108-15 = neuroblastoma glioma hybrid cell line; ORL1 = opioid receptor like-1; p38 STAT3 = signal transducer and activator transcription 3; PBMC = peripheral blood mononuclear cell; PI3K = phosphoinositide 3-kinase; SRC = proto-oncogene tyrosine-protein kinase.

family.[106] JNK activation by GPCRs and opioid receptors has not been examined thoroughly but has been demonstrated for all the opioid receptor subtypes. Like ERK 1 and 2, arrestin 2 and arrestin 3 have been reported to scaffold JNK signaling complexes, and it is believed that arrestin 3 has JNK 3 specificity, although this remains a matter of controversy.[107] The cellular mechanisms of arrestin-dependent JNK at GPCRs remain unresolved.

Opioid-dependent JNK has been demonstrated by only a few groups. DOR causes protein kinase B (Akt)-dependent JNK phosphorylation through a PI3-kinase mechanism,[88] and JNK activity is PI3-kinase independent in others.[108] PI3-kinase is required for μ-opioid–dependent JNK activation. In contrast, U50,488-induced (KOR) JNK activation has been shown to be independent of PI3-kinase.[108] The substrates and *in vivo* effects of opioid-induced JNK activa-

tion are being studied by several groups. KOR (U50,488, dynorphin) agonists activate JNK in a pertussis toxin-sensitive (Gαi) manner.[35,108,109] U50,488-mediated JNK requires focal adhesion kinases and the GTPase Rac in immune cell types. MOR-induced JNK activation recently was shown to require PKC activity.[35]

In two recent studies, KOR- and MOR-induced JNK phosphorylation by norbinaltorphimine and morphine were shown to act as "collateral agonists" to cause JNK phosphorylation and initiate uncoupling of the G protein to block Gαi-mediated transduction.[35,109] The persistent actions of norbinaltorphimine on KOR-agonist–mediated analgesia (21 days) were shown to require JNK because JNK 1 isoform knockout mice show an absence of norbinaltorphimine-dependent 21-day KOR blockade, and selective JNK inhibitors prevented the long-lasting norbinaltorphimine effect. It was also recently identified that the long duration of action of small molecule KOR antagonists *in vivo* is determined by their efficacy in activating JNK 1. The persistent KOR inactivation by these small-molecule collateral agonists did not require sustained JNK phosphorylation,[32] implicating intermediate protein(s) or alternate JNK substrates in this process. In contrast, acute morphine tolerance was shown to require JNK 2 because JNK 2 knockout mice showed an absence of MOR inactivation. How ligand-dependent JNK activation causes receptor uncoupling from Gαi signaling remains unresolved, and proteomic biochemical approaches will need to be used to identify ligand-dependent protein interactions. Together, this work highlights the remarkable nature of opioid receptor sensitivity to a variety of ligand-stabilizing conformations.

p38 MAPK. The p38 MAPK pathway also plays a key role in environmental stress and inflammation and is activated by cytokine production.[110] In glial cells particularly, p38 MAPK activity is required for an array of cellular responses, including interleukin-6 and interleukin-1β production, inhibitory nitric oxide synthase activity, and tumor necrosis factor α secretion. Activation of p38 MAPK is involved in proliferative and chemotactic responses in some systems and has been shown to play a major role in neuropathic pain responses.[77,111]

Opioid receptor-mediated p38 phosphorylation has been most widely demonstrated for the MOR and KOR systems. KOR-induced p38 MAPK has been observed in heterologous expression systems, striatal neurons, astrocytes, and *in vivo*.[2,3,8,77,76,99] KOR-mediated p38 MAPK activation requires serine 369 phosphorylation by GRK-3 and arrestin 3 recruitment.[48,76] μ Opioid receptor internalization recently has been shown to require Rab5 signaling and p38 MAPK. This process seems to be ligand dependent because morphine will not cause p38-dependent receptor internalization, but DAMGO will readily cause internalization.[70] In addition, μ opioid receptor cross regulation of α_{2A}-adrenergic receptors has been shown to require p38 MAPK, and p38 MAPK inhibition blocks DAMGO-induced MOR internalization.[70] This cross activity between MOR and α_{2A}-adrenergic receptors requires arrestin 3, suggesting that arrestin 3 scaffolding of p38 is likely to be conserved across opioid receptors. To date, few studies have identified a role for DOR or ORL1 in mediating p38 phosphorylation. In one report, both ORL1 and DOR were shown to cause p38 phosphorylation through activation of protein kinase A and protein kinase C.[91] Opioid-induced p38 has several potential targets, including modulation of ion channels and transcription factors. Recently, the potassium channel Kir3.1 was demonstrated to become tyrosine phosphorylated *via* KOR-dependent p38 MAPK Src activation.[112] How p38 specifically interacts with various substrates will be an interesting next step and will reveal how such a ubiquitously expressed kinase can selectively modulate the large variety of cellular events.

Protein–Protein Networks and Opioid Receptors

In addition to intracellular signaling and receptor modification by phosphorylation, newer biochemical studies strongly suggest that opioid receptors interact with one another, alternate GPCRs, and a whole host of anchoring and membrane protein sets. These interactions are becoming increasingly appreciated as critical to the ultimate functional role of the opioid receptor families. In many ways, the field of protein–protein interactions is at the forefront of opioid receptor molecular pharmacology, as research moves from previous work in heterologous expression systems to *in vivo* approaches.

Opioid Dimerization. Numerous reports have demonstrated that GPCRs exist as dynamic protein complexes with large interactions between proteins and other receptor types. Several studies have shown that GPCRs can form dimers and oligomers. This oligomerization includes two varieties: homodimers (same receptor) and heterodimers (different receptor type) (fig. 3). The existence of these GPCR homomers and heteromers has been shown in transfected cell line systems, cell lines, and primary cultures and in some cases *in vivo* (for review see Rios *et al.*[113] and Prinster *et al.*[114]). Despite that GPCR oligomerization remains a matter of controversy, it continues to generate interest because opioid receptor dimers may reveal novel targets for the development of new opioid drugs.

Devi and colleagues pioneered research into opioid dimerization and originally identified opioid receptor heterodimers.[115] They found that δ receptors can exist as homodimers, and agonist stimulation causes their dissociation.[115,116] In this seminal work, the authors also found that KOR and DOR form heterodimeric complexes, which appear to alter the trafficking properties of these receptors. They showed how agonist-induced internalization of DOR receptors is reduced substantially in cells expressing DOR/KOR receptors.[115] Moreover, it was shown that 6′-guanidinonaltrindole, which selectively targets the KOR or DOR heterodimer, generates a unique signaling entity, giving additional evidence for the existence of opioid heterodimers.[117]

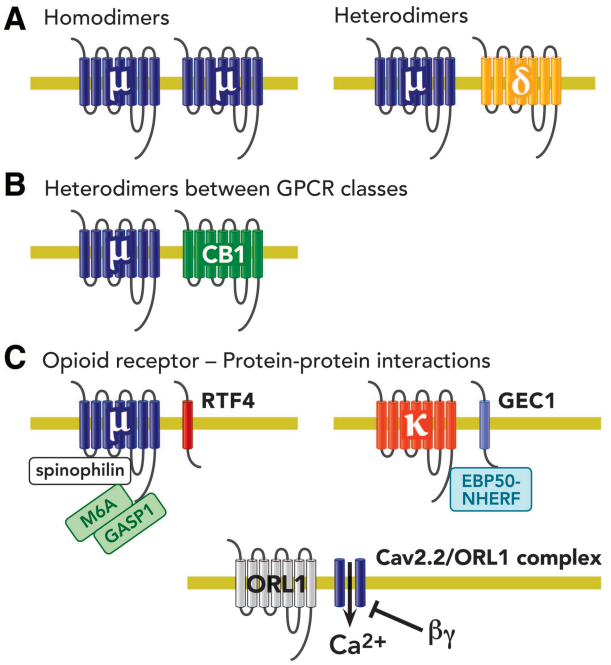

Fig. 3. Opioid dimerization. Figure depicts opioid receptor homodimers and opioid receptor heterodimers (*A*); heterodimers between opioid receptors and other G-protein–coupled receptors (*B*); protein–protein interactions involved in opioid receptor signal transduction (*C*). $\beta\gamma$ = G protein β-γ subunit; μ = μ opioid receptor; δ = δ opioid receptor; CB1 = cannabinoid receptor type 1; GPCR = G protein coupled receptors; κ = κ opioid receptor; ORL-1 = opioid receptor like-1; *arrow* = movement; *T lines* = blockade.

It has been shown that MOR can heterodimerize with ORL1,[118] but the existence of MOR or KOR heterodimers remains a matter of controversy.[115,119] The observation that the antagonism or absence of DOR diminishes the development of morphine tolerance and dependence suggests there may be an interaction between the two receptors, although future biochemical work *in vivo* is needed to validate these concepts. Studies not only identified the existence of MOR and DOR heterodimers but also revealed that MOR and DOR heterodimers have distinct ligand binding and signal transduction properties,[120] suggesting that heterodimerization may represent an alternative mechanism for the cell to tune and control second messenger activity.

It was hypothesized that the mechanisms and/or proteins that modulate the level of MOR or DOR complexes are critical in the development of tolerance[121]; this theory inspired research into the events that lead to dimerization. Devi and colleagues recently identified additional signaling proteins, such as RTP4, that partake in opioid receptor oligomer trafficking from the Golgi to distribute opioid receptor complexes at the cell membrane.[121] In addition, it was found that MOR activation promotes the formation of complexes between RGS9–2 and Gα subunits. It was shown that pharmacological manipulations were able to disrupt RGS9–2 complexes formed after repeated morphine administration.[122] These data provide a better understanding of pharmacologic approaches that can be used to improve chronic analgesic responses and tolerance.

Some studies have shown that opioid receptors can heterodimerize with other classes of GPCR. For example, MOR can interact and potentially heterodimerize with cannabinoid receptor 1 (CB1) (fig. 3).[123,124] Interactions between MOR and cannabinoid receptor 1 receptors appear to modulate their effects, as evidenced by the administration of Δ 9-tetrahydrocannabinol, a cannabinoid receptor 1 agonist, which can enhance the potency of opioids such as morphine.[125] In addition, the concomitant activation of MOR or cannabinoid receptor 1 heterodimers leads to significant attenuation of ERK activity compared with the response after the activation of each individual receptor.[123]

A number of previous studies have noted interesting functional interactions between the MOR and α_{2A}-adrenergic receptor systems.[126–128] These studies reported that the presence of α_{2A} receptors is sufficient to potentiate the phosphorylation of MAP kinases in response to morphine, whereas the combination of ligands abolishes this effect. The interactions between MOR and α_{2A} receptors provide an alternate mechanism for the control of receptor function and could have profound effects in the development of opioid-adrenergic analgesics. Neurokinin 1 and MOR have also been shown to heterodimerize. The interaction between these two receptor types does not alter ligand binding or signal transduction but does change internalization and resensitization.[129] In addition, substance P (neurokinin 1 selective ligand) caused cross phosphorylation and cointernalization of MOR.[130] As neurokinin 1 and MOR coexist in the trigeminal dorsal horn, it has been suggested that they may interact functionally within a signaling complex in these neurons during nociceptive neurotransmission.[130] The functional consequences of opioid receptor oligomerization *in vivo* are largely unknown, unexplored, and matters of controversy. New technological advances in mouse genetics and imaging are crucial in resolving these issues. One major area of continued interest is the *in vivo* demonstration of opioid receptor homo- and heterodimerization, as well as the development of additional biochemical tools to demonstrate unequivocally that these receptor proteins directly interact with one another.

In addition to receptor–receptor interactions, it is increasingly clear that opioid receptors are highly complex systems and that they interact with a whole host of extracellular, intracellular, and membrane proteins. The notion of opioid receptors existing as dynamic signaling complexes sits at the forefront of the future of opioid-based therapeutics. The reasons for this include the notion that different opioid receptor ligands can induce the formation of a diverse array of receptor complexes. In addition, it is increasingly appreciated that the opioid receptor's native environment (*i.e.*, cell type, neural circuit) greatly affects the receptor's ability to signal, traffic, and function. The idea of a binary GPCR as a simple switch mechanism, from off to on, is becoming widely dis-

regarded as new protein–protein interaction networks and ligand-dependent properties are uncovered.[32,33,35,76,95,131]

Other Protein–Protein Interactions. There are multiple lines of evidence pointing to arrestin molecules as crucial proteins that network and engage opioid receptor signal transduction and orchestrate the interaction of proteins within the cellular milieu. The isolation of other opioid-selective protein-interaction networks has been slow, although more studies are examining the many important roles in receptor fate. For one, MOR has been shown to interact with numerous cytoskeletal trafficking proteins, most of which participate in membrane protein endocytosis, including GASP-1, spinophilin, glycoprotein M6A, and tamalin.[132–134] MOR also has been shown to interact with calmodulin, which is a highly sensitive Ca^{2+} binding protein implicated in cytoplasmic enzyme activity, including adenylyl cyclases and CAM kinases.[135] DORs are similar because they also use GASP-1 and glycoprotein M6A for regulating surface trafficking and endocytosis. KORs have been shown to interact with GEC-1 and EBP50-NHERF proteins, potentially acting to enhance receptor recovery and recycling rates.[136,137] Given that ORL1 has not been extensively studied, most of our knowledge about its signaling complex centers around the work of the Zamponi group demonstrating ORL1-Cav2.2 complex formation.[63,138] The increasing specificity and affordability of proteomic technologies, such as tandem affinity purification (TAP tag) approaches,[139] will help to advance our understanding of opioid receptor complexes. Validating protein–protein interactions *in vivo* continues to be a challenge, but it is expected that with newer mouse genetic tools, proteomic dissection of opioid receptor complexes *in vivo* will become an easier task.

Opioid Signaling and Behavior

μ Opioid Receptors. The most common behavioral function linked to opioid receptors has been their ability to mediate analgesic effects. Numerous reports have examined how opioid signaling causes opioid-induced analgesia (see Bodnar[140] and Walwyn *et al.*[3]). It is generally accepted that MOR signaling to pertussis toxin-sensitive Gαi is required for morphine antinociception. In addition, *in vitro* blockade of arrestin 3 expression improves morphine-mediated analgesic responses and acts to prevent morphine tolerance over time.[141] Spinal cord expression of Gβγ is required for MOR coupling to analgesic responses and is thought to play a key role in how MORs mediate antinociception.[142] This is likely to be through the modulation of potassium and calcium channels in the dorsal root ganglion and dorsal horn. Morphine-induced analgesia and tolerance have been linked to numerous signaling pathways, including interaction with adenylyl cyclases AC1, AC8, and AC5.[143,144]

μ Opioid receptor-dependent behavioral studies linked to MAPK signaling have begun to become more common in the literature. ERK 1 and 2 phosphorylation has been shown to be up-regulated by chronic morphine treatment and in opioid withdrawal[145]; consistent with this idea, MOR-induced ERK 1 and 2 activity in the periaqueductal gray region acts as a mechanism to counteract morphine tolerance.[146] Recently, reports have implicated ERK 1 and 2 signal transduction in morphine reward and plasticity, including place preference and psychomotor sensitization.[147,148] ERK 1 and 2 activity in the amygdala was found to mediate anxiety-like behaviors during morphine withdrawal.[149] Together, these reports strongly support the concept that ERK 1 and 2 signaling is an essential mediator of μ opioid-induced plasticity in the brain and spinal cord.

μ Opioid receptors signaling *via* other protein kinases and protein–protein interactions to modulate reward and analgesia, such as PKC or protein kinase A and JNK, has been demonstrated. For example, PKC 1 (also called RACK1) is required for morphine reward in mouse models, and activation of IRS2-Akt signaling in dopaminergic ventral tegmental neurons is required for the behavioral and cellular actions of μ opioids, including morphine.[150] This same group has demonstrated that morphine action on reward requires the activation of transcription factors, including pCREB and DeltaFosB.[151] However, we still lack direct information regarding the substrates for these MOR-dependent transcription factors and kinase-signaling pathways shown to be required in behaviors such as analgesia and reward.

δ Opioid Receptors. Like MOR, DOR signaling research has been focused primarily on mechanisms of opioid analgesia. In addition, DOR research *in vivo* has been more commonly centered around DOR localization and anatomical characterization, with far fewer studies linking DOR signaling with behavioral effects. Ligand-dependent DOR signaling has been an active area of research, with reports suggesting that ligand-mediated trafficking governs agonist-induced analgesic tolerance to δ opioids.[33,152] These studies demonstrated that the DOR agonists SNC80 and ARM390 differ in their ability to cause receptor internalization and down-regulation of DOR-mediated Ca^{+2} channel modulation. DOR antinociception to thermal stimuli requires phospholipase C, and PKC activation also determines DOR-α_{2A} synergistic effects in the spinal cord.[153] DOR agonists have been studied increasingly for their potential antidepressant and anxiolytic effects in rodent behavioral models.[154] However, it is not yet known how or where DOR agonists mediate antidepressant-like behavioral responses.

κ Opioid Receptors. Contemporary studies linking κ opioid receptor signaling and behavior have been centered around the role of κ opioids in stress (Bruchas and Chavkin[155] and Knoll and Carlezon[156]). Stress-induced opioid peptide release has been reported for all of the major opioid systems, and this release causes stress-induced analgesia *via* action at opioid receptors. In a few crucial reports, it was demonstrated that KOR activation after stress cannot only increase analgesic responses but also can modulate numerous behaviors, including reward and depression.[73,157,158]

κ Opioid receptor activation of analgesic responses is thought to require Gβγ signal transduction,[159] whereas KOR-induced potentiation of reward and dysphoria are thought to be mediated by more complex events, including but not limited to MAPK activation.[76] Chartoff *et al.* showed that the KOR agonist salvA has a biphasic effect on reward. The acute administration of salvA decreased the rewarding impact of intracranial self-stimulation; however, repeated KOR activation caused a net decrease in the reward-potentiating effects of cocaine.[75] Both acute and repeated salvA administration increased phosphorylated ERK, but only acute salvA increased c-fos, and only repeated salvA increased cAMP response element-binding protein.[75] These findings provide more information about the effects of KOR activation on the reward-related effects of cocaine and will assist in the dissection of the relationship between activation of KOR- and ERK-signaling pathways. KOR-mediated p38 MAPK activity has been shown to be required for conditioned place aversion and swim-stress immobility responses, whereas cAMP response element signaling is critical for prodynorphin gene induction and depression-like behavioral responses.[70,160] It is thought that KOR modulation of dopamine, serotonin, and noradrenergic systems plays a key role in producing the negative behavioral affective responses.[155,156] Reports include KOR-mediated reductions on dopamine release, along with p38-dependent modulation of serotonergic output.[111,159] It was shown recently that KOR-induced p38α MAPK signaling in serotonergic circuitry is required for stress-induced social avoidance, depression-like behaviors, and reinstatement of drug-seeking behavior. This report went on to show that KOR-induced p38α MAPK causes a hyposerotonergic state through increased surface serotonin transporter expression.[78] The mechanisms and neural circuits in KOR-mediated dysphoria and analgesia are being studied by several groups; it is hoped those studies will greatly assist in the development of potential antidepressant ligands at KOR and novel analgesics that bypass KOR-mediated dysphoria.[161]

Opioids and Genetics

The pathogenesis of addiction involves a series of complex interactions among biological factors, including genetic vulnerability and drug-induced alterations in gene expression and proteins. Despite great efforts, the progress in finding causal variants underlying drug addiction has been somewhat slow. Numerous case–control studies have investigated single nucleotide polymorphisms in opioid receptor genes and their correlation with addiction to opioids. However, these studies often have produced conflicting results. The most extensively researched example is a polymorphism in OPRM1 (A118G, rs1799971), which results in the replacement of asparagine with aspartic acid at codon 40. Three studies found an association with the variant 118G and opioid dependency,[162–164] two studies observed an association with the common allele 118A and opioid dependency,[165,166] and nine studies found no overall association with opioid dependency.[167–175] This polymorphism highlights the conflicting results obtained from genetic studies of opioid receptor genes and drug dependence.

We have summarized the genetic variants that may contribute to vulnerability to develop opioid addiction (table 3). Genetic testing has important clinical applications in the prevention of many diseases, but in the field of addiction, much work remains to be done to understand the associations between these genetic variants and addiction-related phenotypes.

Within the last decade it was learned that the gene encoding the MOR undergoes extensive alternative splicing, resulting in the generation of multiple versions of this receptor protein. However, correlating these splice variants to pharmacologically defined receptors has proven difficult. The relative contribution to the pharmacologic effect of each splice variant could vary from drug to drug and is dependent on each splice variant's potency and efficacy at a particular site. It has been suggested that the difference in the activation efficacies of various μ opioids for the receptor splice variants may help explain the subtle but clear differences among various μ opioids in the clinic. In addition, understanding the functional significance of some of the truncated receptor splice variants will be beneficial because they have been reported to modulate the activity of opioid receptors in other systems (see Pasternak[177,191]).

Conclusions

In this review, we discuss a wide array of molecular, cellular, and *in vivo* studies in opioid receptor pharmacology. We highlight the traditional G protein, βγ signaling pathways, and regulatory mechanisms and discuss recent advances in the subfields of biochemistry, MAPK signal transduction, genetics, and behavior. It is important to note that we have not attempted to discuss all of the fine details regarding the properties of each receptor system.

The most common thread in the reports reviewed is that a large body of our understanding of opioid receptor molecular pharmacology continues to stem from *in vitro* studies. It is also increasingly clear that most molecular and cellular features of opioid receptors remain disjointed and unconnected to any physiological or behavioral effects, which needs to be the focus of future work in this field.

Many of the most important observations and discoveries surrounding opioid receptors have relied on *in vitro* approaches, and they continue to be the starting point for most laboratories in molecular pharmacology. However, given the diverse functionality of the opioid receptor family and the variety of signaling pathways and interacting proteins, our knowledge of how opioid receptors function in animal models and, more importantly, human populations or disease is limited.

Opioid receptor signaling has been a primary focus of researchers in this field since its discovery. The major reason

Table 3. The Association of Polymorphisms in Opioid Receptor Genes with Opioid Addiction and Functional Differences between These Variants

Receptor (Gene)	Polymorphism	Synonymous/ Nonsynonymous	Effects and Associations	Reference
μ Opioid (OPRM1)	A118G (rs1799971)	Nonsynonymous (Asn/Asp, Variant lacks the N glycosylation site in OPRM1 extracellular domain)	118G allele associated with reduced ACTH response to metyrapone	176
			118G associated with increased endorphin- binding affinity and activity	177
			118G allele reduces agonist-induced receptor-signaling efficacy	178
			118G associated with lower OPRM1 expession	179
			118G altered downstream signaling of ERK 1 and 2 and PKA compared with A118	180
			118G associated with opioid dependency	162–164
			118A associated with opioid dependency	165–166
			118A associated with opioid and alcohol dependency	
			No association with heroin dependency	167–175
	C17T (rs1799972)	Nonsynonymous (Ala/Val)	17T allele associated with cocaine dependence	181
			TT genotype associated with cocaine and heroin use in African American women	182
			No association with opiate addiction	162; 165; 172; 174
	A/G (rs510769)	Intron 1	G allele and heroin dependence*	168
	C/T (rs3778151)	Intron 1	T allele and heroin dependence*	
	C/T (rs6473797)	Intron 2	C allele and heroin dependence*	
	A/G (rs569356)	Promoter	G allele significantly higher reporter expression; altered transcription factor binding	183
δ Opioid (OPRD1)	G/T (rs1042114)	Nonsynonymous (Cys27Phe)	Cys27 compromised ATP-induced intracellular Ca^{2+} signaling	184
			Cys27 ↓ HERP	
			Cys27 reduced maturation efficiency and differential subcellular localization	185
	C/T rs2236861	Intron 1	T allele and heroin dependence*	—
	A/G rs3766951	Intron 1	G allele and heroin dependence*	168
	A/G rs2236857	Intron 1	G allele and heroin dependence*	
κ Opioid (OPRK1)	OPRK1 Haplotype	—	No association between OPRK1 haplotype and opioid dependency	186
				187
	G36T (rs1051660)	Synonymous	Association of the T allele with heroin dependency	188
ORL1 (OPRL1)	G501C	Nonsynonymous (Lys167Asn)	167Asn impairs ERK 1 and 2 activation	189
			LDL-induced biosynthesis of LOX-1 receptors is genotype dependent	
	A/G (rs6512305)	Intron	Marginal association with opioid dependence	190
	C206T (rs6090043)	5′UTR	Marginal association with opioid dependence	190

* No association after correcting for multiple testing.

ACTH = adrenocorticotrophic hormone; ATP = adenosine triphosphate; ERK 1 and 2 = extracellular signal-regulated kinases 1 and 2; LDL = low density lipoprotein; LOX-1 = low density lipoprotein-1; ORL1 = opioid receptor like-1; PKA = protein kinase A.

for this interest is that it has been widely accepted that a clear understanding of opioid receptor synthesis, cellular localization, trafficking, and pharmacology will lead to novel therapeutics that either directly act on opioid receptors or modulate opioid receptor signaling pathways. With the advent of conditional genetic approaches, receptor tags, antibodies, fluorescent tools, and optogenetic manipulation of neural circuitry, opioid receptor pharmacology is poised for some major breakthroughs in the next decade. It is hopeful that these new molecular and cellular discoveries will lead to better opioid analgesics in the clinic, with decreased risks of addiction and tolerance. In addition, it is likely that studies at the forefront of molecular and behavioral pharmacology will continue to reveal novel uses for opioids in the treatment of a variety of psychiatric and neurologic diseases.

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