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Kinase Cascades and Ligand-Directed Signaling at the Kappa Opioid Receptor

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Abstract

Background and Rationale—The dynorphin / kappa-opioid receptor (KOR) system has been implicated as a critical component of the stress response. Stress-induced activation of dynorphin-KOR is well-known to produce analgesia, and more recently it has been implicated as a mediator of stress-induced responses including anxiety, depression, and reinstatement of drug seeking.

Objective—Drugs selectively targeting specific KOR signaling pathways may prove potentially useful as therapeutic treatments for mood and addiction disorders.

Results—KOR is a member of the seven transmembrane spanning (7TM) G-protein coupled receptor (GPCR) superfamily. KOR activation of pertussis toxin-sensitive G proteins leads to Gαi/o inhibition of adenylyl cyclase production of cAMP and releases Gβγ, which modulates the conductances of Ca^{+2} and K^{+} channels. In addition, KOR agonists activate kinase cascades including G-protein coupled Receptor Kinases (GRK) and members of the mitogen-activated protein kinase (MAPK) family: ERK1/2, p38 and JNK. Recent pharmacological data suggests that GPCRs exist as dynamic, multi-conformational protein complexes that can be directed by specific ligands towards distinct signaling pathways. Ligand-induced conformations of KOR that evoke β–arrestin-dependent p38 MAPK activation result in aversion; whereas ligand-induced conformations that activate JNK without activating arrestin produce long-lasting inactivation of KOR signaling.

Conclusions—In this review, we discuss the current status of KOR signal transduction research and the data that support two novel hypotheses: 1) KOR selective partial agonists that do not efficiently activate p38 MAPK may be useful analgesics without producing the dysphoric or hallucinogenic effects of selective, highly efficacious KOR agonists and 2) KOR antagonists that do not activate JNK may be effective short-acting drugs that may promote stress-resilience.

Keywords

Kappa opioid receptor; dynorphin; kinase; MAPK; GPCR; ligand-directed signaling; p38; ERK 1/2; JNK; relapse; stress; opioid; arrestin; depression; addiction; therapeutics

Introduction

KORs are widely expressed throughout the central nervous system and are specifically activated by endogenous opioids derived from prodynorphin (Chavkin et al. 1982; Dhawan et al. 1996). Recent evidence has demonstrated that exposure to behavioral stressors causes corticotropin releasing factor (CRF)-induced dynorphin mediated KOR activation (Land et al. 2008). Activation of KOR following stress has been demonstrated to induce dysphoria and is involved in depression-like and anxiety-like behaviors, as well as increasing drug seeking behaviors (Carlezon et al. 1998; Land et al. 2008; Land et al. 2009; Beardsley et al. 2005; Bruchas et al. 2009a; McLaughlin et al. 2003; Redila & Chavkin 2009; for recent reviews see Knoll & Carlezon 2009 and Bruchas et al. 2009b). Dynorphin released during stress exposure produces immobility in the Porsolt forced swim test (Mague et al. 2003;

McLaughin et al. 2003), social defeat behaviors in the Miczek assay (McLaughlin et al. 2006), and potentiation of conditioned place preference (McLaughin et al. 2003, 2006). In addition, activation of dynorphin release during ethanol withdrawal may mediate the dysphoria-induced stimulation of ethanol self-administration (Walker & Koob 2008). Together these results suggest that dynorphin activation of KOR encodes the dysphoric component of stress and plays a critical role in pro-addictive behavioral responses (Bruchas et al. 2009b; Kreek & Koob 1998).

How activation of KOR mediates these complex behavioral responses remains an active area of investigation. In addition, preclinical results from non-human primate and rodent behavioral models suggest that KOR may represent a novel drug target for the treatment of stress-related diseases including depression, anxiety, and addiction. Over the long history of opioid research, the mechanisms by which KORs modulate neuronal activity have been widely investigated. Current studies are focused on understanding the nature of the dynorphin/KOR system in the regulation of intracellular signaling pathways that may mediate psycho-behavioral effects. A better understanding of how KORs translate extracellular stimuli into physiological and molecular responses will be critical as we move towards the potential targeting of KOR systems for the treatment of psychological disorders.

Recent pharmacological data and mathematical modeling strongly support the hypothesis that GPCRs exist as dynamic entities that can occupy multiple conformations and signaling states (Kenakin 2007; Violin & Lefkowitz 2007; Urban et al. 2007; Perez & Karnick 2005; Pineyro et al. 2009). Conformations of a GPCR depend on the ligand and receptor type, and accessory proteins within the signaling complex including associated Galpha proteins (Yan et al. 2008) control binding affinities and responses. Functional selectivity, the ability of a ligand to direct the receptor towards a conformation selectively evoking a specific stimulus response, is now well appreciated in drug discovery and GPCR research fields (Urban et al. 2007) These conserved biochemical properties open new therapeutic horizons for opioid receptors as drug targets and offer intriguing new opportunities for the treatment of pain and stress-related disorders.

This review will focus on kappa opioid receptor signal transduction mechanisms and ligand-directed signaling. We include a brief overview of the foundation of KOR signal transduction research and discuss studies that initially characterized KOR-mediated signal transduction. This review will also include a discussion of the receptor internalization and desensitization properties of KOR, including the variability of different ligands to promote various trafficking-dependent states of KOR. We will also highlight the modern advances in KOR-mediated mitogen- activated protein kinase (MAPK) cascade activation *in vitro* and *in vivo*, as recent reports suggest that KOR activation of this pathway may be important for a wide range of cellular and behavioral processes from cell proliferation to dysphoria. Finally, this review will describe recent efforts to characterize novel ligand-directed signaling properties at KOR, and the potential to take advantage of specific KOR conformations for therapeutic benefit.

Classical KOR Signal Transduction Pathways

Following activation by agonist, such as dynorphin, KOR activates the Gα subunit and Gβγ subunits dissociate and go on to interact with various intracellular effectors. The ability of opioids to couple to G-proteins was initially described by several groups (see Childers & Snyder 1978). It was first demonstrated that guanine nucleotides, including GTP, regulate agonist binding to opioid receptors in rodent brain membranes. This work was followed by work demonstrating that opioids stimulate GTPase activity (Barchfield and Medzihradsky 1984). The activation of opioid receptors was also shown to inhibit cyclic AMP production

(Minneman and Iverson 1976), and this inhibitory action was later found to also be mediated by action of the Gαi subunit (Taussig et al. 1993).

Following G-protein activation, release of the Gβγ from the Gα subunit goes on to directly activate $K_{ir}3$, the G-protein gated inwardly rectifying potassium channel, and channel deactivation occurs when the GTP bound Gα is hydrolyzed to GDP and Gβγ dissociates from the channel (Wickman and Clapman 1995; Sadja et al. 2003). In addition, KOR activation leads to a reduction in Ca^{+2} currents that are sensitive to L-type, N-type and P/Q-type channel blockade (Rusin et al. 1997). The KOR-mediated inhibition of Ca^{+2} conductance is thought to be mediated by direct binding of Gβγ to the channel that reduces voltage-activated channel opening (Figure 1). The evidence for KOR positively coupling to potassium channels and negatively effecting calcium channels has been demonstrated in several cell types ranging from neurons of the hippocampus to the dorsal root ganglia, suggesting that these ion channel effectors are highly conserved targets of KOR activation (see Grudt and Williams 1995).

Interestingly, there are also data to suggest that KOR activation causes intracellular calcium mobilization via the inositol-tris-phosphate (IP3) pathway (Spencer et al. 1997), which can lead to an enhanced hyperpolarization-activated current (I_h) in the rat nucleus raphe magnus (Pan 2003). These different effector systems are summarized in (Figure 1). Kappa-opioids have also been demonstrated to selectively increase intracellular calcium in type-1 astrocytes (Gurwell et al. 1996; Stiene-Martin et al. 1993). It is likely that these effects are also Gβγ mediated. However, it may also be possible that KOR can promiscuously couple to the $G_{q/11}$ G-protein network. The mobilization by KOR of intracellular calcium pools, particularly in glial cell systems, needs further investigation.

The downstream cellular consequences of Gα-mediated decreases in cAMP via $Gα_i$ are not completely understood, although it is likely that adenylyl cyclase inhibition by kappa opioids opposes the functional effects of cAMP increased by stimulatory G-protein coupled receptors in the brain. In addition, several reports have demonstrated that chronic KOR agonist treatment results in an upregulation of adenylyl cyclase, and this supersensitivity may contribute to heterologous desensitization, tolerance and physical dependence mechanisms (Avidor-Reiss et al. 1995; Raynor et al. 1994). The mechanisms of opioid receptor tolerance *in vivo* via upregulation of cAMP production or a loss of receptor coupling to the inhibition of cAMP are unresolved and controversial. Excitatory actions following KOR activation have been also been demonstrated. Dual coupling to inhibitory and excitatory G-proteins (Gi and Gs) has been reported for all of the opioid receptor systems (Crain and Shen 1990), and it is suggested by these studies that KOR under some circumstances can couple to stimulatory G-proteins.

KOR Desensitization Mechanisms

Agonist-induced receptor phosphorylation is thought to be one factor in opioid tolerance and is a well established process whereby GPCRs are desensitized and uncoupled from their G-protein signal transduction networks (Kohout et al. 2003; Liu-Chen 2004). Chronic KOR agonist treatment causes a reduction in KOR binding (Bhargava et al. 1989) and desensitization of U50,488 (a selective KOR agonist) stimulated [^{35}S]GTPγS binding (Li et al. 2002). Agonist-induced KOR phosphorylation was initially shown by immunoprecipitation of ^{32}P-labeled KOR in guinea pig hippocampal slices (Appleyard et al. 1997) and also in heterologous KOR expression systems (Li et al. 2002). It was demonstrated in *Xenopus* oocytes that the selective KOR agonist, U69,593 elicits GRK3-dependent KOR desensitization of receptor coupled Kir3 currents (Appleyard et al. 1999). In AtT-20 cells, McLaughlin et al. (2003) found that KOR-GFP mediated $K_{ir}3$ currents were

desensitized by U50,488 following 1hour of agonist treatment. In this same report a mutant variant form of KOR (serine 369 residue to alanine, called KSA), was not phosphorylated, desensitized, or internalized in response to agonist treatment. These results suggested that serine 369 phosphorylation is required for KOR receptor phosphorylation in the rat/mouse (rKOR) sequence (Figure 1). These data were corroborated by the generation of an affinity-purified polyclonal antisera (KOR-P) raised against a synthetic peptide corresponding to the amino acids 359–372 of the rKOR. This antiserum was later used in subsequent studies (McLaughlin et al. 2004, Land et al. 2008; Land et al. 2009) to show that KOR is phosphorylated *in vivo* following chronic U50,488 treatment or stress-induced dynorphin release. The carboxy-terminus of the human KOR is slightly different, and the critical phosphorylation site for desensitization was found to be serine-358 in hKOR (Li et al. 2002). Using GST-fusion peptide co-precipitation methods, the last 28 amino acid residues in the carboxy-terminus of hKOR have been demonstrated to be critical for β-arrestin binding (Cheng et al. 1998).

Interestingly, like mu-opioid receptor regulation (Blake et al. 1997a), differences in KOR trafficking have been noted for different agonists. For instance, human KOR stably expressed in heterologous cell systems is phosphorylated, desensitized, and internalized by U50,488 and dynorphin 1–17, but not by other agonists such as etorphine or levorphanol (Blake et al. 1997b; Li et al. 2003). Dynorphin A and Dynorphin B have been demonstrated to cause significantly more receptor internalization than alpha-neoendorphin at human kappa opioid receptors (Chen et al. 2007). In addition, Jordan et al. (2000) demonstrated that the N-terminal 7 residues of dynorphin A(1–17) were critical for KOR receptor internalization. A recent study examined three structurally distinct KOR ligands: Salvinorin A, TRK-820, and 3FLB. Although, all three compounds induced receptor internalization in a concentration-dependent manner; Salvinorin A, the high affinity KOR full agonist was 40-fold less potent in causing internalization and down-regulation of KOR than U50,488 (Wang et al. 2005).

Some groups have found conflicting results with regard to agonist-induced KOR-internalization. In Chinese hamster ovary cell (CHO) expression systems, the selective-KOR agonist U50,488, U69,593, etorphine, and bremazocine (0.1 uM for 90 min) did not initiate significant receptor desensitization (Li et al. 1999; Li et al. 2002; Liu-Chen 2004). Whereas, in AtT-20 cells and HEK cells U50,488 causes robust KOR-GFP receptor internalization, phosphorylation, and desensitization of rat KOR-GFP (McLaughlin et al. 2003; Bruchas et al. 2006; Clayton et al. 2009). The discrepancies between these results is likely due to cell-type specific factors possibly including levels of GRK or arrestin expression. Nevertheless, these groups all have demonstrated that KORs are phosphorylated, internalized, and desensitized by the endogenous ligand dynorphin and that the magnitude of this response can vary depending on the ligand, drug concentration, and duration of treatment.

KOR and Mitogen-Activated Protein Kinase (MAPK) Cascade Activation

As outlined above, sustained agonist treatment can cause GRK3 phosphorylation in the carboxyl-terminal domain of the KOR initiating arrestin-dependent receptor desensitization and internalization (Figure 1). In the last decade or so, new evidence in GPCR research has demonstrated that this phosphorylated arrestin-bound GPCR is not inactivated, but can also recruit MAPK signaling cassettes (Lefkowitz and Shenoy 2005). The MAPK pathway regulates diverse cellular responses including: cell proliferation, differentiation, embryogenesis, apoptosis, transcription factor regulation, ion channel phosphorylation, and protein-protein interactions (Raman et al. 2007). The MAPK family is composed of at least 12 different genes. The best characterized forms include extracellular signal-regulated kinases 1 and 2 (ERK 1/2), c-Jun N-terminal Kinase (JNK 1–3) and p38 (α,β,γ,δ) stress

kinase. Each MAPK cassette is typically composed of three critical upstream protein kinases: Map Kinase Kinase Kinase (MKKK), Map Kinase Kinase (MKK), and Map Kinase (MEK) that are sequentially activated to ultimately increase MAPK phosphorylation and activation. The MAPKs are unique in they have the capability to respond to a broad array of stimuli and can translate that into a variety of diverse intracellular signals via protein-protein interactions and phosphorylation cascades (Karandikar and Cobb 1999). This process is tightly regulated by the kinetics of activation, phosphatase activity (inactivation), and cellular distribution of the upstream stimulus and downstream target substrates (Raman et al. 2007; Karandikar and Cobb 1999).

Originally, activation of ERK MAPKs was found to be directly linked to receptor tyrosine kinase (RTK) transactivation, such as epidermal growth factor receptor (EGF), or brain derived neurotrophic factor (BDNF) receptors (also called TrkB receptors) (Pierce and Lefkowitz 2001). However, reports of GPCRs directly activating MAPKs began to surface, and it is now well accepted that most, if not all GPCRs, can directly activate one or more of the MAPK pathways. In some cases the events that cause RTK and GPCR-mediated MAPK activation converge via Ras activation and tyrosine phosphorylation of the adaptor protein SHC. Some GPCRs cause “transactivation” of RTKs by direct or indirect phosphorylation leading to initiation of the MAPK pathway (Pierce et al. 2001). Furthermore, MAPK activation by GPCRs has been demonstrated in a number of contexts to require Src, c-Raf, and phospholipase C (PLC) (Belcheva et al. 2005; Chan et al. 2005; Della Rocca et al. 1999) further supporting the idea that GPCRs and RTK networks share some common kinases and substrates.

The best characterized opioid-induced MAPK network to date is ERK 1/2. Initial studies by Coscia and colleagues have been integral in developing our understanding of the relationship between opioid receptor stimulation and ERK 1/2 signal transduction *in vitro*. Acute mu and kappa opioid stimulation was shown to cause ERK 1/2 phosphorylation in astrocyte cultures; however, the kinetics of ERK 1/2 activation by these two receptor systems differs. Delta-opioid receptors also activate ERK 1/2 via Gβγ and Ras (Belcheva et al. 1998). In immortalized astrocytes, Mu-opioid receptor mediated ERK 1/2 phosphorylation is mediated by PKCε, whereas Kappa-opioid mediated ERK 1/2 phosphorylation requires PI3-Kinase, PKCζ, and calcium (Belcheva et al. 2005). These results suggest that opioid receptors couple to ERK 1/2 via different signaling pathways and strengthens the hypothesis that ERK 1/2, like other MAPK signaling modules, is tightly regulated and depends on the stimulus and environmental context. Interestingly, kappa-opioid receptor activation of ERK1/2 was found to direct embryonic stem cell fate decisions (Kim et al. 2006) and to stimulate proliferation of astrocytes (McLennan et al. 2008). In a recent report it was determined that KOR-induced ERK 1/2 phosphorylation occurs in a multi-phase manner with the early phase happening between 5–15 minutes and late phase after 2 hours of agonist treatment. Like other GPCRs (Gesty-Palmer et al. 2006), the bi-phasic ERK 1/2 activation profile for KOR has an arrestin-independent early phase, and an arrestin-dependent late phase (McLennan et al. 2008). These authors found that Gβγ was required for the early phase of ERK 1/2 and arrestin3 was required for the late phase of ERK activation.

In vivo, few studies have investigated KOR-mediated ERK 1/2 activation. Recently, we showed that a repeated swim-stress, a paradigm sufficient to induce dynorphin release (McLaughlin et al. 2003), causes GRK3-independent ERK 1/2 and pCREB phosphorylation in the nucleus accumbens of mice (Bruchas et al. 2008). These effects were dependent on kappa-opioid receptors; however, the behavioral consequences of stress-induced KOR-mediated ERK 1/2 activation are unresolved. Potential effects of ERK 1/2 activation include increasing AMPA receptor insertion into the plasma membrane, promoting dendritic spine

growth, and synapse stabilization in CA1 pyramidal cells (Sweatt 2004; Thomas and Huganir 2004). In addition, ERK 1/2 is well known as a major upstream kinase involved in CREB activation, a highly conserved transcription factor that controls gene expression of several proteins. pCREB has been shown to tightly regulate dynorphin gene expression (Kreibich and Blendy 2004; Carlezon et al. 2005).

p38 MAPK

The p38 MAPK pathway has been demonstrated to play a key role in environmental stress and inflammation, including the activation of cytokine production (Tibbles and Woodgutt 1999; Lopez-Ilasaca et al. 1997). In some cell systems activation of p38 MAPK is involved in proliferative and chemotaxic responses (Hunton et al. 2005). In astrocytes, p38 MAPK activity is critical for several cellular responses including: production of interleukin1-β, interleukin-6, tumor necrosis factor-α, and inducible nitric oxide synthase (Ashwell, 2006). In the pain response p38 MAPK has been demonstrated to have a critical role in modulating chronic pain states (Watkins et al. 2001), and KOR-induced p38 MAPK activation in astrocytes has been implicated in cellular reorganization following nerve injury (Xu et al. 2007). Blockade of KOR-induced p38 attenuates nerve injury induced hyperalgesia and mechanical allodynia (Xu et al. 2007).

KOR-induced p38 MAPK phosphorylation has been demonstrated in several systems including heterologous expression systems, striatal neurons and astrocytes, spinal cord astrocytes, and *in vivo* (Bruchas et al. 2006; Xu et al. 2007; Bruchas et al. 2007a). KOR-induced p38 MAPK activation requires receptor phosphorylation at serine 369 by GRK3, and subsequent arrestin3 recruitment in heterologous expression systems, primary cultures, and *in vivo*. The data showing that KOR-induced p38 requires GRK3/arrestin (Bruchas et al. 2006) contrasts with KOR-mediated ERK 1/2 phosphorylation which has both arrestin-independent (Bruchas et al. 2006; Bruchas et al. 2008) and dependent phases (McLennen et al. 2008).

Kappa-opioids induce p38 MAPK phosphorylation following behavioral stress *in vivo.* In addition, the selective p38 inhibitor SB203580 was shown to block kappa-opioid-induced conditioned place aversion (CPA) and stress-induced immobility (Bruchas et al. 2007a). The mechanisms whereby p38 mediates these KOR-dependent effects are unknown, and possible substrates of KOR-activated p38 MAPK are numerous. ERK and p38 MAPK have opposing effects on AMPA-R surface expression (Rumbaugh et al. 2006), suggesting that p38 regulates synaptic plasticity. Additionally, p38 MAPK has been shown to phosphorylate sodium channels (specifically Na_V 1.8) to increase current density in these neurons (Hudmon et al. 2008). Recently, the potassium channel $K(ir)_{3.1}$ was demonstrated to become tyrosine phosphorylated via a KOR-dependent p38 MAPK induced Src activation (Clayton et al. 2009). In this report, KOR activation caused phosphorylation of Y12-K(ir)3.1 and channel inhibition through a GRK3-, p38 MAPK- and Src-dependent mechanism. It was proposed that reduced inward potassium current following KOR activation may increase neuronal excitability and may contribute to KOR-mediated behavioral responses (Clayton et al. 2009). Hence, p38 MAPK may cause a synaptic depression in some regions while increasing excitation in others. Other potential substrates of KOR-induced p38 include the serotonin transporter (SERT), since it has been reported that p38 is involved in increasing reuptake of serotonin via PP2A (phosphatase) and p38-dependent SERT modification (Zhu et al. 2005; Prasad et al. 2005; Samuvel et al. 2005; Steiner et al. 2008), and KOR-activated p38 MAPK in the dorsal raphe nucleus, a serotonergic nucleus, is required for KOR-mediated dysphoric behaviors (Land et al. 2009). The regulation of transcription factors such as zif268 by p38 MAPK activation has also been described and may be involved in the behavioral effects induced by p38 MAPK activity (Bruchas et al. 2007a). Because p38 MAPK, is nearly ubiquitously expressed in the mammalian brain, it will be interesting to see

how the application of genetic tools such as viral techniques, and conditional knockout strategies better define the role of KOR-induced p38 in behavioral responses.

c-Jun N-terminal Kinase (JNK)

Similar to p38 MAPK, the JNK pathway has been linked to environmental stress and inflammatory signals, including cytokine activation, and more recently this pathway has been implicated in mouse models of pain and mouse behavioral responses (Zhuang et al. 2006; Minden and Karin 1997). JNK activation causes phosphorylation of specific sites on the amino-terminal trans-activation domain of c-Jun a critical transcription factor in the AP-1 complex. JNK is well known to be activated by cytokines such as Tumor Necrosis Factor, and interleukin-1β (Baker and Reddy 1996; Minden and Karin 1997). Following cytokine receptor activation, the JNKs are activated by two Ha-Ras related GTP binding proteins in the Rho family. These small GTP binding proteins have been examined for their role in maintenance of the actin cytoskeleton (Nobes and Hall 1994).

JNK activation by GPCRs has not been examined as extensively as for the other GPCR-MAPK signaling modules; however, there is still strong evidence that GPCRs cause JNK activation. As with ERK 1/2 and p38 MAPKs, arrestin2 and arrestin3 have been reported to scaffold JNK. There is some evidence that arrestin3 has a unique specificity for JNK3 (McDonald et al. 2000); however, other reports have suggested otherwise (Song et al. 2006) and allude to the possibility that all arrestin isoforms have the capacity to scaffold JNK. Furthermore, the cellular consequences of GPCR-arrestin mediated JNK scaffolding has only recently begun to be explored.

There are only a few reports describing opioid receptor dependent activation of JNK. The delta-opioid receptor causes protein kinase B (Akt) dependent JNK phosphorylation through a PI3-kinase mechanism (Shahabi et al. 2005) in some systems, and JNK activity is PI3-kinase independent in others (Kam et al. 2004a). PI3-kinase is required for Mu-opioid dependent JNK activation. In contrast, U50,488-induced (KOR) JNK activation has been shown to be independent of PI3-kinase (Kam et al. 2004a). The substrates and *in vivo* effects of opioid induced JNK activation is under active investigation by several groups.

The agonists U50,488, U69,593, and Dynorphin B all activate KOR to cause JNK phosphorylation (Kam et al. 2004a; Kam et al. 2004b; Bruchas et al. 2007b). U50,488 and U69,593-induced JNK phosphorylation are mediated through pertussis toxin sensitive $G\alpha_i$-activation (Kam et al. 2004b; Bruchas et al. 2007b). Furthermore, KOR-induced Src stimulation and GTPase Rac-dependent activation of focal adhesion kinases were demonstrated to be critical for KOR-mediated JNK activation in immune cell types (Kam et al. 2007). The identification of the isoform of JNK that KOR activates remains unresolved, as available antibody reagents lack specificity; however, recent data using JNK knockout mice suggest that the JNK1 isoform mediates KOR effects (Melief et al. unpublished).

In another report, KOR agonist-induced JNK phosphorylation was insensitive to norBNI, the selective KOR antagonist; instead it was reported that norBNI acted as a “collateral” agonist to cause JNK phosphorylation via KOR, in a pertussis toxin insensitive manner (Bruchas et al. 2007b). In the same report, this “collateral agonist” JNK effect was replicated with other structurally unrelated long-acting KOR antagonists JDTic and GNTI (Negus et al. 2002; Carroll et al. 2004). The persistent actions of norBNI and related ligands were found to require JNK activation, since blockade of JNK signaling reversed the long-lasting effects of norBNI on other KOR-agonist mediated analgesia. In contrast to other studies in immune cells, KOR-dependent JNK activation was not sensitive to the small GTPase Raf inhibitor, Src inhibitor treatment, or pertussis toxin, suggesting that norBNI-induced JNK may be

initiated via a novel signaling pathway that is different than U50,488-induced JNK phosphorylation (Figure 1).

We hypothesize that the long-acting KOR antagonists inactivate the KOR signaling complex *in vivo* by promoting a high affinity association between KOR and a hypothetical JNK-substrate that sterically blocks G-protein association (Bruchas et al. 2007). This putative 'jammer' (JMR, JNK Modulated Regulator) protein becomes activated by KOR-activated JNK to disrupt signaling. An alternative possibility is that a hypothetical 'linker' (LKR) scaffolding protein exists that is required for physical association of KOR and its effector; if the putative LNK is inactivated by JNK efficient coupling between KOR and its effectors would be disrupted. Future studies are underway to resolve these mechanisms and hopefully identify these hypothetical JNK substrates. However, these studies will also need to explain why JNK activation by conventional KOR agonists (e.g. U50,488) does not also inactivate KOR signaling. We presume that this will be found to be an example of ligand-directed signaling, but this remains speculation at this point. Ultimately, this distinction between different KOR ligands may be another, very interesting example of functional selectivity.

Ligand-directed signaling at KOR

Ligand-selective activation of MAPK has been proposed for several GPCR classes in a number of cell and tissue types (Marinissen and Gutkind 2001). As seven transmembrane spanning proteins, the GPCR is an ideal candidate for an allosteric protein that can exist in numerous conformations (see Figure 1). The idea that the receptor-signaling complex is a linear process whereby all possible receptor-mediated cytosolic behaviors is now becoming modified and adapted. A new concept in receptor theory, termed "collateral efficacy" (Kenakin 2007) "functional selectivity" (Pineyro 2009), or "biased agonism" is gaining in both theoretical and experimental arenas (Urban et al. 2007; Violin and Lefkowitz 2007).

The early work of Clark (1926), Ariens (1954), and Stephenson (1956) defined efficacy as the property of a drug to produce a physiological or pharmacological response. Furthermore, they defined a ligand having affinity for a receptor (i.e. bind tightly), but may not initiate a response (termed competitive antagonist). In this foundational work, and for decades thereafter, pharmacologists used assays such as tissue bath preparations and electrophysiological recordings, where there was only one observable response variable following a particular drug-receptor interaction. However, in the last several years, new pharmacological approaches and enhanced bioassay sensitivity have enabled pharmacologists to uncover a whole host of novel ways to look at efficacy (Kenakin 2007), and we now think of receptors as having a full myriad of conformations and effectors (Figure 1). One can imagine a situation or class of ligands that do one, but not all of the following: 1) Occupancy and receptor internalization (no receptor activation) 2) Occupancy and G-protein activation, phosphorylation and desensitization 3) Occupancy and G-protein activation 4) Occupancy and G-protein independent MAPK activation (Kenakin et al. 2007). These and other potential receptor conformations can direct the GPCR towards one particular output signaling response. What is particularly intriguing is that these conformations may never actually be stabilized by the endogenous ligand, but as pharmacologists and chemists we can take full advantage of their existence for therapeutic goals. This has been clear for synthetic antagonists and partial agonists, but newer forms of collateral agonists may be developed in the future to exploit these possible functional differences.

As evident from the above overview, KOR couples to many different signaling cascades through a variety of ligand types, so there is ample evidence to corroborate the recent findings evident from other GPCRs. KORs exist as dynamic entities and different ligands

tune towards specific signaling states. For example, we propose that receptor-selective KOR partial agonists that do not activate p38 MAPK because they do not efficiently recruit β-arrestin, would not cause dysphoria yet might retain sufficient analgesic activity for the treatment of pain-related disorders. This hypothetical ligand would be unable to cause p38 activation, a prerequisite for KOR-mediated place aversion (Bruchas et al. 2007a; Land et al. 2009), and thus it would provide analgesic relief without the side effect of dysphoria. Presumably, these analgesics would not activate the dopamine-reward system in the way that mu agonists do because the cellular distributions of MOR and KOR in brain are non-overlapping.

The recent discovery of the naturally occurring, high affinity psychoactive KOR agonist Salvinorin A (Roth et al 2002; Chavkin et al. 2004) holds exciting promise for medicinal chemists and pharmacologists because this compound can be structurally modified to act as a KOR agonist to selectively target specific KOR signaling pathways. Some groups have begun to modify the salvinorin A compound to make high affinity, longer acting (pharmacokinetically stable) MOR and KOR agonists (Lozama and Prisinzano 2009;Yan et al. 2009). For example, MOM-Sal B (2-methoxymethyl-salvinorin B) has selectivity for KOR, and a significantly longer duration of action *in vivo* that Salvinorin A, which has been reported to last for anywhere between 1560 minutes *in vivo* (Wang et al. 2008). Interestingly, this modified salvinorin-type compound has been found to have stimulus-specific signal transduction effects at KOR. In contrast to other “classical” full agonists at KOR (i.e. U69,593), MOM-SALB stimulation of KOR causes only “early phase” ERK 1/2 phosphorylation. In addition, while MOM-SALB does initiate ERK 1/2 phosphorylation via KOR differently, it still causes astrocyte proliferation like U69,593-induced KOR activation (McLennan et al. 2008). Further study is warranted to determine if MOM-SALB holds more information about unique kappa-opioid receptor conformations.

Ligands like norBNI, GNTI, and JDTic have long durations of action (Horan et al. 1992; Bruchas et al. 2007b; Negus et al. 2002; Carroll et al. 2004), yet do not cause phosphorylation-mediated receptor internalization (McLaughlin et al. 2004), ERK 1/2 activation (Bruchas et al. 2007b), or stimulation of GTPγS binding. The “collateral agonist” effect of this class of KOR antagonists differs greatly from buprenorphine or naloxone, two other well known “short-acting” opioid receptor ligands that act as competitive antagonists at KOR. The induction of a ligand-dependent conformation in KOR that can activate JNK phosphorylation resulting in long term receptor inactivation posits the idea that this effect may represent a unique receptor state that is perhaps shared with other opioid receptors or GPCR types in general. The receptor conformation-selective recruitment of a JMR protein may represent a novel way to target GPCRs for inactivation in specific ways. This is very plausible given that some muscarinic receptor antagonists display similar pseudo-noncompetitive characteristics (Kukkonen et al. 1998), and rispiradone, a 5HT7-selecitive antagonist, has strikingly similar properties believed to depend on cytosolic-receptor interactions (Smith et al. 2006; Urban et al. 2007). This new defined class of ligands warrants further exploration, but nevertheless, understanding why some ligands have long-acting effects *in vivo*, will help for the potential development of KOR antagonists for therapeutic treatment of stress-related disorders including depression and addiction.

Future Investigations

There is growing body of work demonstrating that stress causes dynorphin release (via extra hypothalamic CRF system activation) and subsequent KOR activation to induce behavioral responses in rodents that include model systems for anxiety, depression, and addiction-like behaviors. Little is known about the functioning of the dynorphin KOR systems in human populations experiencing anxiety, depression, or addiction. Kreek and colleagues have

determined that chronic stress and binge cocaine exposure can increase prodynorphin gene expression in rodents and humans (see Kreek et al. 2009). In addition, based on the predictive value of rodent models, the conservation of the dynorphin gene in primate evolution (Rockman et al. 2005), and the robust effects that KOR agonists have on human psychology (Pfeiffer et al. 1986), it is likely that KOR does play a critical role in how we respond to stressful stimuli. The culmination GPCR receptor signal transduction research, and more recently KOR second messenger studies, has helped to gain deeper insight into how KOR through various intracellular cascades to modify a wide array of behavioral responses.

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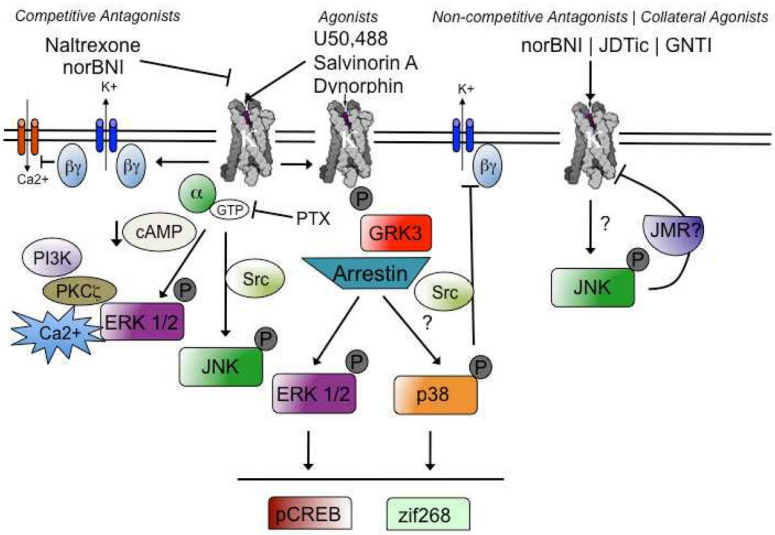

Figure 1. KOR-mediated Signal Transduction

Cartoon depicting the current status of Kappa-opioid receptor (κ) signal transduction pathways. Receptor activation by a variety of KOR-selective ligands, can result in activation of several kinase cascades. Arrows refer to activation steps, T lines refer to blockers or inhibition of function. Abbreviations are as follows: α = G-protein alphai subunit, arrestin = phosphorylation dependent GPCR scaffold, βγ = G-protein beta-gamma subunit, cAMP = cyclic adenosine monophosphate, ERK 1/2 = extra-cellular signal regulated kinase, GRK3 = G-protein coupled receptor kinase3, JAM = JNK Activity Modifier, JNK = c-Jun N-terminal Kinase, p38 = p38 MAPK, P = phosphorylation, pCREB = phospho-cyclic AMP response element binding protein, PI3K = phosphoinositol 3-kinase, PKCζ = protein kinase C zeta, PTX = pertussis toxin, Src = short for sarcoma, member of the src family tyrosine kinases, zif268 = transcription factor, also called Egr-1.

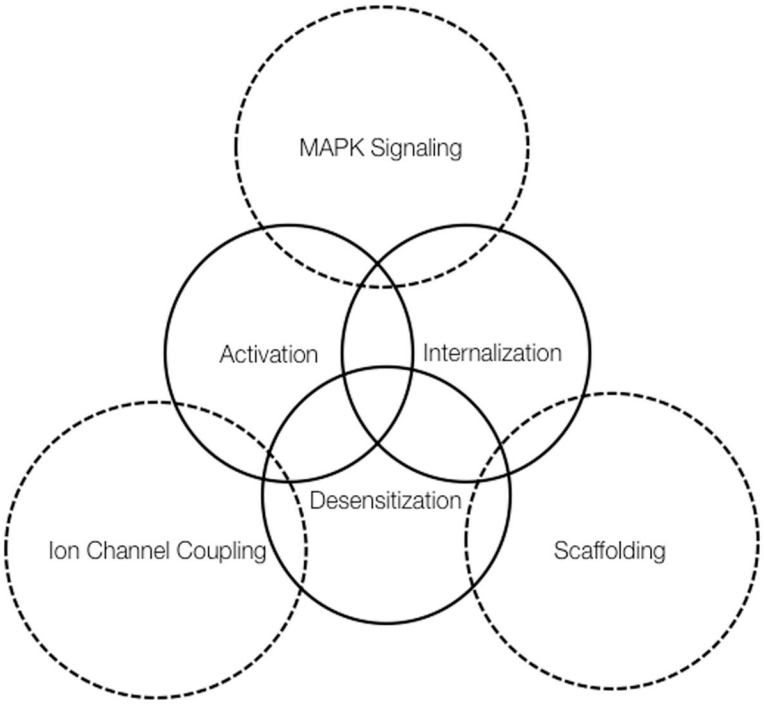

Figure 2. Conceptualization of Collateral Efficacy at Opioid Receptors

Simplified Venn diagram depicting the potential divergence and overlap of various kappa opioid receptor ligand-directed outputs. Depending on the ligand type or bioassay used for detection, opioid receptor ligand interactions can cause the receptor to engage a number of different outputs. For example, the activation of the receptor G-protein might lead to coupling to an ion channel, while also initiating the desensitization machinery. Other ligands may initiate MAPK activation if the ligand causes sufficient receptor phosphorylation, and arrestin recruitment. Finally, some ligands may act on the receptor, not cause activation, but might recruit scaffolds which block the receptor's ability to couple to G-proteins.