Molecular Regulation of Opioid Receptor Signaling

Selena Schreiber Schattauer

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

2012

Reading Committee:

Charles Chavkin, Chair

Neil M. Nathanson

Paul E.M. Phillips

Program Authorized to Offer Degree:

Pharmacology

University of Washington

Abstract

Molecular Regulation of Opioid Receptor Signaling

Selena Schreiber Schattauer

Chair of the Supervisory Committee:

Professor Charles Chavkin

Pharmacology

The varied behavioral effects of kappa opioid receptors (KOR) are mediated through different signaling cascades. KOR activation of G protein-dependent signaling results in analgesia, whereas the dysphoric effects are mediated by a different pathway involving G protein-coupled receptor kinase (GRK) and arrestin. Therefore, a partial KOR agonist that does not efficiently activate arrestin-dependent signaling may produce analgesia without dysphoria. No selective KOR partial agonists are currently available, and preclinical assessment is complicated by sequence differences between rodent (r) and human (h) KOR. KOR antagonists are also of therapeutic interest for their potential anxiolytic and antidepressant effects, but many KOR antagonists have long durations of action resulting from selective activation of cJun kinase (JNK).

In this thesis, I compared the signaling events initiated by agonist stimulation of hKOR and rKOR. Although a partial agonist at both hKOR and rKOR, pentazocine was more potent at activating p38 MAPK in hKOR than rKOR expressed in HEK293 cells. In contrast, pentazocine was equally potent for arrestin-independent activation of ERK1/2 in hKOR and rKOR. There were no potency differences

between hKOR and rKOR in U50,488 activation of ERK1/2 or p38 MAPK. hKOR lacks the Ser369 phosphorylation site in rKOR required for GRK/arrestin-dependent p38 activation, but mutation of the Ser358 to asparagine in hKOR blocked p38 activation without affecting the arrestin-independent activation of ERK1/2. Although pentazocine is dysphoric in humans, an analgesic dose of pentazocine failed to produce KOR-dependent aversion in C57Bl/6 mice, consistent with its lower potency of p38 activation of rKOR. This study shows that hKOR activates p38 MAPK through a phosphorylation and arrestin-dependent mechanism; however ligand directed signaling differences have important implications for preclinical screening of partial KOR agonists.

This thesis also includes contributions to other studies on opioid receptor signaling. This includes: characterizing an antibody selective for MOR phosphorylated at Thr370 and Ser357, which are implicated in MOR desensitization; radioligand binding studies demonstrating that norBNI does not change the drug binding site of KOR in vivo; and development of lentiviral vectors expressing KOR which were used to show that KOR expression in the dorsal raphe is sufficient for KOR-mediated analgesia and aversion.

Table of Contents

List of Figures

List of Tables

Acknowledgements

First I would like the thank Dr. Charles Chavkin for his mentorship and support. I would also like to thank my supervisory committee for their advice and especially my reading committee. I would also like to thank my fellow lab members, past and present, and especially those who have been co-authors with me. Additionally, I'd like to thank my parents and my siblings who have supported me throughout my life. Finally I'd like to thank my husband, Chris Schattauer, whose love and support I've benefited from, in addition to his ability to resuscitate dying computers and salvage files when needed.

Introduction to opioid receptors and regulation of G protein coupled receptor signaling

The most common primary complaint at medical appointments is pain, with pain medication sales over $8.0 billion annually in the US. Opioid analgesics are the benchmark against which other drugs are measured for the treatment of moderate to severe pain (Kivell 2010). Cultivation of opium poppy dates to at least 3000 BC in Mesopotamia, and records of medical use date to at least 1500 BC in Egypt. By the 13th century AD opium had spread throughout Europe and Asia, with records of abuse as early as the 1500s (Brownstein 1993). Morphine was isolated from opium in 1804; since then one of the aspirations of medicinal chemistry has been development of a nonaddictive opioid. Heroin, now DEA schedule I, was synthesized in the late 1800s as a result of these efforts and initially marketed as a nonaddictive alternative to morphine. With the realization that there was in fact more than one opioid receptor (Martin 1976), focus shifted onto the effects of the different opioid receptors. Kappa opioid receptor agonists have been of interest as a potential nonaddictive opioid analgesics since their discovery, but although they lack the addictive potential of mu opioid receptor agonists such as morphine, their clinical potential has been limited by dysphoric and hallucinatory effects (Millan 1990, Kivell 2010).

Introduction to opioid receptors

The opioid receptors (μ, κ, and δ) are members of the ɣ-subfamily of class A (rhodopsin-like) G protein coupled receptor (GPCR) (Fredriksson 2003). The opioid receptors share approximately 60% amino acid sequence homology overall, with the greatest similarity in the transmembrane domains (~75%) and the greatest divergence in the extracellular domains (~35-40% sequence homology) (Satoh 1995). All three opioid receptors are primarily Gi/Go coupled, decreasing cell excitability. Activation of all three types of opioid receptors produces analgesia but also produces additional and sometimes opposing effects, including changes in mood, anxiety state, and stress response.

Their endogenous ligands are opioid peptides processed from the precursors prepro-opiomelanocortin (β-endorphin and met-enkephalin), preproenkephalin (leu-enkephalin and met-enkephalin), and preprodynorphin (the dynorphins and leu-enkephalin) (Akil 1984). β-endorphin has higher affinity for the μ opioid receptor (MOR) than the δ opioid receptor (DOR), and the lowest affinity for the κ opioid receptor (KOR). The enkephalins have higher affinity for DOR than MOR, and little affinity for KOR. The dynorphins (dynorphin A, dynorphin B, big dynorphin, α-neo-endorphin, and β-neo-endorphin) and have the highest affinity for KOR, although they also have lower affinity at DOR, MOR, and the NMDA receptor (Chavkin 1982).

The opioid receptors are expressed throughout the central and peripheral nervous system but differ in their distribution within these regions. KOR is found in the cortex, hippocampus, amygdala, thalamus, midbrain, brainstem, and basal ganglia (Mansour 1987, 1996). KOR is expressed in the peripheral nervous system in nerve terminals of sensory neurons and in cell bodies of the dorsal root ganglia and trigeminal ganglia (Peckys 1999, Rau 2005).

In addition to analgesia, MOR produces euphoria, constipation, and respiratory depression. While most clinically used opiates act primarily through MOR, the high potential for abuse of MOR agonists and side effects of constipation and respiratory depression limit safety and compliance (Bohn 2006, Vanderah 2010). In addition to producing analgesia, DOR selective agonists are anxiolytic (Baamonde 1992, Jutkiewicz 2006) and proconvulsive (Tortella 1983, Jutkiewics 2006). KOR-selective agonists produce also produce analgesia, but do not produce constipation, respiratory depression, or euphoria at analgesic doses and are thought to have a lower abuse potential (Porreca 1983, 1984, Shippenberg 1986, Unterwald 1987, Field 1999). Clinical use of KOR agonists as analgesics has been limited however by their propensity to cause sedation, dysphoria, anxiety, and perceptual disturbances (Pfeiffer 1986, Rimoy 1994, Walsh 2001). The anxiogenic and aversive effects of KOR agonists have also been observed in a variety of rodent models, including the elevated plus maze, conditioned place aversion, intracranial self-stimulation reward threshold, and the forced-swim test (Mucha 1985, Iwamoto

1985, Mague 2003, Todtenkopf 2004, Carlezon 2006, Knoll 2007). These models have provided useful tools to probe the neurocircuitry and molecular signaling underlying these effects. If the negative effects of KOR activation could be dissociated from the analgesic effects, KOR agonists would have significant clinical potential.

Classic G-protein coupled receptor signaling

Over 25% of drugs target rhodopsin family GPCRs (Overington 2006). Classically, activation of GPCRs are thought to activate downstream signaling cascades via activation of heterotrimeric G proteins. Agonist binding to the receptor promotes a conformational shift from an "off" state to an "on" state, allowing the receptor to act as a guanine nucleotide exchange factor (GEF), catalyzing exchange of GTP for GDP bound to the Gα subunit (Johnston 2007). This results in dissociation of the Gα subunit from the Gβγ subunits, allowing them to modulate downstream effectors. The Gβγ subunits form a high affinity dimer and only dissociate under denaturing conditions.

To date, 21 Gα, 6 Gβ, and 12 Gγ subunits have been identified. The Gα subunits fall into four classes: the Gαi family (including Gαi, Gαo, Gαt, Gαgust, and Gαz) which regulate potassium and calcium channels and decrease cAMP via inhibition of adenylyl cyclase and activation of phosphodiesterase; the Gαs family (including Gαs and Gαolf) which increase cAMP via activation of adenylyl cyclase; the Gαq family (including Gαq, Gα11, and Gα14-16) which increase intracellular calcium via activation of phospholipase C (PLC); and the Gα12/13 family, which activate members of the Rho family of small GTPases (Oldham 2008). The functional differences between the different Gβ and Gγ subunits are not well characterized but have been implicated in regulating downstream event including G protein-coupled receptor kinase (GRK) recruitment (Daaka 1997).

The heterotrimeric G proteins are post-translationally modified with fatty acid chains to promote membrane association. With the exception of Gαt, the Gα subunits are palmitoylated. Gαi subunits are also myristylated, which is irreversible unlike palmitoylation. Gα subunits are comprised of a GTPase

domain, which also serves as the interface for interaction with Gβγ, GPCRs, and effector proteins, and a helical domain which acts a cap over the nucleotide binding pocket. The Gβ subunit is a β-sheet propeller structure with 7 WD40 sequences. The N termini of the Gβ and Gγ subunits forms a coiled-coil, while the C terminus of the Gγ subunit binds part of the Gβ β-propeller. The Gγ subunit is prenylated, promoting membrane association of the Gβγ dimer. GPCR activation catalyzes release of GDP from the nucleotide binding pocket of the Gα subunit, which is rapidly replaced by GTP due to the higher amount of GTP within cells relative to GDP. GTP binding leads to a conformational change in the Gα subunit, leading to dissociation from the GPCR and Gβγ subunits and exposing portions of Gα subunit which associate with and regulate downstream effectors. There is little evidence of a major conformational change in Gβγ, and activation of signaling is thought to be a result of exposure of effector binding domain which were occluded by Gα binding. (Johnston 2007, Oldham 2008.)

The freed Gβγ dimers also recruit GPCR kinases 2 and 3 (GRK2/3) to the cell membrane. GRK2/3 then phosphorylates activated receptors, usually at multiple sites within the carboxyl tail of the receptor, allowing recruitment of arrestin and initiating receptor desensitization. The general structure of the GRK family of proteins consists of an amino terminal 25 amino acid region specific to the GRK family, a regulator of G protein (RGS) homology domain, an AGC kinase family serine/threonine kinase domain, and a domain which regulates the membrane association and varies between different GRK proteins. In GRK2/3 this carboxyl-terminus includes a 125 amino acid sequence which interacts with the freed Gβγ subunits which are localized to the plasma membrane near activated receptors. Unlike most AGC kinase proteins, the GRK kinase domain is opened and disordered; it is thought that this domain is rearranged to form an active kinase domain upon binding to active GPCRs. This rearrangement partially underlies the specificity of GRK phosphorylation for active receptors. (Gurevich 2012, Hubbell 2003.)

The non-visual arrestins, arrestin-2 and arrestin-3, are ubiquitously expressed members of the arrestin protein family and function as critical regulators of GPCRs. Canonically, arrestins bind to

phosphorylated receptors in the active state, uncoupling G protein signaling from GPCRs by sterically blocking further G protein binding and activation by the GPCR (Krupnick 1997). Arrestin binding also promotes receptor internalization by coupling the receptor to endocytic machinery, including clathrin (Goodman 1996), clathrin adaptor protein-2 (Laporte 1999), and N-ethylmaleimide-sensitive fusion protein (McDonald 1999). Receptor endocytosis results in either receptor degradation and downregulation or receptor resensitization and recycling back to the surface (Luttrell 2002).

Arrestin recruitment to the GPCR

Arrestin has two receptor sensors, an activation state sensor and a phosphorylation state sensor, each of which can promote association with receptor at low affinity, but association of both sensors causes a conformational change in arrestin leading to a high affinity association between arrestin and the active phosphorylated receptor (Gurevich 1993, 1995). Arrestins therefore act as molecular coincidence detectors, generally requiring both receptors to be in an active conformation and phosphorylated for a high affinity association between arrestin and the GPCR, although the difference in affinity between inactive unphosphorylated receptor and active phosphorylated receptor is not as high for the non-visual arrestins (arrestin-2 and arrestin-3) as for the visual arrestins (arrestin-1 and arrestin-4) (Gurevich 2006).

Arrestin is comprised of two domains comprised largely of β-sheets, with extensive interactions between the domains including hydrophobic interactions, a polar core, and a three-element interaction between the carboxyl tail and β-strand I and α-helix I of the amino domain. Crystal structures show that in its basal inactive state arrestin is an elongated molecule, but based on current models the two domains are thought to move during the conformational change upon receptor binding. Upon relief of the constraints in the basal state, the flexibility of the position of the amino and carboxyl terminal domains of arrestin relative to one another allows the molecular flexibility necessary for small family of arrestins proteins to bind a large variety of GPCRs (Hirsch 1999, Vishnivetskiy 1999).

The amino domain β-strands V and VI and carboxyl domain β-strands XV and XVI function as the activation sensors of arrestin and bind intracellular parts of the receptor which undergo conformational changes upon receptor activation (Gurevich 2003, Vishnivetskiy, 2004). This association presumably weakens the interaction between the two domains of arrestin. Lysines within the β-strand 1 of arrestin bind the phosphates attached to the receptor C tail (Vishnivetskiy 2000), destabilizing the three-element interaction and guiding the phosphates to the polar core where they bind the positively charged residues within the polar core. This destabilizes the polar core by leaving only the negatively charged amino acids of the polar core which repulse each other and drives further movement of the arrestin domains. These binding events and conformational changes combine to create a high affinity between the arrestin molecule and the agonist bound active phosphorylated receptor which is primarily destabilized by agonist dissociation and subsequent shift to an inactive receptor state.

The phosphorylation sensor of arrestin recognizes negative charges in close proximity (Gurevich 1993, 1995, Mendez 2000). This usually consists of two or more phosphates within the carboxyl tail of receptors, but phosphorylated residues within other intracellular domains are capable of recruiting arrestin to some receptors. Further, spatially constrained acidic amino acids have also been shown to contribute to the negative charge required to destabilize the polar core of non-visual arrestins, and in the case of some receptors such as the substance P and lutropin receptors (Mukherjee 1999, Richardson 2003) a high concentration of acidic amino acids within the carboxyl tail paired with agonist activation is sufficient for arrestin recruitment. In addition to residues within GPCRs which mediate binding to the phosphate and activation sensors of arrestins, motifs have been identified within some GPCRs which act as brakes to inhibit binding to arrestin and often require additional phosphorylation events to relieve this inhibition (Whistler 2001, Kim 2004).

In addition to their canonical role in uncoupling GPCRs from G protein signaling and promoting receptor internalization and recycling, the non-visual arrestin also function as scaffolds for a variety of kinase signaling cascades including non-receptor tyrosine kinases and components of the MAPK cascades

(Gurevich 2003, Lefkowitz 2005). By serving as adaptors linking these cascades to GPCRs, arrestins serve as signal transducers initiating GPCR signaling pathways distinct from those initiated by heterotrimeric G proteins. In addition to coupling additional cascades to GPCRS, arrestin-mediated signaling regulates the substrates of those cascades (Tohgo 2002, 2003, Luttrell 2003). The particular mechanism by which the GPCR activates a kinase cascade affects the substrates of that cascade. In addition, the same receptor-arrestin complex may be capable of initiating different events, depending on the arrestin conformation induced. In the cases of the AT(1A) angiotensin receptor and the V2 vasopressin receptor, phosphorylation by GRK2 and GRK3 is required for arrestin-dependent receptor desensitization while phosphorylation of GRK5 and GRK6 is required for arrestin-dependent ERK1/2 activation (Kim 2005; Ren 2005)

Numerous sequences within arrestin-2 and arrestin-3 have been identified which are involved in interactions of downstream effectors. Both arrestin-2 and arrestin-3 scaffold Raf1, MEK1, and ERK1 and ERK2, to promote ERK1/2 activation (Luttrell 2001). The amino terminus of arrestin-2 contains proline rich motifs which bind to Src (Luttrell 1999). The resulting activation of Src leads to dynamin phosphorylation and regulation of endocytosis. Arrestin-mediated Src activation also leads to Ras-dependent ERK1/2 activation. Arrestin-3, but not arrestin-2, scaffolds Ask1, MKK4, and JNK3 to promote JNK activation (McDonald 2000, Song 2009).

KOR signaling pathways

KOR activation leads to the activation of classic G protein-mediated signaling cascades. These downstream effects include decreased cAMP via Gαi-mediated inhibition of adenylyl cyclase (Konkoy 1989), and decreased cellular excitability through modulation of plasma membrane ion channels. KOR decreases cellular excitability through inhibition of voltage-sensitive calcium channels (Macdonald 1986, Gross 1987, Attali 1989) and Gβγ-mediated stimulation of G protein-coupled inwardly-rectifying potassium channels (North, 1987; Sadja, 2002). Although KOR inhibits calcium channels on the plasma

membrane, KOR activation has also been shown to increase intracellular calcium through mobilization of intracellular calcium stores (Spencer 1997; Pan 2003).

Following sustained activation, KOR undergoes desensitization similar to other GPCRs, including uncoupling from G protein cascades and receptor internalization. KOR activation results in GRK3-dependent phosphorylation of rodent KOR at the Ser369 residue within the C tail (McLaughlin 2003, 2004). Desensitization of KOR has been shown to require GRK3 and arrestin-3 in heterologous expression systems (Appleyard 1999; McLaughlin 2003), and genetic deletion of GRK3 reduces the development of analgesic tolerance to KOR agonists (McLaughlin 2004). Mutation of KOR(Ser369Ala) also prevents desensitization of KOR (Appleyard, 1999; McLaughlin 2003) in heterologous expression systems.

KOR also activates multiple MAPK cascades, including ERK1/2, p38, and JNK. KOR agonists promote phosphorylation of ERK1/2 within 5 minutes via a Gβγ-dependent mechanism which also requires PI3-Kinase and PKCζ (Fukuda, 1996, Belcheva, 1998, 2005; Bruchas, 2006). Similar to other GPCRs, ERK1/2 activation via KOR is biphasic and also includes a later phase of ERK1/2 activation which requires arrestin-3 (McLennan 2008). The relative contribution of the early Gβγ-dependent and late arrestin-dependent phases to the overall activation of ERK1/2 via KOR depends on the cell system. The functional difference in ERK1/2 signaling via activation of these two mechanisms is unclear. KOR activation of ERK1/2 has been demonstrated to regulate embryonic stem cell differentiation (Kim 2006) and astrocyte proliferation (McLennan 2008), but the potential effects of KOR-mediated ERK1/2 activation have not been fully explored, given the wide range of cellular processes ERK1/2 has been shown to regulate.

Stimulation of KOR has been shown to result in activation of p38 MAPK through arrestin-dependent pathways rather than G protein signaling, and requires GRK3 phosphorylation of KOR at Ser369 and arrestin-3 recruitment (Bruchas 2006). This signaling pathway is not required for the

analgesic effects of KOR agonists, which are primarily G-protein mediated. In contrast, p38 activation mediates several therapeutically undesirable KOR induced changes in cellular signaling and in vivo.

KOR induced p38 activation is required for the increase in GFAP immunoreactivity and astrocyte proliferation in the dorsal horn of the spinal cord following sciatic nerve ligation (Xu 2007). Activation of p38 via KOR also results in a Src-dependent phosphorylation of the Tyr12 residue of the Kir3.1 subunit of GIRK, resulting in GIRK desensitization, reduced potassium conductance through channel. This phosphorylation event is also observed following sciatic nerve ligation, and may results in an increase in cellular excitability (Clayton, 2009). KOR-induced p38 activation has been implicated in the development of allodynia and hyperalgesia following sciatic nerve ligation (Xu 2007), possibly through the mechanisms described above.

Disruption of the GRK3/arrestin-3/p38 signaling cascade via genetic deletion of GRK3 or pharmacological inhibition of p38 also prevents the development of KOR-induced conditioned place aversion and KOR-induced increases in swim-immobility, suggesting that p38 plays a role in the dysphoric effects of KOR agonists (Bruchas 2007). Furthermore, lentiviral expression of KOR or mutant KOR(Ser369Ala) specifically in the dorsal raphe of KOR-/- mice is sufficient to recover KOR thermal analgesia in the warm water tail withdrawal assay. Lentiviral expression of wild type KOR, but not the mutant KOR(Ser369Ala), recovers KOR conditioned place aversion, and this place aversion is blocked by injection of the KOR antagonist norBNI into the nucleus accumbens. These experiments demonstrate that activation of KOR specifically in dorsal raphe neurons projecting to the nucleus accumbens is sufficient to produce KOR conditioned place aversion and confirm that phosphorylation of KOR at Ser369, which results in p38 activation, is required for the aversive but not analgesic properties of KOR agonists (Land 2009).

Agonist stimulation of KOR has been demonstrated to cause cJun-Kinase (JNK) activation in several heterologous expression systems and in vivo (Kam 2004; Bruchas 2007). JNK activation by KOR

agonists like U50,488 is pertussis-toxin sensitive but mediated by the Gβγ subunit rather than Gαi. Studies in COS-7 cells also implicate the tyrosine kinases Src and FAK, the Rho-subfamily GTPases Rac and Cdc42, and the guanine nucleotide exchange factor Sos (Kam 2004). The downstream effects of KOR agonist induced JNK activity are not currently understood.

Unexpectedly, the KOR selective antagonist norBNI has been shown to activate JNK in heterologous expression systems and in vivo (Bruchas 2007, Melief 2011). This is in contrast to studies showing that norBNI acts as an antagonist, rather than agonist, at KOR in multiple assays, including GTPγS binding, adenylyl cyclase inhibition, ERK1/2 phosphorylation, p38 phosphorylation, and GIRK conductance. NorBNI activation of JNK is not sensitive to pertussis toxin or inhibitors of Src or the small GTPase Raf, demonstrating that norBNI activates JNK by different mechanism than prototypical KOR agonists like U50,488.

Furthermore, JNK activation has been shown to be required for the unusually long-lasting effects of norBNI. Despite evidence from in-vitro radioligand binding studies that norBNI is a competitive antagonist (Takemori 1988, Smith 1990), norBNI blocks KOR agonist analgesia for over two weeks after a single administration (Horan 1992, Bruchas 2007). This long lasting effect of norBNI is prevented by pretreatment with short-acting antagonists which do not activate JNK, co-administration of JNK inhibitors with norBNI, or genetic deletion of the JNK1 isoform (Bruchas 2007, Melief 2011). Therefore norBNI is not simply a competitive antagonist as originally thought, but a collateral agonist which selectively activates JNK. This collateral agonist activity at JNK has also been identified for other long lasting KOR antagonists including JDTic and GNTI, which are structurally distinct from norBNI (Bruchas 2007, Melief 2011). This leads to the hypothesis that norBNI-like antagonists uncouple KOR from signaling via a JNK-mediated cascade resulting in a high affinity association between KOR and another, currently unidentified, protein. The mechanisms of JNK activation by norBNI and receptor uncoupling by norBNI-stimulated JNK remain unresolved, as does the question of why KOR agonists such as U50,488 do not inactivate KOR through this mechanism.

Based on the studies to date, KOR can activate at least three distinct signaling pathways. The first is the canonical heterotrimeric G protein pathway with a myriad of effectors downstream of Gαi and Gβγ. This pathway regulates cellular cAMP, ion channel conductance, JNK activation by classic KOR agonists such as U50,488, and rapid activation of ERK1/2. This is also the pathway which mediates the analgesic properties of KOR agonists. The second pathway uses arrestin as a signaling scaffold, rather than signaling through G proteins. Arrestin-dependent signaling cascades include p38 and the late phase of ERK1/2 activation. Although the significance of the late phase of ERK1/2 activation is not well understood, arrestin-dependent p38 activation has been implicated in the aversive effects of KOR agonists as well as in the development of allodynia and hyperalgesia resulting from chronic KOR activation after injury. Finally the most recently identified and least characterized pathway is G protein-independent activation of JNK by collateral agonists/antagonists (i.e., norBNI) which results in receptor inactivation. It is significant that different effects of KOR activation are mediated by distinct pathways, since ligands which could selectively specific pathways might have therapeutic value. In particular, "biased agonists" which activate G-protein signaling without effectively activating arrestin-mediated pathways would have potential as analgesics lacking both the addictive properties of MOR agonists and the aversive properties of traditional KOR agonists.

Ligand directed signaling

In traditional receptor theory, a receptor has a single active state which produces all signaling events. Agonists and partial agonists are defined according to the degree to which they stabilize the receptor active state after binding, but all stabilize (or not) the same receptor active state, while antagonists bind without stabilizing the inactive state (Stephenson 1956, De Lean 1980, Black 1983). A key concept in traditional receptor theory is intrinsic efficacy – the stimulus per receptor induced by a ligand binding, and that this characteristic should be consistent for a ligand for all second messengers. Apparent differences in downstream signaling are based on differences in the degree of receptor and upstream effector activation required for an effect (threshold), the degree of coupling of that pathway to

the receptor in the particular system being studied, and the amount of activated receptor required for maximal signaling for a given pathway before a ceiling effect is reached (spare receptor). Furthermore, the relative potencies and efficacies of different agonists should be consistent for all second messengers.

Increasingly data has amassed which does not fit this model. It is becoming increasingly evident that GPCRs can have multiple active states which are differentially coupled to different second messengers. Some active states may effectively promote signaling through all possibly second messengers for a receptor, while other active states may only effectively activate a subset of these pathways. Different ligands may stabilize different receptor active states, resulting in different intrinsic efficacies for different signaling pathways depending on the particular active state stabilized. This concept has several names: functional selectivity, biased agonism, agonist-directed trafficking of receptor stimulus, protean agonism, and ligand directed signaling (Kenakin 2003, 2007; Urban 2007).

Examples of ligand directed signaling have been identified for a variety of GPCRs, and can be achieved in diverse ways, highlighting the complexity of GPCR signaling (Urban 2007). Ligands may differentially activate specific subtypes of heterotrimeric G proteins by stabilizing GPCR conformations which preferentially couple to that subtype; this may include switching of G protein usage from the typical G protein for that receptor. Ligands may also preferentially couple the receptor to G-protein signaling, arrestin signaling, or other G protein and arrestin-independent signaling pathways. Ligands may promote different conformations of arrestin bound to receptor, as a result of different receptor phosphorylation events, with the result of downstream signaling. These events may be a result of ligands inducing different conformations of the GPCR, or a result of ligands preferentially binding and/or preferentially activating specific GPCR complexes (i.e. heterodimers).

The CB1 cannabinoid receptor can couple to Gs in addition to Gi, but agonist-specific differences have been identified in coupling to the two systems. Differences have been identified between agonists in their ability to couple CB1 to the different G proteins, with some agonists acting as partial agonists for

both Gi and Gs activation and some acting as full agonists for both, but some agonist acting as full agonists for one but partial agonists for another (Bonhaus 1998, Glass 1999). Furthermore, CB1 ligands vary in their activity at specific Gi subtypes, and with some ligands acting as agonists for some Gi subtypes but inverse agonists for other Gi subtypes (Mukhopadhyay 2005).

The efficacy of some D2 dopamine receptor agonists is also dependent on the assay used. Dihydrexidine is a potent full agonist when measuring Gi-mediated inhibition of adenylyl cyclase, but a partial agonist with low potency for stimulation of GIRK currents or inhibition of dopamine release. S-(+)-N-propylnorapomorphine in contrast is effective at stimulation of GIRK currents and inhibition of dopamine release but has low efficacy for inhibition of adenylyl cyclase (Kilts 2002). Similar differences between D2 agonists have been observed when comparing adenylyl cyclase inhibition and ERK1/2 activation (Gay 2004).

The 5HT2C serotonin receptor activates phospholipase C and phospholipase A2 independent of each other, and couples to inotisol phosphate production via phospholipase C activation and arachidonic acid production via phospholipase A2 activation. The relative efficacy of agonists depends on which pathway is measured, with some drugs acting as full agonists for arachidonic acid production but partial agonists for inotisol phosphate production, while others are full agonists for inotisol phosphate production but only partial agonists for arachidonic acid production (Berg 1998). These two signaling pathways have also been shown to be differentially desensitized by different agonists (Stout 2002). Ligand directed signaling through the 5HT2C receptor has been further shown to be isoform specific, with some RNA edited isoforms of the receptor not having these properties (Berg 2001). Additionally, the 5HT2C receptor are generally thought to couple to Gq/11, they also can couple to Gi/o, and the ability 5HTC2C serotonin receptors to couple to Gq/11 versus Gi/o is differently effected by the ligand (Cussac 2002).

The β2 adrenergic receptor stimulates adenylyl cyclase and ERK1/2 via Gs. Propranolol and ICI118551 are traditionally considered inverse agonists, but have been shown to act as partial agonists for

ERK1/2 activation despite their inverse agonists effect at cAMP accumulation assays (Azzi 2003, Galandrin 2006). Furthermore stimulation of ERK1/2 by propranolol via β2 has been shown to cause changes in gene transcription (Baker 2003). These drugs also activate ERK1/2 through a different mechanism from traditional β2 agonists and do not require heterotrimeric G proteins. Surprisingly, propranolol and ICI118851 still recruit arrestin to the receptor and arrestin is required for the activation of ERK1/2 (Azzi 2003), indicating that arrestin can be recruited to the receptor independent of heterotrimeric G protein activation. Studies using FRET technologies to assess cAMP activation, arrestin recruitment, and receptor internalization of β2 adrenergic receptor agonists have also found that while the efficacy for these pathways is proportional; for most agonists at the β2 receptor, there are a few ligands with higher efficacies at arrestin-dependent pathways than would be predicted based on their efficacy at G-protein dependent pathways (Drake 2008).

Cases of arrestin recruitment independent of heterotrimeric G protein activation have been identified for other receptor systems as well. An analog of angiotensin II has been identified which fails to activate G protein signaling via the AT(1A) angiotensin receptor, but still causes receptor phosphorylation and association with arrestin-2 (Qian 2001). This analog has also been shown to stimulate arrestin-3 and AT(1A)-dependent chemotaxis (Hunton 2005). Ligand/receptor systems with the reverse result have been observed as well. For instance the endogenous chemokines CCL19 and CCL21 are both efficacious agonists with similar potencies for Ca2+ mobilization via the CCR7 chemokine receptor, but only CCL19 is effective and inducing receptor phosphorylation, recruitment of arrestin 3, and receptor desensitization (Kohout 2004).

Ligand directed signaling has also been observed for opioid receptors, in addition to the case of norBNI-like antagonist/collateral agonists described above for KOR. Morphine exhibits biased signaling, preferentially activating G protein pathways via MOR but lacking efficacy at promoting GRK phosphorylation and arrestin recruitment. Morphine fails to stimulate MOR phosphorylation, arrestin recruitment, and receptor internalization, and morphine-induced tolerance is independent of receptor

phosphorylation and arrestin. This is not a result of low efficacy in general, as morphine is not only a highly efficacious analgesic, but equally efficacious to and in some cases more efficacious than other MOR agonists which promote arrestin-dependent desensitization and internalization (i.e., fentanyl and methadone) when G-protein dependent assays like GTPγS binding are used to measure efficacy. (Whistler 1999, Chu 2008, Keith 1996, Johnson 2006, Zhang 1998.)

Differences in MAPK signaling have also been observed between MOR agonists. Both morphine and fentanyl cause Gi-dependent JNK activation via MOR, but fentanyl activation of JNK requires arrestin, whereas morphine activation of JNK is arrestin-independent (Melief, 2010). Morphine tolerance in mice is also attenuated in JNK2 knockout mice or when JNK is inhibited, but fentanyl tolerance is unaffected. Given the sequence similarities between MOR and KOR, it seems plausible that it may be possible to identify KOR agonists which activate G-protein signaling and promote analgesia, but to not activate the arrestin-dependent cascades which have undesirable effects.

Species differences in KOR

The majority of studies examining the signaling mechanisms of KOR-mediated aversion have used mouse models, and most studies examining arrestin-dependent signaling by KOR have used cell lines expressing the rat KOR sequence. The rat and mouse KOR sequences are highly homologous (98.7% homology) with no differences in the intracellular or transmembrane domains, and the amino acids at all but one sites of differences are highly similar in properties (i.e., charge and hydrophobicity). They have lower homology to the human KOR however, with 94% homology between rat and human KOR.

These differences between the human and rodent sequence include six amino acids within the transmembrane domains which are different but similar in properties and six amino acids different in the C tail of KOR of which three are non-conservative amino acid changes. The human KOR contains a serine at the 358 position instead of an asparagine, a tyrosine at the 369 position instead of a serine, and

an aspartate at the 374 position instead of a glycine. These differences change the GRK phosphorylation site of KOR and add an additional negative charge to the C tail in the human receptor. The rodent KOR is phosphorylated by GRK3 at Ser369, but in the human sequence this residue is a tyrosine, which is not a substrate for GRK proteins. The human receptor has been shown to be phosphorylated by GRKs at Ser358 instead (Li 2002); in rodents this site is an asparagine and not a GRK substrate. While it is not fully understood how the change in phosphorylation site affects the conformation of the KOR-GRK or KOR-arrestin complex, the alteration of the molecular environment of the GRK site has the potential to alter the interaction of KOR with GRK and arrestin.

Most studies examining GRK phosphorylation of the human receptor and subsequent arrestin recruitment have focused on KOR desensitization and internalization (Liu-Chen 2004). Agonist stimulation of human KOR induces GRK3 phosphorylation at KOR(Ser358) and arrestin-dependent receptor desensitization and internalization (Li 1999, 2002), similar to rodent KOR, but the efficacy of agonists for inducing desensitization and internalization is different for rodent and human KOR. Higher concentrations of the agonists dynorphin-A(1-17) and U50,488 are required to induce desensitization and internalization of rodent KOR than human KOR (Li 1999; Jordan 2000, Zhang 2002). It is not known if these differences extend to arrestin-mediated signaling events or how these differences affect KOR induced p38 activation.

In this thesis I examine the regulation of opioid receptor signaling, with a primary focus on non-classical mechanisms of KOR signaling and regulation outside the traditional model of heterotrimeric G protein-mediated signaling which is inactivated by receptor phosphorylation by GRK and arrestin recruitment. In particular this thesis is concerned with extending our understand of how these signaling and regulatory cascades are affected by the differences in human and rodent KOR sequences.

Ligand directed signaling differences between rodent and human kappa opioid receptors

This chapter has been submitted to the Journal of Biological Chemistry:

Ligand Directed Signaling Differences Between Rodent and Human Kappa Opioid Receptors. Selena S. Schattauer, Mayumi Miyatake, Haripriya Shankar, Chad Zietz, Jamie R. Levin, Lee-Yuan Liu-Chen, Vsevolod V. Gurevich, Mark J. Rieder, Charles Chavkin. Submitted to *Journal of Biological Chemistry* in May 2012.

Introduction

Kappa opioid receptor (KOR) agonists are effective analgesics, and are thought to have a lower addictive potential than mu opioid receptor (MOR) agonists (Fraser 1964, Martin 1965, 1983). However, KOR agonists also have dysphoric effects in humans (Pfeiffer 1986) and produce aversion in experimental animals (Mucha 1985, Carlezon 1998, Mague 2003, McLaughlin 2003, Land 2008), unlike MOR agonists, which are euphorigenic. These negative affective responses to available KOR agonists have limited their therapeutic use (Rosow 1987, Wadenberg 2003). The cellular and molecular mechanisms of kappa opioid-induced dysphoria (measured operationally as conditioned place aversion in rodents) are not completely clear, but recent results suggest that KOR activation of p38α MAPK in serotonergic neurons innervating the ventral striatum is required (Bruchas 2006, Land 2009, Bruchas 2011). KOR activation of p38 MAPK follows from G protein receptor kinase 3 (GRK3) phosphorylation of the Serine369 residue within the C terminal tail of the rodent sequence (rKOR) and subsequent recruitment of non visual arrestins, that act as a scaffold linking a kinase signaling cascade to p38 MAPK phosphorylation (McLaughlin 2003, Bruchas 2006). Disruption of this signaling pathway in mice through receptor mutation (KORS369A), GRK3 deletion, or conditional deletion of p38α MAPK blocks the aversive effects of KOR agonists without reducing their analgesic effects (Bruchas 2007, Land 2009, Bruchas 2011). These findings have potentially important therapeutic implications, because a partial KOR

agonist that does not efficiently activate arrestin dependent signaling might produce analgesia without significant dysphoria (Chavkin 2011). This type of "ligand directed signaling" or "biased agonism" has been demonstrated for other receptor systems (Urban 2007) including MOR; some highly efficacious MOR agonists such as fentanyl effectively recruit arrestin while others such as the partial agonist morphine do not, but both are highly effective analgesics (von Zastrow 2003).

Development of biased KOR agonists for human treatment is complicated by sequence differences between rKOR and hKOR. Whereas hKOR and rKOR share 94% amino acid sequence homology overall, the amino acid residues involved in GRK/arrestin signaling are not conserved (Fig 2.1). In hKOR the residue at 369 is a tyrosine instead of a serine, and is not a GRK substrate. The residue responsible for arrestin recruitment to hKOR and desensitization is Ser358 (Li 2002), but residue 358 is an asparagine in rKOR and not a GRK substrate. The significance of these differences in GRK/arrestin dependent signaling has not been established, and previous studies examining GRK/arrestin dependent signaling pathways other than desensitization have used rKOR. In this study we asked whether GRK phosphorylation of hKOR Ser358 produces equivalent signaling events to GRK phosphorylation of rKOR S369.

Interestingly, a variant (rs34369022) has been reported for hKOR at the Ser358 residue (dbSNP). In the NCBI dbSNP database, this variant (G>Y at the cDNA position 1308) is reported in one of the five Celera donors, and would result in a serine to isoleucine mutation at protein position 358 (Ser358Ile). If this SNP was not an error in sequencing or a rare, novel individual variant, this would suggest that a sub-population of individuals may express a variant of KOR that would not be regulated by GRKs and arrestins, and may therefore alter these individuals' responses to stress as well as to kappa analgesics.

There are no KOR selective partial agonists currently available, but there are non-selective partial KOR agonists used clinically, including butorphanol, nalbuphine, and pentazocine. Pentazocine is a mixed acting opioid analgesic, with agonist activity at the kappa opioid receptor and sigma receptor and antagonist or partial agonist activity at the mu opioid receptor (Emmerson 1996, Zhu 1997). The side effect profile of pentazocine in people includes classic KOR agonist effects such as dysphoria and

hallucinations, but individual responses vary, with some individuals reporting euphoric rather than dysphoric effects. The euphoric effects are more apparent at lower doses while dysphoria becomes more prominent with increasing dosage (Beaver 1966, Hamilton 1967, Jasinski 1970, Zacny 1998). Administration of the opioid antagonist naltrexone at doses selective for the mu opioid receptor (MOR) effectively block the euphoric effects of pentazocine in people and unveils a stronger aversive dysphoric response that can be blocked by higher doses of naltrexone sufficient to antagonize KOR (Preston 1993). Because it is not clear how partial agonists produce dysphoria, in this study we compared pentazocine-induced signaling through arrestin-dependent and independent pathways to that of the selective full KOR agonist U50,488.

Materials and Methods

Chemicals/Reagents. (-)U50,488 (Tocris) was dissolved in water. Stock solution of (-) Pentazocine (NIDA) was dissolved in a DMSO, then diluted as described below prior to use. Naloxone (Sigma) and norBNI (NIDA) were dissolved as described below.

*Animals***.** Male C57BL/6 mice (20-30g) were group-housed and kept on a 12-h light/dark cycle with food and water available *ad libitum*. Animal procedures were approved by the Animal Care and Use Committee of the University of Washington and conform to the guidelines on the care and use of animals promulgated by the National Institutes of Health. Homozygous KOR-/- and MOR-/- mice were generated by homologous recombination as previously described (Schuller 1999, Clark 2002).

Conditioned Place Preference/Aversion. C57BL/6 mice were trained in a balanced, unbiased three-chamber conditioning apparatus as described previously (Land 2008). Briefly, mice were given a pretest of 30 min in which they were allowed to explore the entire arena. During this time the amount of time spent in each of the three chambers was video recorded (ZR90, Cannon) and analyzed using Ethovision (v.3.0, Noldus, Wageningen, The Netherlands). Mice were assigned to either vertical or horizontal striped chambers based on pretest times. The morning of each of the two training days was paired with vehicle (10 mL/kg i.p. of 15% DMSO, 5% cremophor & 80% sterile saline), and the afternoon session with drug

(pentazocine 10 mg/kg i.p., pentazocine 10 mg/kg + norBNI 10 mg/kg i.p., or pentazocine 10 mg/kg + naloxone 5 mg/kg i.p.) with a minimum of 4 hr elapsing between training sessions. On the fourth day, allowing the mice to roam freely between all three compartments and recording the amount of time spent in each compartment measured. Test sessions took place midday. Conditioned place preference or aversion (CPP/CPA) scores were calculated by subtracting the time spent in the drug-paired side during the post-test from time spent in the saline-paired side during the post-test.

Locomotor activity. Locomotor activity during conditioned place preference training was video recorded (ZR90, Cannon) and analyzed using Ethovision (v.3.0, Noldus, Wageningen, The Netherlands). Scores represent total distance moved (cm) during the 30 min session.

Antinociceptive Testing. Antinociceptive responses were measured using the warm-water tail-withdrawal assay (McLaughlin 2003). The response latency for an animal to withdraw its tail after being immersed in 52.5°C water was measured before treatment with (-)Pentazocine (10 mg/kg, i.p.). After drug administration, responses were measured every 10 min for the first 30 min and every 30 min after that for 3 hr post drug administration. A 15 sec maximal immersion duration was used as a cut off to prevent tissue damage.

Cell Culture. HEK293 cells expressing rKOR-GFP were generated as described previously (McLaughlin 2004). HEK293 cells stably expressing flag tagged hKOR or mutant hKOR (S358N) were generated by transfecting HEK293 cells using Fugene 6 according to manufacturer instructions. Plasmids expressing flag tagged human KOR or flag tagged human KOR (S358N) were previously described (Li 2002). HEK293 cells were maintained in Dulbecco's modified medium/F12 with 10% fetal bovine serum. HEK293 cells expressing KOR were grown in media supplemented with G418 (200 μg/ml). HEK293 cells expressed comparable levels of receptor: 1.6 pmol and 4.1 pmol [^{3}H]-U69,593 (2 nM) specifically bound per mg protein, rKOR and hKOR, respectively.

siRNA. HEK293 were transfected with siRNA directed against arrestin-2 or arrestin-3 or scrambled siRNA control (Dharmacon Research) using Lipofectamine RNAiMAX (Invitrogen) according to

manufacturer instruction. Transfected cells were passaged onto 6 well plates after 48 hr, and 24 hr later (72 hr post transfection) treated as described.

Dominant positive arrestin. HEK293 expressing hKOR(S358N) were transiently transfected with phosphorylation independent arrestin-2(R169E) or arrestin-3(R170E) (Kovoor 1999, Celver 2002) tagged with mCherry at the C-terminus as described (Walther 2010) using Fugene HD (Promega) according to manufacturer instructions. Transfected cells were passaged onto 6 well plates after 48 hr, and 24 hr (72 hr post transfection) treated as described.

Immunoblotting. HEK293 cells were serum starved 6 hr and then treated as described in Results and lysed in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, phosphatase and protease inhibitors). Lysates were sonicated and centrifuged (15,000 × *g*, 20 min, 4°C), and the supernatant was stored at –20°C. Total protein concentration was determined by BCA assay (Pierce) with bovine serum albumin standards before loading 30 µg (phospho-ERK1/2) or 45µg (phospho-p38 MAPK) onto 10% Bis-Tris precast gels (Invitrogen) and running at 130 V for 1.5–2 hr. Blots were transferred to nitrocellulose (Whatman, Middlesex, UK) for 1.5–2 hr at 30 V. The nitrocellulose was blocked with 5% BSA-TBS 1 hr at room temperature and stained overnight at 4 °C for phospho-ERK1/2 or phospho-p38 (Cell Signaling, 1:1000) and actin (Abcam, 1:5000) in 5% BSA-TBS. Blots were incubated in IRdye secondary (Li-Cor Biosciences, 1:10,000 for phospho-p38 and phospho-ERK1/2 or 1:20,000 for actin) in 1:1 Odyssey buffer (Li-Cor) and 5% milk-TBS 1 hr at room temperature then scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences). Band intensity was measured using the Odyssey software and expressed as phospho-ERK1/2 or phospho-p38 MAPK band intensity over actin band intensity. Data were normalized to percentage of control sample (basal, 100%) and plotted using GraphPad Prism. Statistical significance ($p<0.05$) was determined by Student's t-test or analysis of variance followed by Bonferroni's post-hoc test.

Dose Response Curves. Dose response curves for p38 and ERK1/2 phosphorylation were calculated in GraphPad Prism, using a three parameter least squares nonlinear regression with a 1/Y weighting and a

bottom constrained at 100%. Significant differences between EC50s and Emax were determines by extra sum-of-squares F test.

SNP analysis. To identify common nucleotide sequence polymorphisms, we performed re-sequencing on DNA samples from a discovery panel of 95 individuals from several diverse populations (Afr. American, Caucasian, African, Hispanic, Asian). Polymerase chain reaction (PCR) and sequencing methods for SNP discovery have been published previously (Livingston 2004). For the present study, all of the coding regions of *OPRK1* were resequenced using M13-tailed PCR amplification of overlapping amplicons. PCR primer sequences are available upon request. All amplicons were purified, diluted and used in standard Big-Dye terminator sequencing reactions under standard conditions (v3.1 Cycle Sequencing Kit Protocol Manual; Applied Biosystems, Foster City, CA, USA), and run on an ABI 3730XL. Each chromatogram was trimmed of low-quality sequence (Phred quality score < 25), assembled, and edited in Consed to ensure accuracy. SNPs were identified using the PolyPhred program (version 5.0) (Strephens 2006). Putative polymorphic sites were flagged by PolyPhred and reviewed for genotype accuracy to remove false positives.

Results

Do hKOR and rKOR differ in their activation of p38? HEK293 cells stably expressing rKOR or hKOR were treated 30 min with either U50,488 or pentazocine at concentrations from 100 pM to 10 µM prior to lysis and phospho-p38 was measured by Western blot (Fig 2.2). The EC50s for pentazocine-induced p38 phosphorylation for rKOR and hKOR were 26 nM (confidence interval 2.2 nM to 310 nM, n=4-9) and 0.53 nM (85 pM to 3.4 nM, n=8-12), respectively. The 50 fold greater potency of pentazocine in activating p38 MAPK in cells expressing hKOR was statistically significant ($p<0.05$). The EC50s for U50,488 induced p38 phosphorylation for rKOR and hKOR were 28 nM (confidence interval 5.5 nM to 140 nM, n=4-8) and 8.6 nM (1.9 nM to 39 nM, n=4-12) respectively. In contrast to pentazocine, the potencies of U50,488 stimulation of phospho-p38 for hKOR and rKOR were not significantly different. Neither pentazocine nor U50,488 at the highest concentration tested increased p38 MAPK

phosphorylation in untransfected HEK293 (Fig 2.2). Consistent with previous in vivo assays (Zhu 1997, Remmers 1999), pentazocine also appears to be a partial agonist in activating the p38 pathway (having about 60% of the activity of U50,488 in both hKOR and rKOR). Pentazocine increased p38 phosphorylation to 167% and 150% of basal levels in hKOR and rKOR expressing cells, whereas stimulation with U50,488 resulted in p38 phosphorylation of 241% and 260% over basal. This difference in Emax was significant for both hKOR ($p<0.001$) and rKOR ($p<0.001$).

Are the differences between hKOR and rKOR pathway dependent? Activation of KOR has been reported to increase phospho-ERK1/2 by two separate mechanisms: a G protein mediated early phase of ERK1/2 phosphorylation, and a slower arrestin mediated late phase (McLennan 2008). To measure the early phase of ERK1/2 activation through the G protein pathway, HEK293 expressing rKOR or hKOR were treated for 5 min with pentazocine or U50,488 at concentrations from 100 pM to 10 μM prior to lysis, and phospho-ERK1/2 was measured by Western blot (Fig 2.3). The EC50s for pentazocine induced ERK1/2 phosphorylation for rKOR and hKOR were 12 nM (confidence interval 2.3 nM to 59 nM, n=4-10) and 2.8 nM (570 pM to 13 nM, n=4-11), respectively with no significant difference. The EC50s for U50,488 induced ERK1/2 phosphorylation for rKOR and hKOR were 9.1 nM (confidence interval 3.6 nM to 23 nM, n=4-8) and 5.8 nM (2.1 nM to 16nM, n=10-11), respectively with no significant difference. As was observed for p38 pathway activation, pentazocine appears to be a partial agonist (with 52-59% of U50,488 activity) at stimulating the early phase of the ERK1/2 pathway. Stimulation of hKOR and rKOR expressing cells results in ERK1/2 phosphorylation of 263% and 306% of basal levels, respectively, whereas stimulation with U50,488 results in ERK1/2 phosphorylation of 446% and 592% of basal. This difference in Emax was significant for both hKOR ($p<0.001$) and rKOR ($p<0.001$). The difference between pentazocine's effects on p38 and ERK activation in hKOR and rKOR is striking and suggests that the sequence differences between hKOR and rKOR more strongly affect p38 activation than the early phase of the ERK1/2 signaling cascade. Furthermore, because U50,488 affected hKOR and rKOR similarly, the results suggest that the differences in pathway activation were a consequence of pentazocine-KOR interaction differences rather than expression differences.

What is the relevance of in vitro pharmacological data for pentazocine to behavioral effects? The relatively weak p38 activation in rKOR by pentazocine suggests that it may not produce aversion in mice through KOR activation. To assess this, mice were trained in a balanced, unbiased three chamber conditioning apparatus, receiving saline, 10 mg/kg pentazocine, or pentazocine in combination with 10 mg/kg norBNI and/or 5mg/kg naloxone**.** Mice showed a 200 second place preference to pentazocine, which was not affected by pretreatment with the KOR selective antagonist norBNI. Pretreatment with naloxone reversed the preference to pentazocine and unveiled an aversion that was also not blocked by pretreatment with norBNI (Fig 2.4a). These results suggest that pentazocine did not produce a KOR mediated aversion in mice, in contrast to reports of KOR mediated aversive effects in humans.

To assess the analgesic properties of pentazocine in mice, the warm (52°C) water tail withdrawal assay was used (McLaughlin 2003). Wild type, KOR-/-, and MOR-/- mice were injected with pentazocine 10 mg/kg i.p., and tail flick withdrawal latency was measured over 180 min (Fig 2.4b). There was a significant time dependent ($p<0.0001$) increase in tail withdrawal latency and a specific effect of subject pairing ($p<0.0001$), genotype ($p<0.05$) and interaction ($p<0.05$). This anti-nociceptive response was not significantly different between wild type and KOR-/- mice, whereas MOR-/- had significantly reduced analgesia as compared to wild type and KOR-/- mice. To determine the locomotor effects of pentazocine, total distance traveled was measured during drug training sessions for conditioned place preference. Pentazocine produced neither hypolocomoter effects (typical of kappa agonists) nor hyperlocomotor effects (typical of mu agonists) (Fig 2.4c).

Is the Ser358 site of hKOR required for hKOR activation of p38 upon U50,488 stimulation? Since hKOR and rKOR differ in p38 activation and in GRK phosphorylation sites, we next asked if the mechanism for hKOR activation of p38 requires phosphorylation of hKOR by GRK, similar to rKOR. To determine if activation of hKOR induced p38 phosphorylation requires the Ser358 residue, HEK293 expressing hKOR, rKOR, or hKOR(S358N) were treated with 10μM U50,488 for 5 – 120 min at 37°C. This resulted in a significant increase in phospho-p38-ir by 30 min in both hKOR and rKOR expressing HEK293, whereas no increases in p38 phosphorylation were detected in HEK293 expressing

hKOR(S358N) or in untransfected HEK293 (Fig 2.5). These data suggest that hKOR activation promotes p38 phosphorylation in a receptor phosphorylation dependent manner as has previously been reported for rKOR (Bruchas 2006).

Is arrestin required for hKOR to activate p38? Phosphorylation of p38 in rKOR requires recruitment of arrestin-3 following receptor phosphorylation by GRK3 (Bruchas 2006, 2007). In order to determine if hKOR stimulated p38 phosphorylation also requires arrestin, we transiently transfected hKOR expressing HEK293 with siRNA against arrestin-2, arrestin-3, or a scrambled siRNA control. 72 hr following transfection, cells were treated with vehicle or 10 μM U50,488 for 30 min, prior to lysis and immunoblotting for phospho-p38. To confirm the selectivity of arrestin knockdown, lysates were immunoblotted against arrestin-2 or arrestin-3 and arrestin expression was compared to expression in cells transfected with scrambled siRNA control. Arrestin-2-immunoreactivity levels were reduced by 43% by arrestin-2 siRNA, but were unchanged by arrestin-3 siRNA. Arrestin-3-immunoreactivity levels were reduced by 53% by of arrestin-3 siRNA, but were unchanged by arrestin-2 siRNA (Fig 2.6a-c). Knockdown of arrestin-3 but not arrestin-2 prevented the U50,488 stimulated increase in p38 phosphorylation (Fig 2.6d).

To confirm that the absence of p38 phosphorylation in hKOR(S358N) was due to inability of the phosphorylation mutant receptor to recruit arrestin, hKOR(S358N) expressing HEK293 were transiently transfected with constructs expressing arrestin-2(R169E) or arrestin-3(R170E). Mutation of the Arg169 in arrestin-2 or the Arg170 in arrestin-3 to a glutamate allows the arrestin molecule to bind to active GPCRs in the absence of receptor phosphorylation (constitutive activity) (Kovoor 1999, Celver 2002). Cells were treated for 30 min with 10 μM U50,488 or vehicle 72 hr after transfection prior to lysis for immunoblot analysis (Fig 2.6e). Phosphorylated p38 was measured by immunoblot and the amount of phospho-p38 was normalized to the level in transfection reagent control (no DNA) cells treated with vehicle. Treatment with U50,488 had no significant effect on phospho-p38-immunoreactivity in transfection reagent control cells but a significant increase in phospho-p38-ir was observed after U50,488 in hKOR(S358N) cells transiently transfected with arrestin-3(R170E). No basal increase was observed in vehicle treated cells

transfected with arrestin-3(R170E). In contrast, a basal increase in p38 phosphorylation was observed following transfection with arrestin-2(R169E), with no significant further increase in p38 phosphorylation after U50,488. These data show that expression of phosphorylation independent arrestin-3 was sufficient to allow agonist-induced p38 phosphorylation by hKOR lacking the GRK phosphorylation site.

Is the Ser358 site of hKOR required for ERK1/2 activation? Rapid agonist-induced, GRK/arrestin-independent ERK1/2 activation has been previously reported for both hKOR and rKOR, and a slower GRK/arrestin dependent phase of phosphorylation after rKOR stimulation has been reported in some cell lines (McLennan 2008). We therefore sought to assess the time course of ERK1/2 phosphorylation after hKOR stimulation and to determine if the Ser358 site plays a role in ERK1/2 phosphorylation following hKOR activation. ERK1/2 phosphorylation levels were analyzed by Western blot following treatment with 10 μM U50,488 for 5 – 120 min at 37°C. This treatment resulted in a rapid and significant increase in ERK1/2 phosphorylation at 5 min in HEK293 expressing rKOR, hKOR, or hKOR(S358N), but not in untransfected HEK293 (Fig 2.7). While there was no significant difference in ERK1/2 phosphorylation between hKOR, rKOR, or hKOR(S358N) at 5 min, ERK1/2 phosphorylation was significantly higher for hKOR compared to hKOR(S358N) at 15 and 30 min after U50,488. Surprisingly, ERK1/2 phosphorylation also remained significantly higher for hKOR as compared to rKOR. ERK1/2 phosphorylation remained significantly elevated in hKOR but not rKOR or hKOR(S358N) expressing HEK293 up to 60 min after agonist stimulation. This suggests that in addition to the established rapid, receptor phosphorylation-independent, phase of ERK1/2 phosphorylation, hKOR has a slower phase of ERK1/2 phosphorylation in HEK293 that requires the Ser358 site. Further, this late phase of ERK1/2 phosphorylation was more evident in HEK293 expressing hKOR than rKOR.

Is arrestin required for hKOR to activation of late phase ERK1/2? Arrestin recruitment has been implicated in the late phase of ERK1/2 activation by rKOR (McLennan 2008). We hypothesized that arrestin is also required for the late phase of hKOR stimulated ERK1/2 phosphorylation, which is significantly reduced by mutation of the Ser358 site. In order to address this question, we transiently transfected hKOR expressing HEK293 with siRNA against arrestin-2, arrestin-3, or a scrambled siRNA

control. 72 hr following transfection, cells were treated with vehicle or 10 μM U50,488 for 30 min, prior to lysis and immunoblotting for phospho-ERK1/2. The 30 min time point after agonist stimulation was selected because the greatest difference in ERK phosphorylation between hKOR and hKOR(S358N) expressing cells was detected then (Fig 2.5a). Knockdown of either arrestin-2 or arrestin-3 significantly reduced the level of ERK1/2 phosphorylation (Fig 2.8a). This suggests that both arrestin-2 and arrestin-3 were required for the increased ERK1/2 phosphorylation relative to hKOR(S358N).

To determine if expression of phosphorylation independent arrestin-2 or -3 was sufficient to increase the late phase of U50,488-induced ERK1/2 activation in hKOR(S358N) expressing cells to levels observed in hKOR cells, hKOR(S358N) expressing HEK293 were transiently transfected with constructs expressing mCherry tagged arrestin-2(R169E), arrestin-3(R170E), or a no DNA transfection reagent control. Cells were treated 72 hr after transfection for 30 min with 10 μM U50,488 or vehicle prior to lysis for immunoblot analysis. Phosphorylated ERK1/2 was measured by immunoblot and phospho-ERK1/2 was normalized to the level in transfection reagent control (no DNA) cells treated with vehicle. The results obtained showed that ERK1/2 phosphorylation was increased after U50,488 treatment regardless of transfection condition, but transfection with arrestin-2(R169E) or arrestin-3(R170E) alone did not further increase ERK1/2 phosphorylation to levels observed in cell expressing wild type hKOR (Fig 2.8b). These data show that in the case of the ERK1/2 response, the expression of phosphorylation independent mutants of arrestin-2 or arrestin-3 did not compensate for the lack of phosphorylation at Ser358 of hKOR.

Are polymorphisms at the S358 residue common in the human population? The NCBI Single Nucleotide Polymorphism Database (dbSNP), reports 10 missense mutations for the *OPRK1* gene. This includes a guanine to thymine mutation at mRNA position 1308 (rs34369022), which results in mutation of Ser358Ile, which would prevent receptor phosphorylation at that residue and subsequent activation of the p38 pathway. To assess if this is a common SNP within the human population, we performed resequencing of the *OPRK1* gene in 95 individuals from diverse populations. Of the 14 SNPs identified, 10 were in coding regions. Three non-synonymous SNPs were found, two of which had been previously

reported in dbSNP. The identified missense SNPs were a valine to methionine within the second intracellular loop (not previously reported), a lysine to glutamate within the second extracellular loop, and an aspartate to asparagine in the C tail (Table 2.2). No SNPs were identified within codon 358, suggesting that the previous report is a sequencing mistake or that the reported variant is a rare allele (<1%). To further explore the frequency of this variant, we accessed the NHLBI Exome Sequencing Project, which screened nearly 5,500 individuals. A search of this database reveals 23 non-synonymous SNPs within the coding region of the *OPRK1* gene, but no report of a variant at amino acid position 358, also suggesting that previously reported mutation was extremely rare (<1/10,000) or a false report.

Discussion

The primary findings of this study were that the basic mechanism of p38 MAPK activation is conserved between rKOR and hKOR, but that the potency of different agonists for activation of different pathways is not consistent between rKOR and hKOR. This has important implications for the development of biased KOR agonists for therapeutic use, including a limitation on the ability to assess ligand directed signaling in people based on rodent models.

We found that the nonselective KOR agonist pentazocine is 50-fold more potent for p38 activation in hKOR as compared to rKOR. In contrast, pentazocine was equally potent for arrestin-independent activation of ERK1/2 in hKOR and rKOR and there was no difference in potency for U50,488 stimulated phosphorylation of ERK1/2 or p38. Consistent with other studies examining the efficacy of KOR agonists in GTPγS assays (Zhu 1997, Remmers 1999), we found pentazocine to be a partial agonist for both hKOR and rKOR, in both pathways studied. Based on these results, pentazocine appears to be a biased ligand for rKOR but not hKOR. This may be a result of the difference in the GRK phosphorylation site between hKOR and rKOR and resulting difference in the molecular environment of the phosphorylated residue. Arrestin binding has previously been shown to be affected by the distribution of charged amino acids and other phosphorylatable residues adjacent to a GRK phosphorylation site (Gurevich 2006), which are altered by the differences in location of the GRK site within the KOR C tail. We conclude from

this that mice, rats, and rKOR expression systems are not an appropriate model for screening for biased KOR ligands for human therapeutics.

The effects of pentazocine in mice were complex and not consistent with the model of pentazocine functioning primarily as a KOR agonist. We found that pentazocine produces conditioned place preference and thermal analgesia. Both of these effects were mediated by MOR and unaffected by the disruption of KOR through genetic knockout or administration of the KOR selective antagonist norBNI. In the presence of naloxone, mice demonstrated a conditioned place aversion to pentazocine, but this aversion was not blocked by norBNI, showing that it was not KOR mediated. The mechanism of this aversion is not clear, but may be a result of pentazocine activation of sigma receptors (Hiramatsu 2005, Mori 2011). These findings in mice are distinctly different from human studies on pentazocine, in which pentazocine produced KOR-dependent aversive effects that were enhanced by blocking MOR (Preston 1993). These results may reflect differences in measurements and assays between humans and mice, although other KOR agonists have been shown to cause KOR-dependent conditioned place aversion in mice. It seems likely that the differences between the aversive properties of pentazocine in humans and mice are at least partly due to the sequence differences between hKOR and rKOR, since hKOR and rKOR differ in pentazocine potency of p38 activation, which has been shown to play a role in the aversive properties of KOR agonists. The opioid receptor mediating pentazocine analgesia in mice is not consistent across studies and seems to be dependent on multiple factors including mouse strain and pain modality (Suzuki 1991, Chien 1995, Ide 2011, Shu 2011). Similar to other studies (Ide 2011, Shu 2011), we found that in C57Bl/6 mice pentazocine thermal analgesia is mediated through the partial agonism at MOR rather than KOR. While it has been assumed that pentazocine analgesia is mediated through KOR in humans, few studies have tested this (Levine 1988), and it is not clear to what extent the mechanisms of pentazocine analgesia are similar between mice and humans.

While the ligand directed signaling properties of KOR agonists are not consistent between hKOR and rKOR, the signaling mechanisms are conserved. P38 activation through hKOR requires the GRK phosphorylation site on the C tail and arrestin-3, while the early phase of ERK1/2 phosphorylation is

independent of either. There is also evidence that hKOR has a second, slower phase of ERK1/2 phosphorylation which is receptor phosphorylation and arrestin dependent, as has previously been shown in some cell lines with rKOR (McLennan 2008). Interestingly, our data show that this late phase of ERK1/2 requires both arrestin-2 and arrestin-3, although how arrestin-2 and -3 cooperate in promoting ERK1/2 signaling is not clear.

While we cannot extrapolate biased signaling properties of individual agonists from mice and rats to humans, these results show that our understanding of KOR signaling mechanisms in these models likely applies to humans as well. It will be important to not draw conclusions about the signaling properties of particular KOR agonists in humans based purely on rKOR models, but the approach of identifying partial KOR agonists with lower potency for arrestin mediated pathways than G protein mediated pathways holds promise for developing analgesics which lack the abuse potential of MOR agonists and the aversive properties of classic KOR agonists. It is worth noting that the guinea pig KOR is closer in homology to hKOR, containing the Ser358 residue similar to hKOR. Further studies are needed, but guinea pigs may be a better model system than rats or mice for in vivo screening of biased KOR ligands with human therapeutic potential.

Figure 2.1. Comparison of the amino acid sequences of human and rat KOR. Transmembrane domains are shaded. Transmembrane domains (TM1-7), intracellular loops (IL1-3) and the intracellular carboxyl terminal tail (CT) are labeled above the domain and amino acid numbers indicated on the right. The serine residues in the carboxyl terminal tail implicated in GRK/arrestin mediated signaling and desensitization in human and rat KOR, and corresponding amino acid residues in the rat and human KOR respectively, are indicated by boxes.

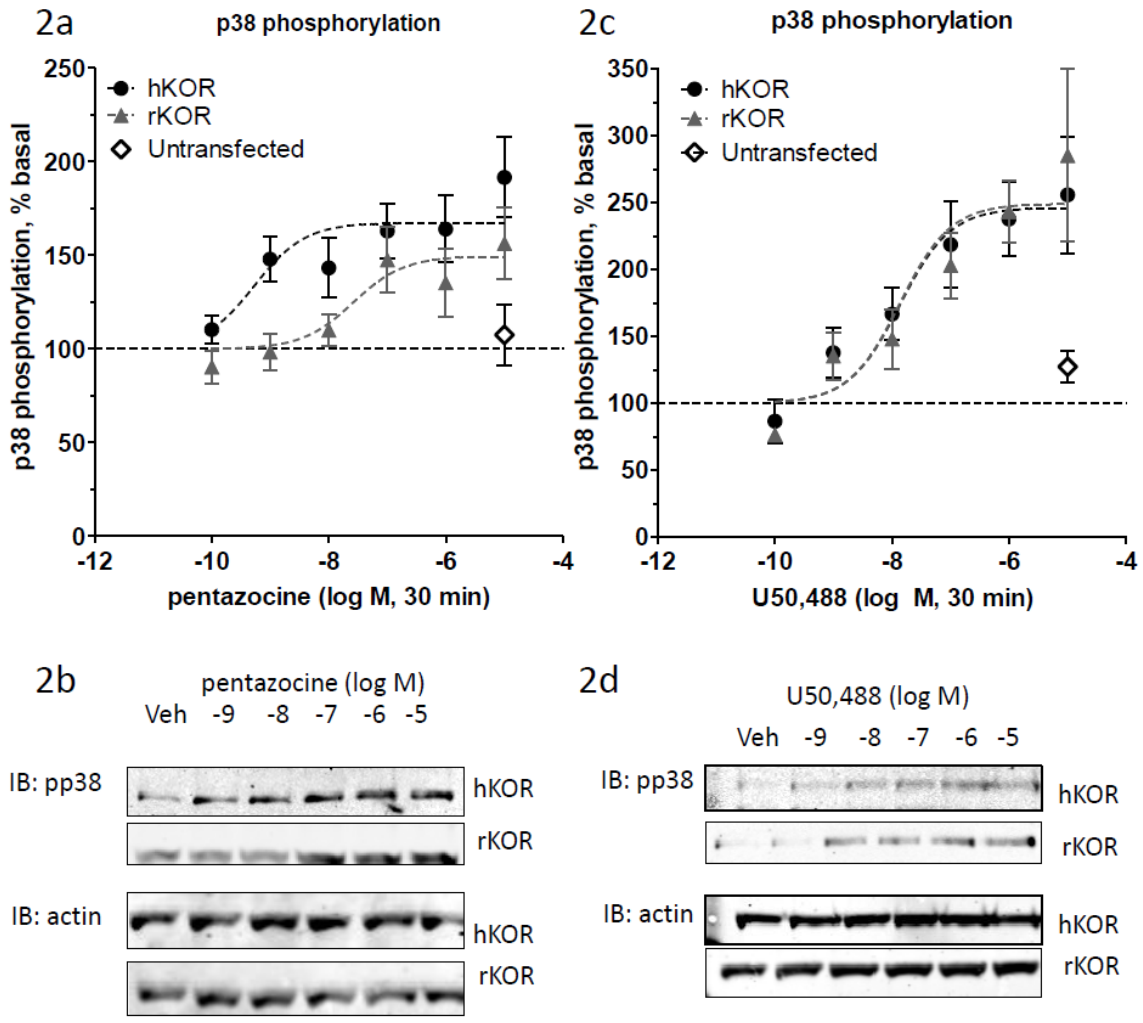


Figure 2.2. Pentazocine is less efficacious than U50,488 in activation of p38. Pentazocine, but not U50,488, is more potent at activation of the p38 pathway in hKOR as compared to rKOR. A,B. HEK293 cells expressing hKOR or rKOR were treated for 30 minutes with indicated concentrations of pentazocine (A) or (-)U50,488 (C) prior to lysis and immunoblotted for phospho-p38 (n=4-12). Dose response curves are shown. C,D. Representative immunoblots for A and B.

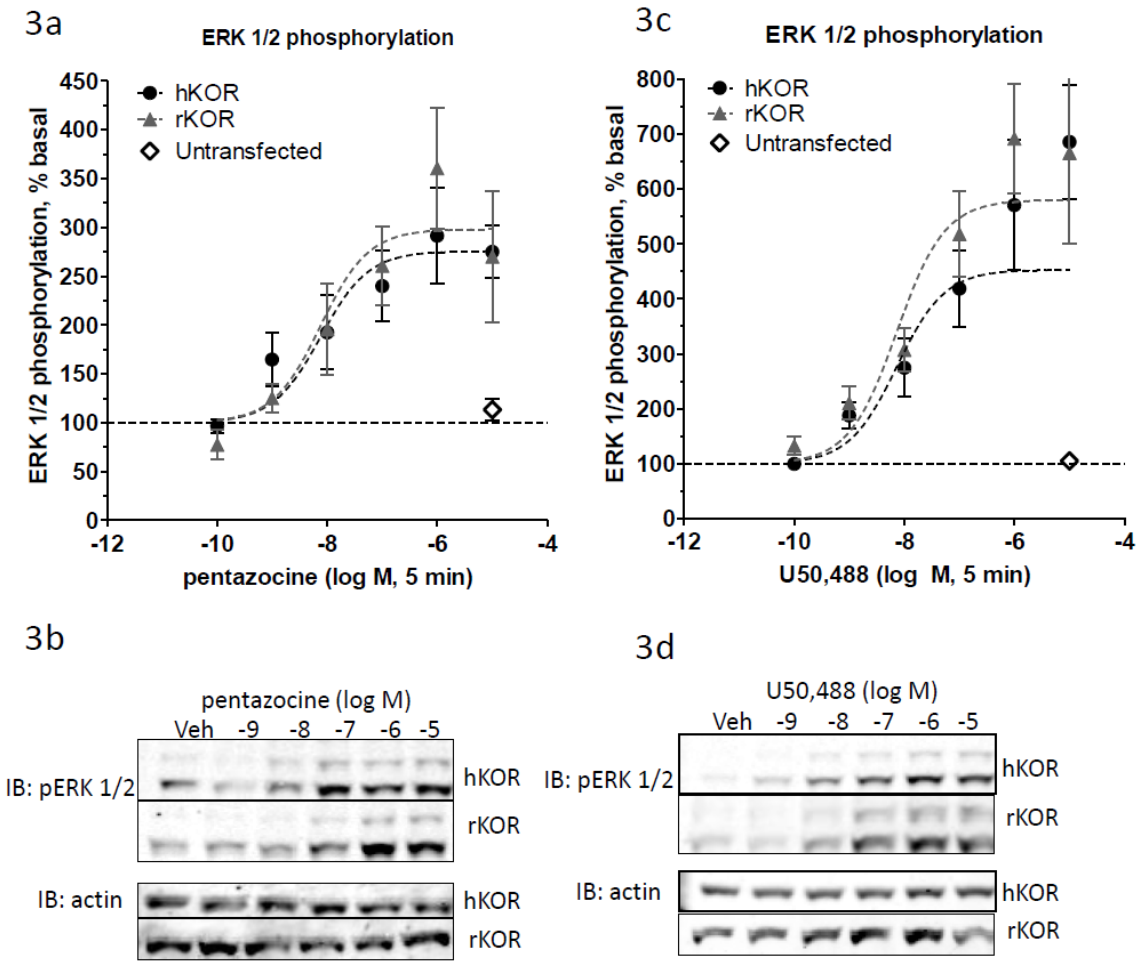


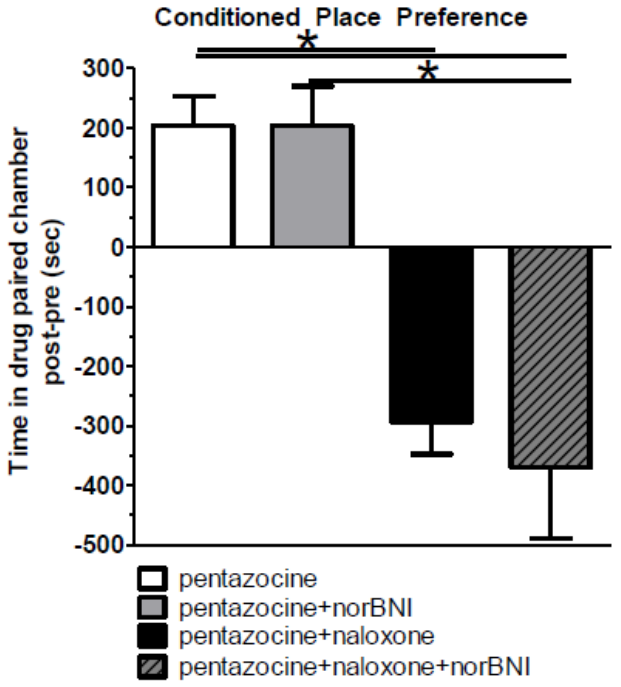
Figure 2.3. Pentazocine is less efficacious than U50,488 in activation of ERK1/2. hKOR and rKOR do not differ in potency at the ERK1/2 pathway. A, B. HEK293 cells expressing hKOR or rKOR were treated 5 minutes with pentazocine (A) or (-)U50,488 (C) prior to lysis and immunoblotted for phospho-ERK1/2 (n=4-10). Dose response curves are shown. C,D. Representative immunoblots for A and B.

	pentazocine induce pERK		pentazocine induced pp38		U50,488 induced pERK		U50,488 induced pp38	
	EC50	C.I.	EC50	C.I.	EC50	C.I.	EC50	C.I.
hKOR	2.8 nM	570 pM to 13 nM	530 pM	85 pM to 3.4 nM	5.8 nM	2.1 nM to 16 nM	8.6 nM	1.9 nM to 39 nM
rKOR	12 nM	2.3 nM to 59 nM	26 nM	2.2 nM to 310 nM	9.1 nM	3.6 nM to 23 nM	28 nM	5.5 nM to 140 nM
	Efficacy	C.I.	Efficacy	C.I.	Efficacy	C.I.	Efficacy	C.I.
hKOR	0.59	0.50-0.69	0.69	0.63-0.76	1.00	0.84-1.16	1.00	0.95-1.16
rKOR	0.52	0.41-0.63	0.58	0.49-0.66	1.00	0.83-1.17	1.00	0.80-1.19

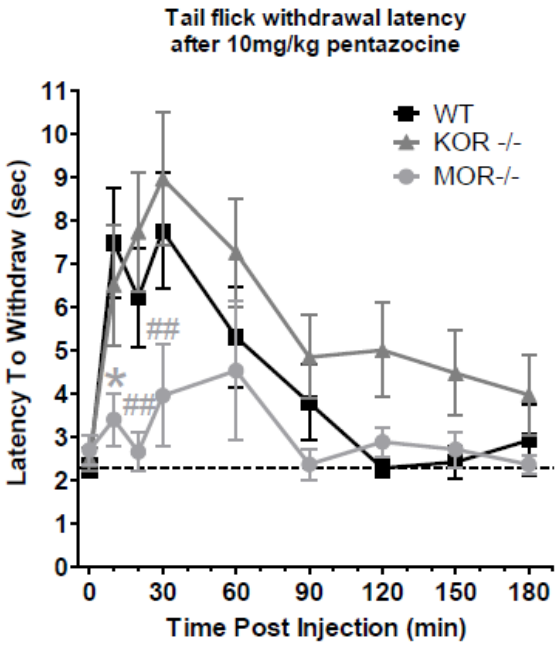
Table 2.1. Summary of pentazocine and U50,488 EC50 and Emax values. EC50 and Emax values and confidence intervals (CI) for p38 and ERK1/2 phosphorylation following pentazocine and U50,488 .

Figure 2.4. Behavioral effects of pentazocine in mice. A. C57BL/6 mice were trained in a balanced, unbiased three-chamber conditioning apparatus. The morning of each of the two training days was paired with vehicle (10mL/kg i.p. of 15% DMSO, 5% cremophor & 80% sterile saline), and the afternoon session with drug (pentazocine 10 mg/kg i.p., pentazocine 10 mg/kg + norBNI 10 mg/kg i.p., pentazocine 10 mg/kg + naloxone 5 mg/kg i.p., or pentazocine 10 mg/kg + norBNI 10mg/kg + naloxone 5 mg/kg). On the test day, mice were allowed to roam freely between all three compartments and the amount of time spent in each compartment was recorded. Scores were calculated by subtracting the time spent in the drug-paired side of the posttest from time spent in the saline-paired side of the posttest. Significant effect of drug treatment based on one way ANOVA ($p<0.0001$, n=4-10), with Bonferonni-Dunn post-hoc test with correction for multiple comparisons. B. Wild type, KOR-/-, and MOR-/- C57/Bl6 mice were injected with pentazocine 10mg/kg i.p. Tail flick withdrawal latency was recorded over 180 minutes post-injection. Two-way repeated measures ANOVA revealed a significant effect of interaction ($p<0.05$), time ($p<0.0001$), genotype ($p<0.05$), and subject matching ($p<0.0001$) (n=11-16). Significance according to Bonferroni-Dunn post-hoc test of the differences as compared to wild type or KOR-/- is indicated * or # respectively ($p<0.05$ for one mark, $p<0.01$ for two marks). C. Total distance traveled during training sessions for conditioned place preference testing in A was recorded. Significant effect of the specific drug treatment ($p<0.01$) and drug vs vehicle ($p<0.01$) based on two way repeated measures analysis of variance. Significance according to Bonferroni-Dunn post-hoc test of the differences for drug vs vehicle ($p<0.05$) is indicated by *.

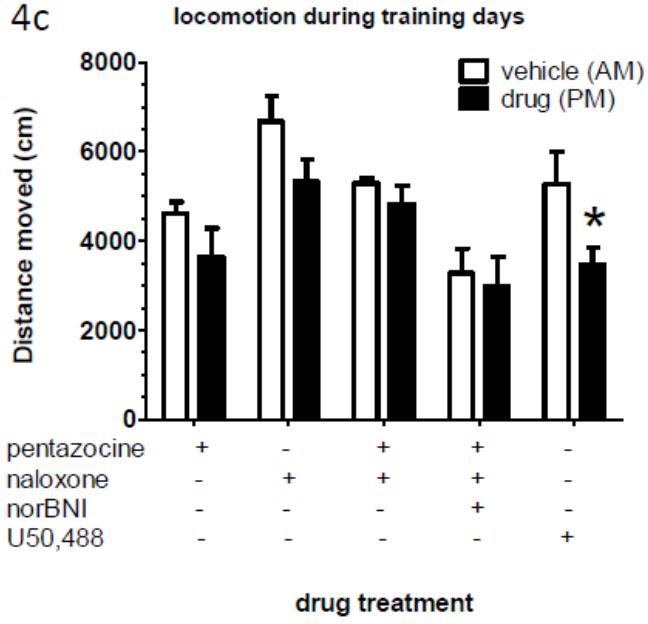
4a


Conditioned Place Preference
Time in drug paired chamber post-pre (sec)
300
200
100
0
-100
-200
-300
-400
-500
*
*
pentazocine
pentazocine+norBNI
pentazocine+naloxone
pentazocine+naloxone+norBNI

4b


Tail flick withdrawal latency after 10mg/kg pentazocine
Latency To Withdraw (sec)
11
10
9
8
7
6
5
4
3
2
1
0
WT
KOR -/-
MOR-/-
*
##
##
0
30
60
90
120
150
180
Time Post Injection (min)

4c


locomotion during training days
Distance moved (cm)
8000
6000
4000
2000
0
vehicle (AM)
drug (PM)
*
pentazocine + - + + -
naloxone - + + + -
norBNI - - - + -
U50,488 - - - - +
drug treatment

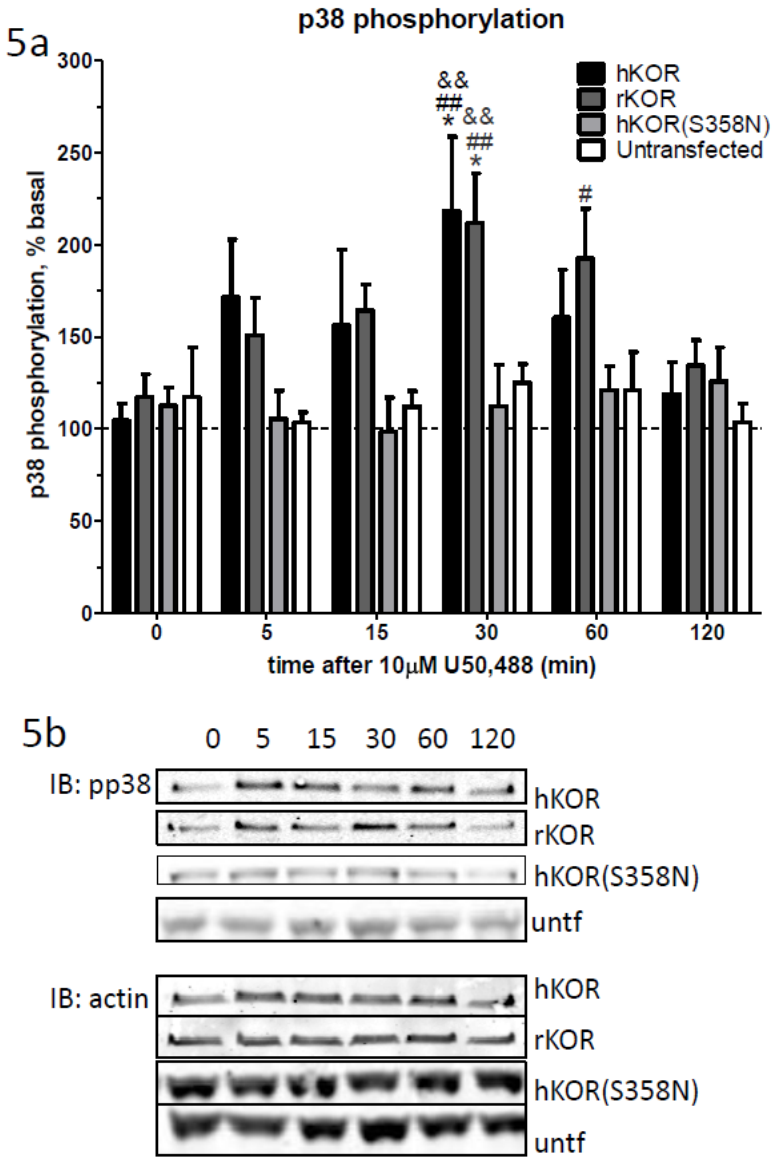
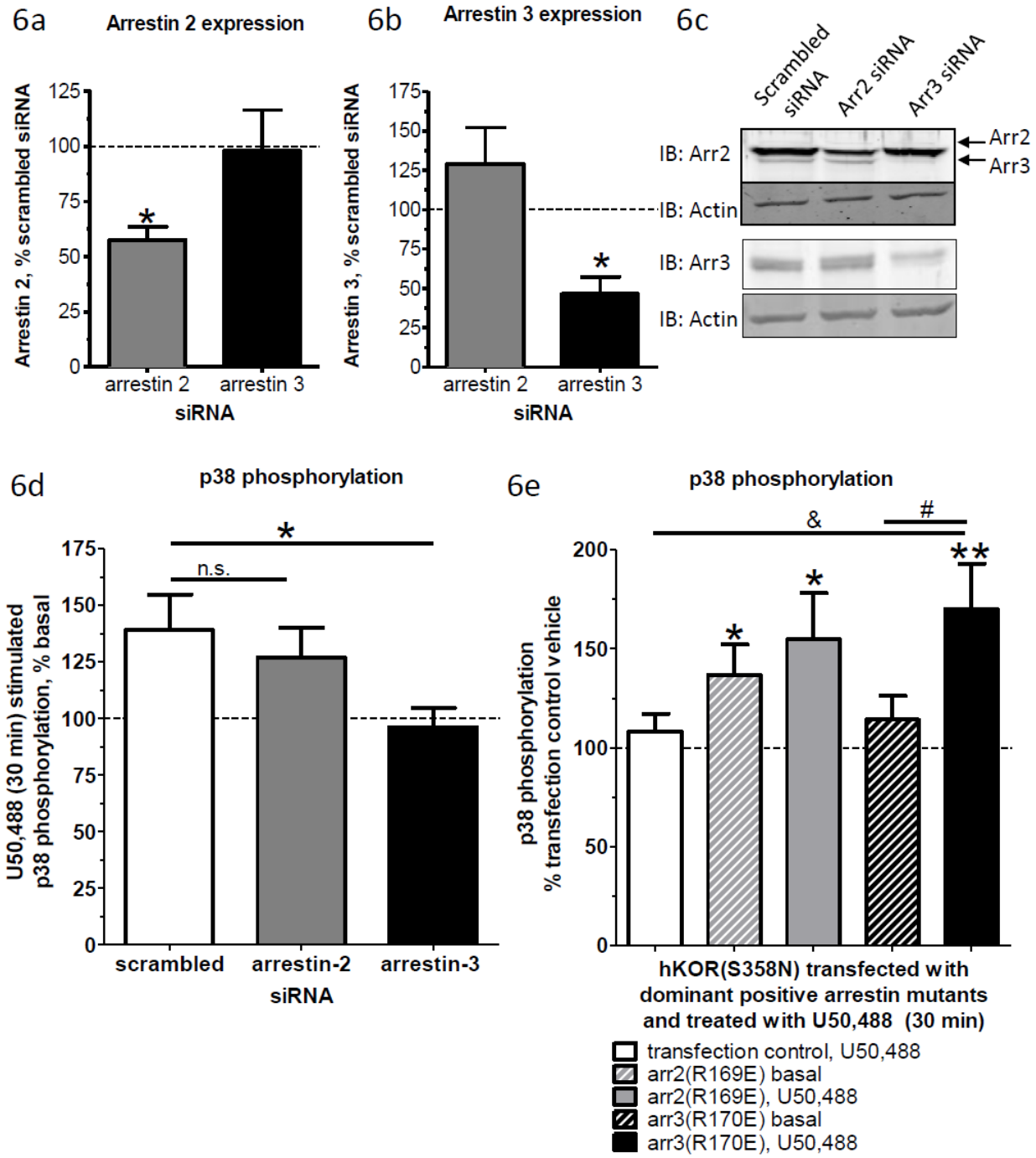


Figure 2.5. The S358 residue is required for hKOR activation of p38. A. HEK293 cells expressing hKOR, hKOR(S358N), rKOR, or untransfected wild type HEK293 were treated 5- 120 minutes with U50,488 (10μM) prior to cell lysis and immunoblotted for phospho-p38. Values represent mean ± SEM. Significant effect of cell type ($p<0.05$) and U50,488 treatment ($p<0.01$) based on two-way analysis ANOVA (n=8-16). Significance according to Bonferroni-Dunn post-hoc test, as compared to hKOR(S358N) , untransfected, or vehicle are indicated by *, &, or # respectively ($p<0.05$ for single mark or $p<0.01$ for double mark). B. Representative immunoblots for A.

Figure 2.6. Arrestin-3 is required for hKOR induced p38 phosphorylation. A. HEK293 cells expressing hKOR were transiently transfected with siRNA against arrestin-2, arrestin-3, or scrambled siRNA control. After 72 hours, cells were treated with U50,488 (10μM) 30 minutes prior to cell lysis and immunoblotted for arrestin-2. Significant decrease in arrestin-2 expression as compared to scrambled siRNA controls is indicated by *, one sample t-test ($p<0.001$, n=9). B. Cells were treated as in A, but immunoblotted for arrestin-3. Significant decrease in arrestin-3 expression as compared to scrambled siRNA controls is indicated by *, one sample t-test ($p<0.01$, n=5). C. Representative immunoblots for A and B. D. Cells were treated as in A, but immunoblotted against phopho-p38. Significance of the decrease in U50,488 induced p38 phosphorylation as compared to scrambled siRNA controls is indicated by *, student's t-test ($p<0.05$, n=14-16). E. HEK293 cells expressing hKOR(S358N) were transiently transfected with arrestin-2(R169E), arrestin-3(R170E), or no DNA (transfection reagent control). After 72 hours, cells were treated with U50,488 (10μM) 30 minutes prior to cell lysis and immunoblotted for phospho-p38. Significant increase in p38 phosphorylation relative to vehicle treated transfection reagent control is indicated by *, one sample t-test ($p<0.05$ for single mark or $p<0.01$ for double mark, n=21-23). Significant increase in p38 phosphorylation relative to U50,488 treated transfection reagent control or vehicle treatment for same transfection is indicated by & or # respectively, student's t-test ($p<0.05$, n=21-23).


6a
Arrestin 2 expression
Arrestin 2, % scrambled siRNA
125
100
75
50
25
0
*
arrestin 2
arrestin 3
siRNA
6b
Arrestin 3 expression
Arrestin 3, % scrambled siRNA
175
150
125
100
75
50
25
0
*
arrestin 2
arrestin 3
siRNA
6c
Scrambled siRNA
Arr2 siRNA
Arr3 siRNA
IB: Arr2
IB: Actin
IB: Arr3
IB: Actin
Arr2
Arr3
6d
p38 phosphorylation
U50,488 (30 min) stimulated p38 phosphorylation, % basal
175
150
125
100
75
50
25
0
*
n.s.
scrambled
arrestin-2
arrestin-3
siRNA
6e
p38 phosphorylation
p38 phosphorylation % transfection control vehicle
200
150
100
50
0
&
#
*
*
**
hKOR(S358N) transfected with dominant positive arrestin mutants and treated with U50,488 (30 min)
transfection control, U50,488
arr2(R169E) basal
arr2(R169E), U50,488
arr3(R170E) basal
arr3(R170E), U50,488

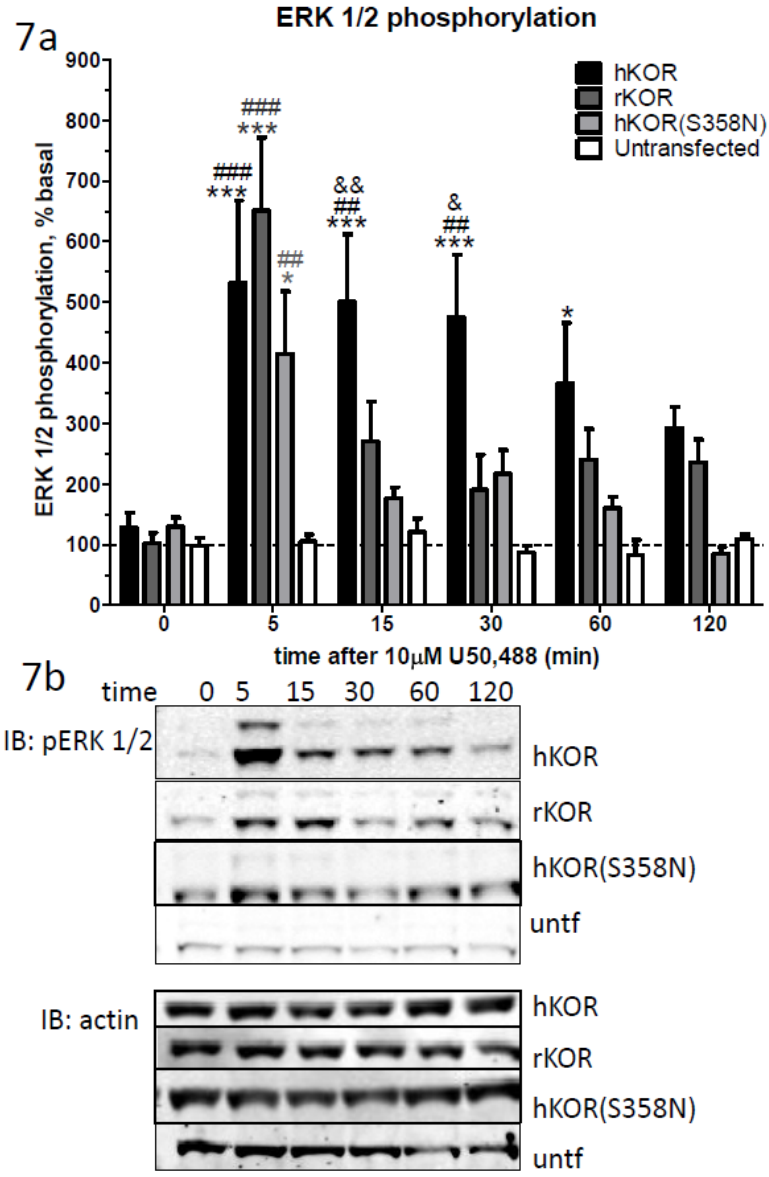


Figure 2.7. S358 is required for late phase, but not early phase, ERK1/2 activation. A. HEK293 cells expressing hKOR, hKOR(S358N), rKOR, and untransfected wild type HEK293 were treated 5- 120 minutes with U50,488 (10μM) prior to cell lysis and immunoblotted for phospho-ERK1/2. Significant effect of cell type ($p<0.01$) U50,488 treatment ($p<0.01$), and interaction ($p<0.05$) was revealed by two-way ANOVA (n=6-11). Significance of the differences, according to Bonferroni-Dunn post-hoc test, as compared to hKOR(S358N) , untransfected, or vehicle are indicated by *, &, or # respectively ($p<0.05$ for single mark, $p<.01$ for double mark, or $p<0.001$ for triple mark). B. Representative immunoblots for A.

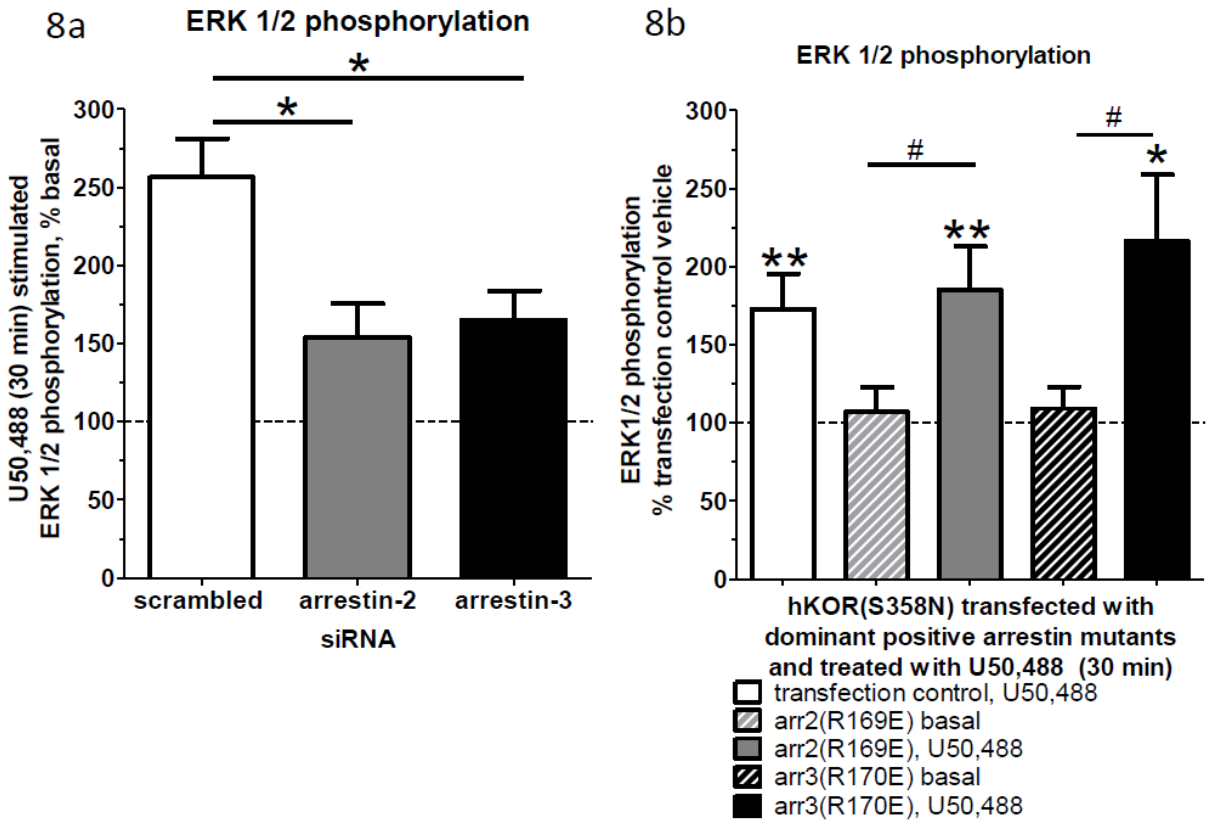


Figure 2.8. Both arrestin-2 and arrestin-3 are required for late phase hKOR activation of ERK1/2. A. HEK293 cells expressing hKOR were transiently transfected with siRNA against arrestin-2, arrestin-3, or scrambled siRNA control. After 72 hours, cells were treated with U50,488 (10μM) 30 minutes prior to cell lysis and immunoblotted for phospho-ERK1/2. Significance decrease in U50,488 induced ERK1/2 phosphorylation as compared to scrambled siRNA controls indicated by *, student's t-test ($p<0.01$, n=15-16). B. HEK293 cells expressing hKOR(S358N) were transiently transfected with arrestin-2(R169E), arrestin-3(R170E), or no DNA (transfection reagent control). After 72 hours, cells were treated with U50,488 (10μM) 30 minutes prior to cell lysis and immunoblotted for phospho-ERK1/2. Significant increase in p38 phosphorylation relative to vehicle treated transfection control is indicated by *, one sample t-test ($p<0.05$ for single mark or $p<0.01$ for double mark, n=20-22). Significant increase in ERK1/2 phosphorylation relative to vehicle treatment for same transfection is indicated by #, student's t-test ($p<0.05$, n=21-23).

Polymorphisms identified in the human kappa opioid receptor gene

Chromosome 8 Position	cDNA Position	SNP	Amino Acid Position	Effect	Minor Allele Frequency	Variant ID
54163669	intron	C>T	---	---	0.038	rs9282806
54163562	271	G>T	12	synonymous	0.108	rs1051660
54147470	694	C>T	153	synonymous	0.053	rs7815824
54147451	**713**	**G>A**	**160**	**val>met**	**0.005**	**---**
54147368	796	A>G	187	synonymous	0.005	---
54147331	**833**	**A>G**	**200**	**lys>glu**	**0.005**	**rs77476023**
54147250	intron	G>A	---	---	0.005	---
54147242	intron	G>A	---	---	0.021	rs16918900
54142157	1078	A>G	281	synonymous	0.258	rs702764
54142154	1081	C>T	282	synonymous	0.047	rs16918875
54142148	1087	C>T	284	synonymous	0.005	rs60353047
54142052	1183	C>T	316	synonymous	0.016	rs78517449
54141880	**1355**	**G>A**	**374**	**asp>asn**	**0.016**	**rs9282808**
54141824	1411	G>A	3' UTR	---	0.245	---

Table 2.2. Polymorphisms identified in KOR in human population

Contributions to other published studies on opioid receptor signaling

Antibody specificity controls for immunostaining demonstrating phosphorylation of the mu opioid receptor at Thr370/Ser375 following receptor activation.

Petraschka M, Li S, Gilbert TL, Westenbroek RE, Bruchas MR, **Schreiber S**, Lowe J, Low MJ, Pintar JE, Chavkin C. (2007) The absence of endogenous beta-endorphin selectively blocks phosphorylation and desensitization of mu opioid receptors following partial sciatic nerve ligation. *Neuroscience*. **146**(4):1795-1807.

The mu opioid receptor is the primary target of most clinically used opioid analgesics, but the consequences which limit their use include dependence and tolerance. Desensitization of MOR is once possible mechanism which may underlie opioid tolerance. Desensitization of MOR requires GRK3 and arrestin-2 (Kovoor 1998, Zhang 1998, Bohn 2000). Several potential phosphorylation sites within the C tail of MOR have been implicated in receptor desensitization and internalization. Site directed mutagenesis of MOR combined with heterologous expression in oocyte, HEK293, and AtT20 cell models have suggested a role for the Thr370 and Ser375 sites in MOR internalization and desensitization (El Kouhen 2001, Celver 2004, Schulz 2004). The Thr180 site within the second intracellular loop of MOR has also been implicated in MOR desensitization (Celver 2001, 2004). These studies suggest that phosphorylation of these residues may be important for MOR desensitization.

In order to investigate the role of phosphorylation at Thr180, Thr370, and Ser375 in vivo, antibodies selective for phosphorylation at these sites were developed. These antibodies were MORP1, generated against a peptide corresponding to MOR residues 171-186 phosphorylated at Thr180, and MORP2, generated against a peptide corresponding to MOR residues 364-378 phosphorylated at Thr370 and Ser375. No specific MOR phosphorylation was detected using the Thr180 antibody in cell expression systems or in vivo, under any condition tested. In contrast, an increase in MORP2 immunostaining was detected in the striatum of mice following fentanyl treatment. Increased MORP2

immunostaining was also detected in the striatum of wild type, but not β-endorphin knockout mice, following partial sciatic nerve ligation. In addition, partial sciatic nerve ligation produced tolerance to morphine in wild type but not β-endorphin knockout mice. These data showed that sciatic nerve ligation induced β-endorphin release, leading to desensitization of MOR, and that MOR activation in vivo, as a result of treatment with MOR agonists or stimulated of endogenous opioid release, results in phosphorylation of MOR at Thr370 and Ser375.

I contributed immunocytochemistry data outlined below to this study. The following studies demonstrate the specificity of the MORP2 antibody. These studies were previously published.

Methods

Cell Culture and Receptor Mutagenesis Stably transfected AtT20 Cells and HEK cells expressing the green fluorescent protein (GFP) conjugated MOR were used as described previously (Celver et al., 2004). Receptor mutants were created by designing PCR primers to introduce either a double amino acid (T370A/S375A) mutation in the C-terminal tail of the rat MOR cDNA or a C-terminal truncation after isoleucine-352. The constructs were ligated into pcDNA3-GFP37 vector provided by Dr. Kenneth Mackie (University of Washington, Seattle, WA) or pTARGET (Promega Biosciences, San Luis Obispo, CA), sequenced to confirm the mutagenesis and transfected into both HEK 293 and AtT20 cells using Lipofectamine (Invitrogen, Carlsbad, CA). Cells were maintained in DMEM supplemented with 2 mM L-glutamine, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 10% horse serum (AtT20 Cells) or DMEM /F12 supplemented with 2 mM L-glutamine, Penicillin/Streptomycin, and 10% Fetal Bovine Serum (HEK 293 cells). Cells were grown at 37°C in 5% CO2 and were passaged when 90% confluent.

Confocal Imaging Cells were plated at 75% confluency on poly-D-lysine coated cover slips 24 hours prior to treatment. Cells were treated as described in the text, fixed in 4% paraformaldehyde and incubated in 5 μg/ml primary antibody overnight at 4°C. Cover slips were incubated with a 1:10,000 dilution of Rhodamine conjugated anti-rabbit antibody (Jackson Immunoresearch Labs, West Grove, PA)

for 2 hours at room temperature. Cover slips were allowed to air-dry, were then mounted to slides using Vectashield (Vector Labs, Burlingame, CA) and imaged with a Leica TCS SP/MP Laser Scanning Confocal Microscope.

Results

HEK 293 cells and AtT20 cells transfected with MORGFP failed to show an increase in MORP1 immunoreactivity after 15, 30, 60 or 120 min treatment with agonist (0.1, 1 or 10 μM DAMGO or 0.1, 1 or 10 μM fentanyl) (data not shown). The results suggest that either the MORP1 antibody does not recognize the full-length receptor or that this site does not change phosphorylation state following agonist treatment. In contrast, transfected HEK or AtT20 cells treated with 1 μM DAMGO showed a robust increase in MORP2 immunoreactivity and translocation of the GFP-tagged MOR from plasma membrane into intracellular compartments, consistent with receptor internalization (Fig. 3.1). The increase in MORP2 immunoreactivity was blocked by pretreatment with 1 μM naloxone, was not evident after treatment with 1 μM naloxone alone, and was not evident in cells treated with 1 μM DAMGO but not incubated with primary antibody (data not shown). Site directed mutagenesis of MOR at Thr370 and Ser375 MOR(T370A/S375A) abolished the DAMGO-induced increase in MORP2 immunoreactivity (Fig. 3.1e). MORP2 immunoreactivity was not increased for DAMGO treated cells expressing MOR truncated after isoleucine-352 MOR(Δ352)-GFP (Fig. 3.1f). Similarly, AtT20 cells expressing MOR(S375A)-GFP did not increase MORP2 immunoreactivity following DAMGO treatment (data not shown). The single mutant construct MOR(T370A) did not efficiently express cell surface labeling in either AtT20 or HEK293 cells for reasons that we did not identify (data not shown). The results suggest that the MORP2 antibody was able to recognize phosphorylated MOR following agonist treatment.

Radioligand Studies demonstrating that norBNI does not alter KOR binding sites in vivo

Bruchas MR, Yang T, **Schreiber S**, Defino M, Kwan SC, Li S, Chavkin C. (2007) Long-acting kappa opioid antagonists disrupt receptor signaling and produce noncompetitive effects by activating c-Jun N-terminal kinase. *J Biol Chem*. **282**(41):29803-29811.

The selective KOR antagonist norBNI has been used extensively in research since its synthesis was first reported in 1987 (Portoghese 1987a,b), but has unusual pharmacological properties. In vitro competition binding assays indicate that norBNI is a competitive antagonist for KOR (Takemori 1988; Smith 1990), but norBNI has a long duration of action in vivo, with antagonist effects lasting up to three weeks in species ranging from mice to monkeys (Horan 1992, Jones 1992, Butelmann 1993, Broadbear 1994). These atypical properties are not unique to norBNI. The KOR antagonists JDTic and GNTI have since been synthesized (Jones 2000, Thomas 2001). Like norBNI, these ligand are competitive antagonists based on in vitro assays, but have long durations of action in vivo (Negus 2002, Carroll 2004).

The study examined the mechanism underlying the long lasting effect of norBNI and other long lasting antagonists. Three basic explanations were proposed for these effects: norBNI is not cleared from the site of action but instead remains trapped in the lipid membranes; norBNI is biotransformed in vivo into a metabolite which covalently binds KOR resulting in long lasting antagonism; norBNI uncouples the KOR signaling complex resulting in a loss of KOR signaling ability. The duration of norBNI antagonism of KOR agonist (U50,488) induced analgesia, measured by the warm water tail withdrawal assay, was used to measure norBNI actions in vivo.

Pretreatment with the short-acting, rapidly cleared KOR antagonists naloxone and buprenorphine prior to norBNI treatment prevented the long lasting antagonism of norBNI. These receptor protection experiments demonstrated that the duration of action of norBNI was not a result of poor clearance, as the

norBNI antagonism of KOR analgesia would be expected to continue when the short acting drugs had cleared. Saturation radioligand binding was performed on membranes prepared from mice treated with norBNI one week prior. At this timepoint, KOR analgesia was still inhibited, but no changes in receptor binding sites or affinity observed. These results were consistent with prior in vitro studies showing KOR is a competitive antagonist, and shows that in vivo KOR is not covalently bound by norBNI or a norBNI metabolite. It was found that norBNI, GNTI, and JDTic all activate JNK via KOR, despite having antagonist activity in a wide range of other assays. Pretreatment with a JNK inhibitor prior to norBNI treatment blocked the long lasting antagonism of KOR, showing that this collateral agonist activity at JNK was required for the long duration of action of these antagonists.

I contributed the saturation radioligand binding data outlined below to this study. These experiments demonstrate that neither norBNI nor the receptor protection by buprenorphine alter KOR binding sites in vivo. These studies were previously published.

Methods

Radioligand Binding Whole mouse brains with the cerebellum removed were homogenized in ice-cold 50 mM Tris buffer, pH 7.5, followed by centrifugation at 26,000 × g for 30 min at 4 °C. Three brains were pooled for each independent replicate with the pellet collected as the membrane fraction. Homogenization and centrifugation were repeated twice more to wash the membrane fractions and remove residual ligand present in the preparation. After the final centrifugation step, excess buffer was removed, and the membranes were stored at −80 °C until use. Membranes were resuspended in ice-cold Tris buffer. Protein concentrations were determined by BCA assay. Samples were incubated for 90 min at room temperature with the KOR ligand [3H]U69,543. Nonspecific binding was determined in the presence of 10 μM U50,488. GF/B glass fiber filters (Brandel) were preincubated for 90 min at room temperature with Tris buffer, 0.3% polyethyleneimine. After 90 min of incubation at room temperature, samples were placed on ice and collected with the filters with a Brandel 24 well harvester. Filters were

washed 3× with cold Tris buffer and counted in 5 ml of Ecoscint scintillation fluid. For saturation binding, [3H]U69,543 was tested at concentrations ranging from 0.156 to 20 nM. Radioligand concentrations were confirmed by scintillation count of free ligand. Saturation binding isotherms were fit using non-linear regression (Prism 4.0), and Scatchard plots were fit using linear regression analysis. Statistical significance was taken as $p < 0.05$ or $p < 0.01$ as determined by the Student's t test or ANOVA followed by a Bonferroni post hoc test where appropriate.

Results

Single injections of norBNI followed by saturation binding experiments 1 week later were performed to assess whether norBNI exposure affected KOR receptor agonist affinity or KOR receptor density. Mice were injected with norBNI (10 mg/kg intraperitoneally), and brain membranes were prepared on day 8 for radioligand binding. Using the selective agonist [3H]U69,593, we found no significant difference in the total receptor density (Bmax) or the affinity for κ agonist (Kd) between norBNI-treated (Bmax = 21 ± 2 fmol/mg of protein, Kd = 1.65 ± 0.4 nM) and saline-treated (Bmax = 21 ± 1 fmol/mg of protein, Kd = 1.0 ± 0.2 nM) mouse brain membranes taken 7 days after injection of norBNI (Fig. 3.2A and Table 3.1). These data are consistent with previous reports demonstrating that norBNI does not form a covalent bond to the receptor and does not decrease apparent receptor number. The effect of receptor protection on membranes from norBNI-treated mice was also measured. In this experiment mice were pretreated with buprenorphine (3 mg/kg intraperitoneally) 1 h before injection of norBNI (10 mg/kg intraperitoneally), and membranes were prepared on day 8 after these initial injections. There was also no significant change in either the Bmax or Kd of [3H]U69,593 (Fig. 3.2B and Table 3.1), further supporting the conclusion that the long duration of action of norBNI cannot be attributed to changes in receptor density or affinity.

Development of lentiviral expression systems as tools to study the function of KOR expressed in specific regions of the central nervous system and the role of phosphorylation of KOR at Ser369 in vivo.

Land BB, Bruchas MR, **Schattauer S,** Giardino WJ, Aita M, Messinger D, Hnasko TS, Palmiter RD, Chavkin C. (2009) Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. *Proc Natl Acad Sci U S A.* **106**(45):19168-19173.

Activation of KOR by agonist treatment stress produces a conditioned place aversion. KOR agonist conditioned place aversion requires p38 activation (Bruchas 2007), and phosphorylation of KOR at Ser369 by GRK3 is required for arrestin recruitment and p38 activation (McLaughlin 2003, Bruchas 2006). Stress induces release of the endogenous KOR ligand dynorphin, activating KOR in regions that include the ventral tegmental area and dorsal raphe, implicating these dopaminergic and serotonergic nuclei (Land 2008). The dopaminergic system has been implicated in drug abuse and addiction (Wise 2004), but dopamine deficient mice still display conditioned place preference to morphine and cocaine (Hnasko 2005, 2007), suggesting that other regions and neurotransmitters also play important roles. Serotonergic systems regulate stress and mood (Amat 2005, Zhao 2007), suggesting a possible role for serotonergic projections from the dorsal raphe.

This study examined the role of the dorsal raphe in KOR-mediated behaviors. Local injection of norBNI into the nucleus accumbens blocked U50,488 conditioned place aversion, but dopamine deficient mice stilled displayed conditioned place aversion to the KOR agonist U50,488. Local injection of norBNI into the dorsal raphe blocked U50,488 conditioned place aversion in addition to KOR analgesia and stress-induced reinstatement of cocaine conditioned place preference. To determine if KOR in cells originating in the dorsal raphe is sufficient for analgesia and conditioned place aversion in response to KOR agonists, a lentivirus expressing KOR was injected into the dorsal raphe of KOR knockout mice. Expression of lenti-KOR in the dorsal raphe was sufficient to recover U50,488 conditioned place aversion

and analgesia. In contrast, expression of lenti-KOR(Ser369Ala) in the dorsal raphe recovered U50,488 analgesia but not U50,488 conditioned place aversion. Injection of norBNI into the nucleus accumbens blocked U50,488 conditioned place aversion in mice expressing lenti-KOR in the dorsal raphe. These experiments show that KOR activation and phosphorylation at Ser369 in dorsal raphe neurons projecting to the nucleus accumbens is sufficient for conditioned place aversion to KOR agonists.

My contribution to these studies was the development of the lentiviral vectors used in the studies. This reagent was crucial to the experiments below demonstrating that KOR expression in the dorsal raphe is sufficient for analgesic and aversive responses to KOR activation and that the Ser369 site of KOR is required in vivo for aversion. These studies were previously published.

Methods

Viral Vector Design and Production Three constructs were developed based on the lentiviral construct expressing the β2 subunit of the nicotinic acetylcholine receptor under the mouse phosphoglycerol kinase (PGK) promoter (Maskos 2005). The lenti-KOR vector is a bicistronic construct expressing KOR and GFP. Lenti-KOR(S369A) is a monocistronic construct containing a mutant form of the KOR where serine 369 was mutated to alanine. A construct expressing only eGFP was used as a negative control. In each case, gene expression was under the control of the PGK promoter. The integrated virus was rendered replication incompetent by deletion of the U3 region of the 3′ long terminal repeat (Zufferey 1998, Sirven 2001). Sequences were incorporated to enhance RNA stability, transgene expression, and infection of nondividing cells (Zennou 2001), and the viral expression plasmid was inserted into the pUC18 plasmid. Viral particles were produced by the Fred Hutchinson Cancer Research Center, Seattle, WA. Briefly, viral particles were produced by cotransfection of the vector plasmid with a packaging plasmid and the VSV-G envelope plasmid; at 24–72 h following transfection, media were collected, and viral particles were isolated by filtration and ultracentrifugation. Virus was tested for replication competency by ELISA against the p24 capsid protein over a course of at least 3 weeks.

Microinjection Procedure (norBNI and Lentiviral Constructs) Isoflorane-anesthetized mice were mounted on a stereotaxic alignment system (David Kopf Instruments). A bevel-tipped Hamilton syringe was lowered into the dorsal raphe (x = 0.0, y = −4.65, z = −3.85 mm from bregma) or bilaterally into the NAc (x = 0.75, y = +1.45, z = −5.00 mm from bregma) and either norBNI (2.5 μg/side) or one of the three viral constructs (dorsal raphe only) were injected at a rate of 100 nL/min for 10 min (1 μL total). The syringe remained in place for at least 3 min after the infusion was finished and was then slowly removed. Animals were sutured with 5–0 polyviolene sutures (Sharpoint) and allowed to recover for at least 5–7 days before behavioral testing began. Virally injected animals recovered for at least 3 weeks, allowing adequate time for expression and distribution of protein.

Conditioned Place Aversion. Mice were trained in an unbiased, balanced three-compartment conditioning apparatus as described (Land 2008). Briefly, mice were pre tested by placing individual animals in the small central compartment and allowing them to explore the entire apparatus for 30 min. Time spent in each compartment was recorded with a video camera (ZR90; Canon) and analyzed using Ethovision software (Noldus). Mice were assigned to saline and drug compartments and received saline in the morning (10 mL/kg, i.p.) and U50,488 (2.5 mg/kg, i.p.) in the afternoon at least 4 h after the morning training on 3 consecutive days. CPA was assessed on day 4 by allowing the mice to roam freely in all three compartments and recording the time spent in each. Scores were calculated by subtracting the time spent in the U50,488-paired compartment post test minus the pre test.

Tail-Flick Analgesia The response latency for the mouse to withdraw its tail from immersion into 52.5 ± 1 °C water was measured using a stopwatch before and 30 min after U50,488 administration (10 mg/kg, i.p.).

Results

We next assessed whether KOR activation in the DRN was also sufficient for this behavior. We generated a bicistronic lentiviral vector based on a construct developed by the Changeux group (Maskos

2005) to express KOR-GFP under a PGK promoter (lenti-KOR) (Fig. 3.3A, Upper) in KO mice lacking KOR (KOR KO) (Hough 2000). As a control, we also engineered a lenti-vector to express KOR(S369A), a serine to alanine point mutation at position 369 in the C-terminal domain that allows the receptor to activate Gαi-mediated responses, but is not phosphorylated by GRK3 (schematically diagramed in Fig. 3.3A, Lower) (McLaughlin 2003). GRK3 phosphorylation of KOR is necessary for β-arrestin-dependent activation of p38 mitogen-activated protein kinase (MAPK) (Bruchas 2006), and p38 MAPK activation is required for KOR-dependent aversion (Bruchas 2007). A second control lentiviral vector containing only eGFP was generated to assess nonspecific effects of viral-mediated gene transfer on behavior (Fig. 3.3A).

Three weeks after injection of lenti-KOR into the DRN of mice lacking the endogenous receptor (KOR KO), systemic U50,488 administration increased KORp-immunoreactivity and phospho-p38-ir in the DRN, whereas mice injected with either lenti-KOR(S369A) or lenti-eGFP vectors showed no such increase (Fig. 3.3B). These results are consistent with previous in vitro results using these KOR constructs in heterologous expression systems. This enhanced KORp-immunoreactive staining was visualized only in cells that also expressed eGFP (Fig. 3.4A) and strongly co-localized with serotonergic DRN cells expressing tryptophan hydroxylase (TPH) (Fig. 3.4B). Greater than 90% (± 3%) are both KOR and TPH positive inside the "fountain" of the DRN, whereas 75% (± 5%) are both KOR and TPH positive throughout the DRN. Although unable to increase KOR-P-immunoreactivity or phospho-p38 MAPK-immunoreactivity, lentiviral KOR(S369A) expression restored robust ERK activation (p-ERK) in DRN neurons following U50,488 administration (Fig. 3.3B). These results confirm that the Gαi functionality of KOR was intact (Bruchas 2007), in concordance with reports showing that KOR-mediated ERK activation does not require GRK3 or arrestin expression (Belcheva 1998, Bruchas 2006).

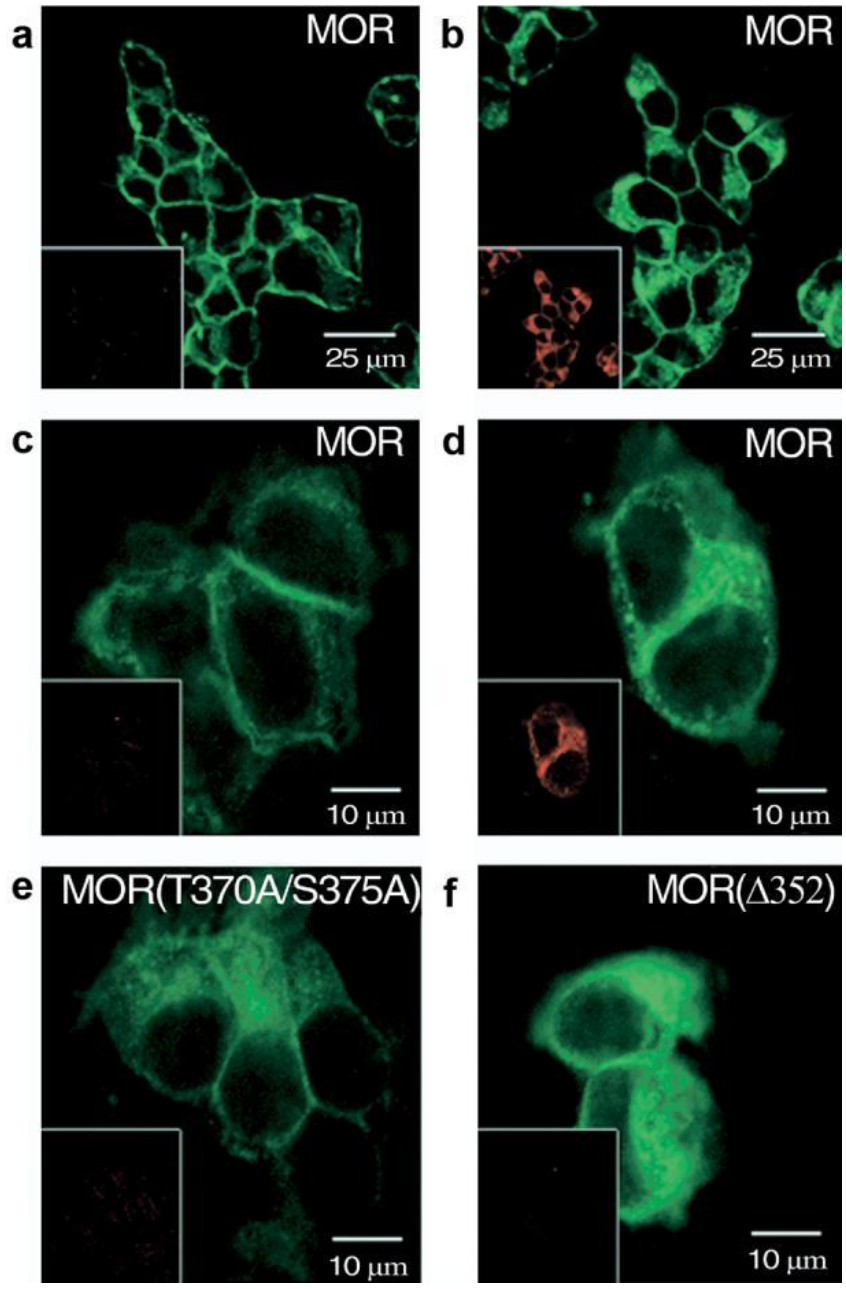
KOR KO mice did not develop place aversion after systemic U50,488 injection; whereas KOR wild-type (WT) littermate mice developed aversion (Fig. 3.5A). Lenti-KOR injection into the DRN of KOR KO mice completely rescued the KOR-agonist-induced CPA (Fig. 3.5A), whereas neither lenti-KOR(S369A)- nor the lenti-eGFP-injected mice developed U50,488-induced place aversion (Fig. 3.5A).

As a behavioral control for receptor functionality, lenti-KOR(S369A)-injected mice showed analgesic responses comparable to lenti-KOR-injected mice following U50,488 (Fig. 3.5B). In contrast, control lenti- eGFP injection in DRN of KOR KO mice did not restore the antinociceptive response to U50,488 (Fig. 3.5B). The selective rescue of the antinociceptive response by KOR(S369A) further supports the conclusion that KOR activation in the DRN regulates pain responses. The ability of the KOR(S369A) receptor to restore the analgesic, but not aversive response is consistent with a GRK3/phospho-p38-dependent role of KOR in mediating the aversive response to U50,488 (Bruchas 2007). These data also suggest that KOR activation of p38 MAPK in the DRN is sufficient to produce CPA. The combination of local lentiviral expression in KO mice represents an effective technique for determining G-protein-coupled receptor-dependent behaviors.

Serotonergic projections from the DRN innervate nearly all forebrain regions, including the NAc. The NAc has been widely associated with the regulation of hedonic state (Reynolds 2002) and was therefore a plausible projection site for KOR-containing neurons originating in the DRN to affect mood. Consistent with this concept, local injection of norBNI into the rostral NAc (Fig. 3.6B) also significantly attenuated U50,488-induced CPA of WT mice (Fig. 3.6A). To assess whether the site of norBNI action in NAc was pre- or postsynaptic, we injected lenti-KOR into the DRN of KOR KO mice, then injected norBNI or saline into the NAc 3 weeks later (Fig. 3.6C). Mice receiving lenti-KOR in the DRN and saline in the NAc showed CPA to U50,488; whereas KOR KO mice receiving lenti-KOR in the DRN and subsequent norBNI in the NAc did not develop aversion (Fig. 3.6D). This experiment suggests that norBNI injection in the NAc blocked the functioning of KOR expressed on nerve terminals of DRN cells, because only axons from the lenti-KOR-injected DRN cells express KOR in the KO mice.

Figure 3.1. DAMGO treatment increases MORP2 immunoreactivity of wild-type MOR-GFP. (A) Confocal images of GFP-conjugated MOR (green) with Rhodamine labeled MORP2 immunoreactivity images (red, inset). MORP2 antibody specifically labels AtT20 cells transfected with the mu-opioid receptor in the presence (B) of 1 μM DAMGO for 30 min at 37°C. HEK293 cells transfected with wild-type MOR that were untreated (C) or treated with DAMGO as above (D) showed increased MORP2 immunoreactivity (inset). A MOR construct with double amino acid substitution of MOR eliminating the two phosphorylation sites (T370A/S375A) was transfected into HEK 293 cells and showed no labeling of healthy cells in the presence of agonist (E). The C-terminal truncation mutant MOR-(Δ352) transfected into HEK 293 cells also showed no DAMGO-induced staining (F)

.


a
MOR
25 μm
b
MOR
25 μm
c
MOR
10 μm
d
MOR
10 μm
e
MOR(T370A/S375A)
10 μm
f
MOR(Δ352)
10 μm

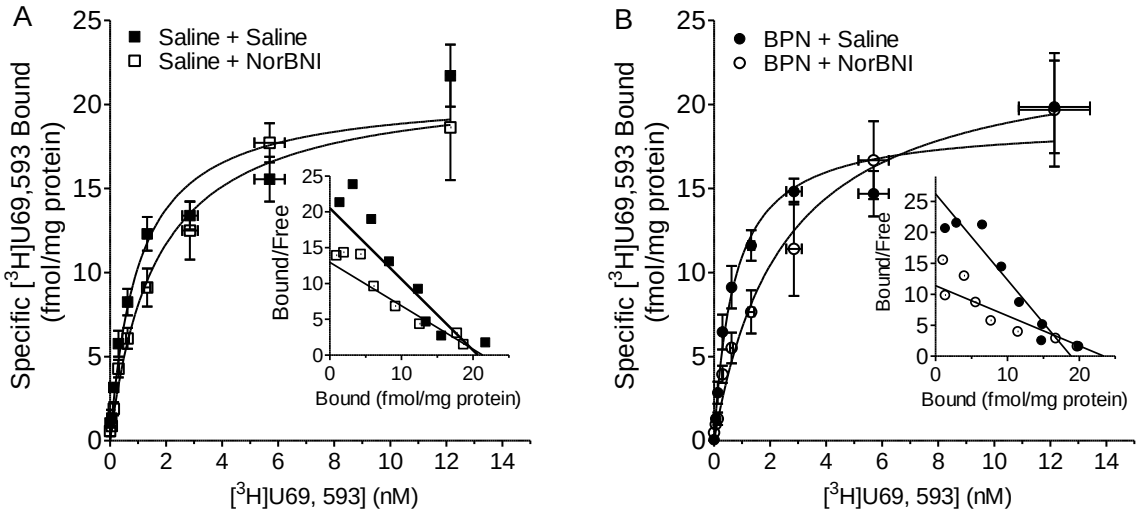


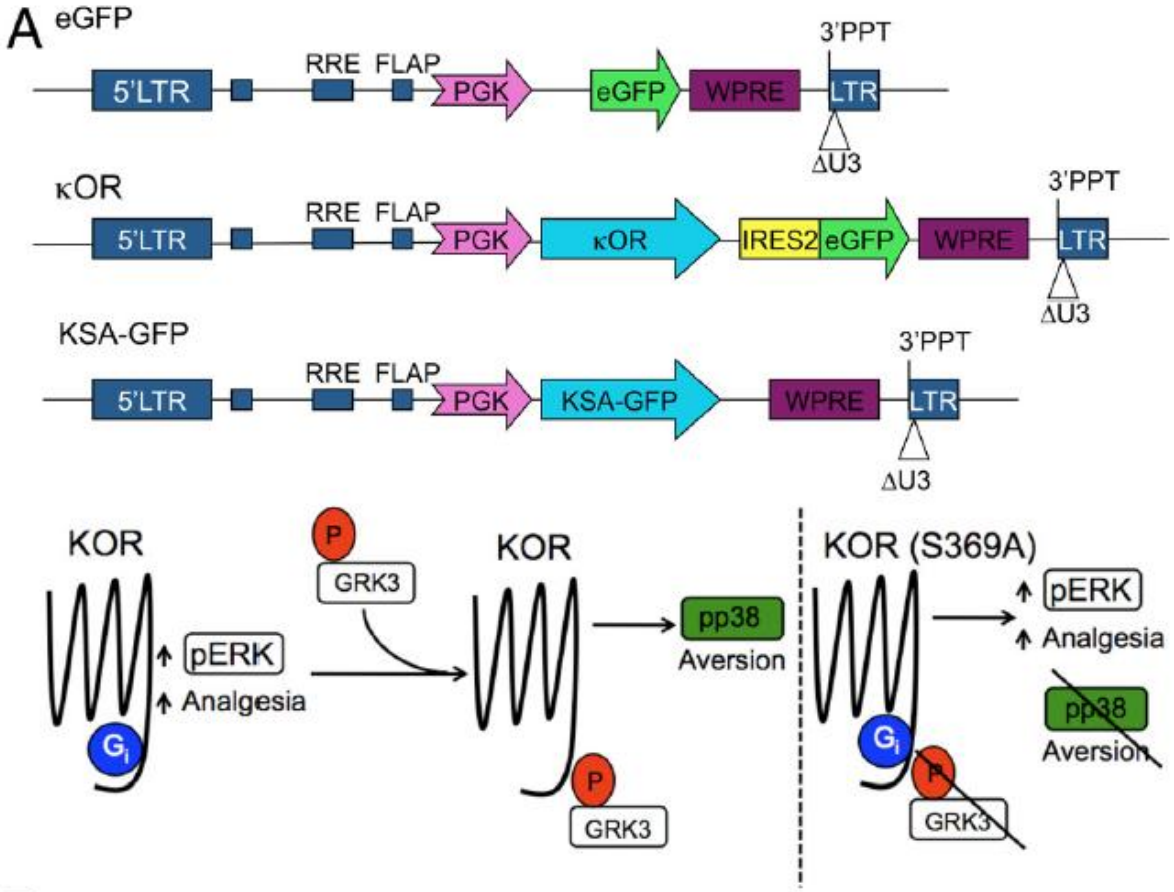
Figure 3.2. NorBNI does not alter KOR binding characteristics. *A*, mean saturation binding isotherms for specific [^{3}H]U69,593 (a KOR-selective agonist) in membranes from mouse brain on day 8 after norBNI (10 mg/kg, intraperitoneal) or saline. Concentrations of total [^{3}H]U69,593 ranging from 0.156 to 20 nM were incubated with membranes in the presence (nonspecific binding) and absence (total binding) of 10 μM U50,488 as described under "Experimental Procedures." Specific binding was determined by subtracting nonspecific from total binding counts and converted into fmol/mg of protein. Non-linear regression of five individual binding curves show that [^{3}H]U69593 bound to a homogenous receptor population and had similar affinity and receptor density in both the norBNI group (B_{max} = 21 ± 1 fmol/mg of protein, K_d = 1.00 ± 0.20 nM) and the saline-treated group (B_{max} = 21 ± 2 fmol/mg of protein, K_d = 1.65 ± 0.40 nM). The *inset* shows the mean data ± S.E. in Scatchard plots that were derived from the saturation isotherms. n = 5, where each n is a different experiment. *B*, mean saturation binding isotherms for specific [^{3}H]U69,593 and measured as indicated above. Non-linear regression of five individual binding curves show that under conditions known to protect KOR from norBNI, [^{3}H]U69,593 bound to a homogenous receptor population in both the BPN (B_{max} = 19 ± 1 fmol/mg of protein, K_d = 0.73 ± 0.16 nM) and BPN + NorBNI (B_{max} = 23 ± 3 fmol/mg of protein, K_d = 2.50 ± 0.78 nM) groups with similar affinity and total receptor numbers. The *inset* shows the mean data ± S.E. in Scatchard plots that were derived from the saturation isotherms. n = 5, where each n is a different experiment.

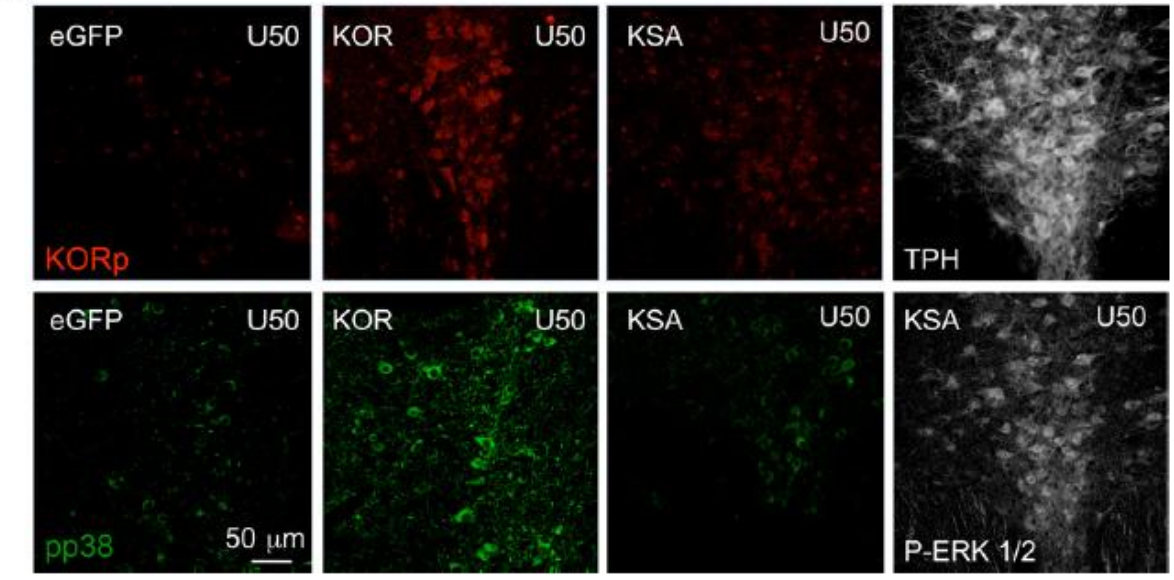
NR, no response.

Group	[^{3}H]U69,593		pJNK	
	K_d	B_{max}	EC_{50}	IA
	nM	*fmol/mg*	*nM*	
Saline	1.00 ± 0.20	21 ± 1		
NorBNI	1.65 ± 0.40	21 ± 2	158 ± 18	0.84
Buprenorphine + saline	0.73 ± 0.16	19 ± 1		
Buprenorphine + NorBNI	2.50 ± 0.78	23 ± 3		
U50,488			1240 ± 180	1.00
Dynorphin B			87 ± 13	0.61
JDTic			5 ± 3	0.57
Buprenorphine			NR	NR

Table 3.1 Affinity values, total binding site densities, and pharmacological data for KOR ligands.

Figure 3.3 Viral injection results in functional expression of KORs. (*A*, *Upper*) Schematic of the three lentiviral constructs used. (*Lower*) schematic of KOR signaling through Gαi and GRK/β-arrestin and the behaviors each is thought to control. KOR(S369A) can only signal through the Gαi upon activation. (*B*) Representative photomicrographs of KORp-immunoreactivity (red) and phospho-p38 (pp38)-immunoreactivity (false-green) in the DRN of KOR KO animals injected with the three lentiviral constructs. Photomicrographs of cells expressing TPH (serotonin cells) and *p*-ERK1/2 in KOR(S369A) (KSA) are also shown in white.


A
eGFP
5'LTR
RRE
FLAP
PGK
eGFP
WPRE
LTR
3'PPT
ΔU3
κOR
κOR
IRES2
KSA-GFP
KSA-GFP
KOR
P
GRK3
pERK
Analgesia
Gi
pp38
Aversion
KOR (S369A)


B
eGFP
U50
KOR
KSA
KORp
TPH
pp38
50 μm
P-ERK 1/2

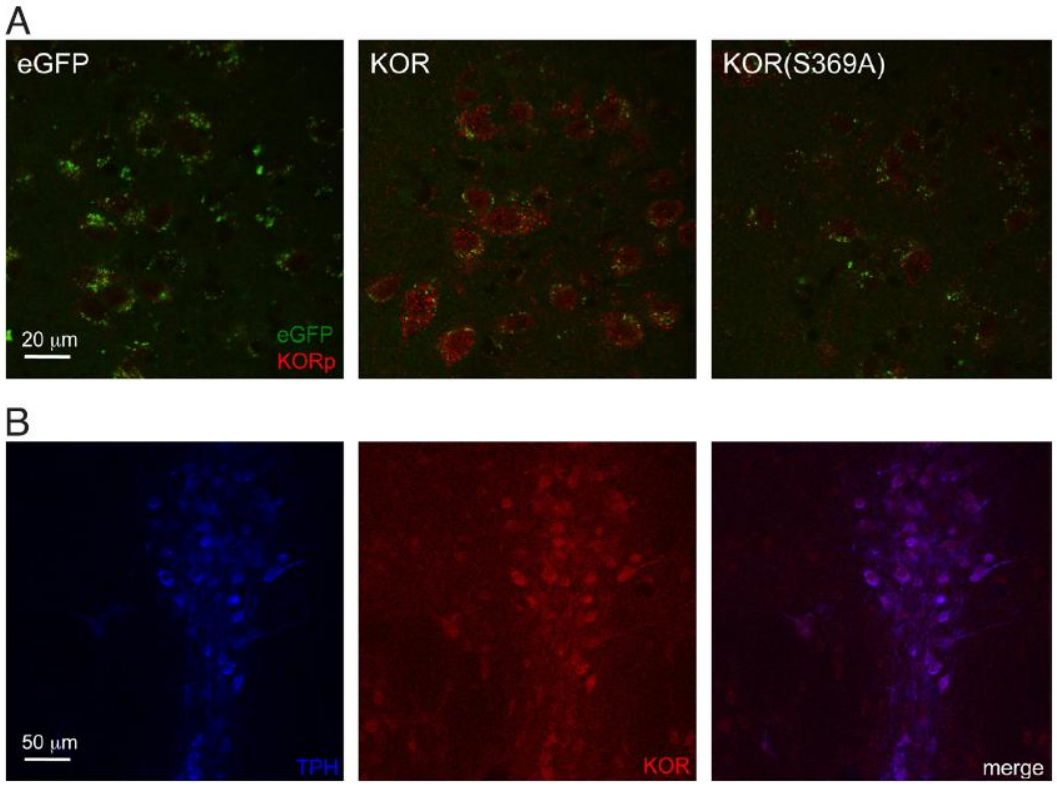


Figure 3.4. Functional KOR is expressed only in neurons containing eGFP and on serotonin neurons. (*A*) Confocal photomicrographs showing eGFP (green) and KORp (red) in the DRN of animals injected with one of the three lentiviral constructs 3 weeks previously. (*B*) Confocal photomicrographs of DRN in a lenti-KOR-injected KOR KO animal, showing overlap of TPH (blue) and KOR (red).

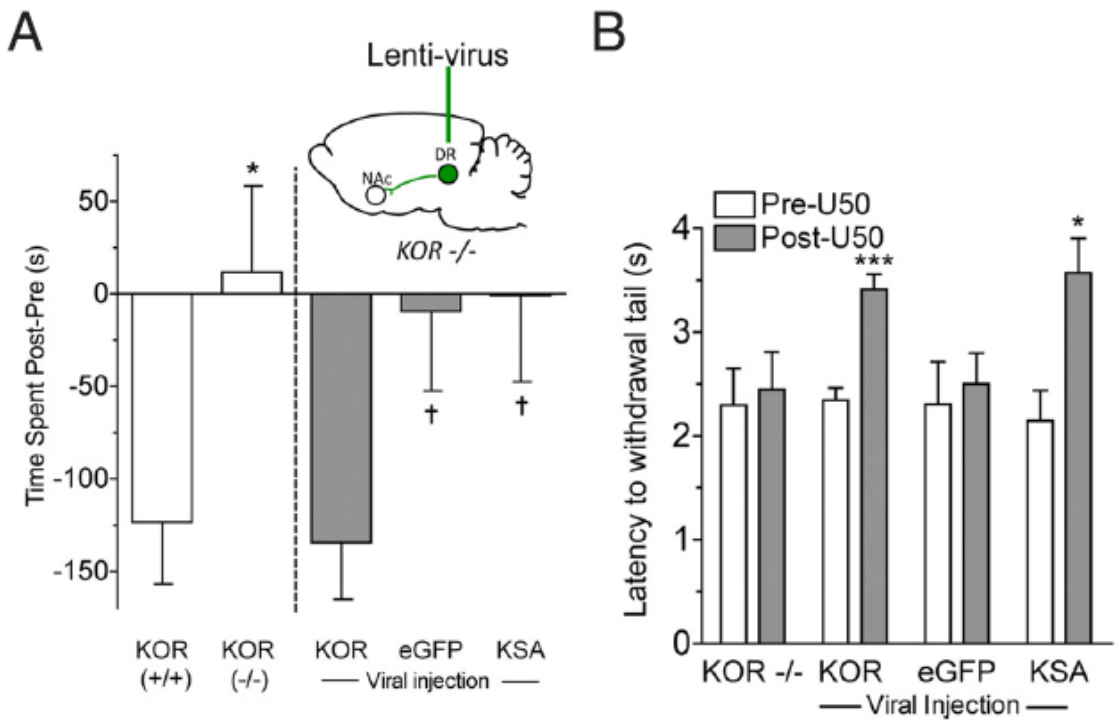


Figure 3.5. Viral expression of KOR in the DRN of KOR KO mice rescues the response to agonist. (*A*) CPA scores induced by U50,488 (2.5 mg/kg) for KOR WT, KOR KO, and lentiviral-injected KOR KO animals. (Inset) Sagittal schematic illustrating the DRN injection site (n = 9–12; *, $P < 0.05$ *t*-test compared to KOR KO; ANOVA $F_{2,31} = 3.55$; † $P < 0.05$ Bonferroni posthoc compared to KOR). (*B*) Tail withdrawal latency before and after a 10 mg/kg U50,488 injection for KOR KO and lentiviral-injected KOR KO animals (n = 4–11; *, $P < 0.05$, ***, $P < 0.001$ *t*-test compared to pre-U50). Bars represent mean ± SEM.

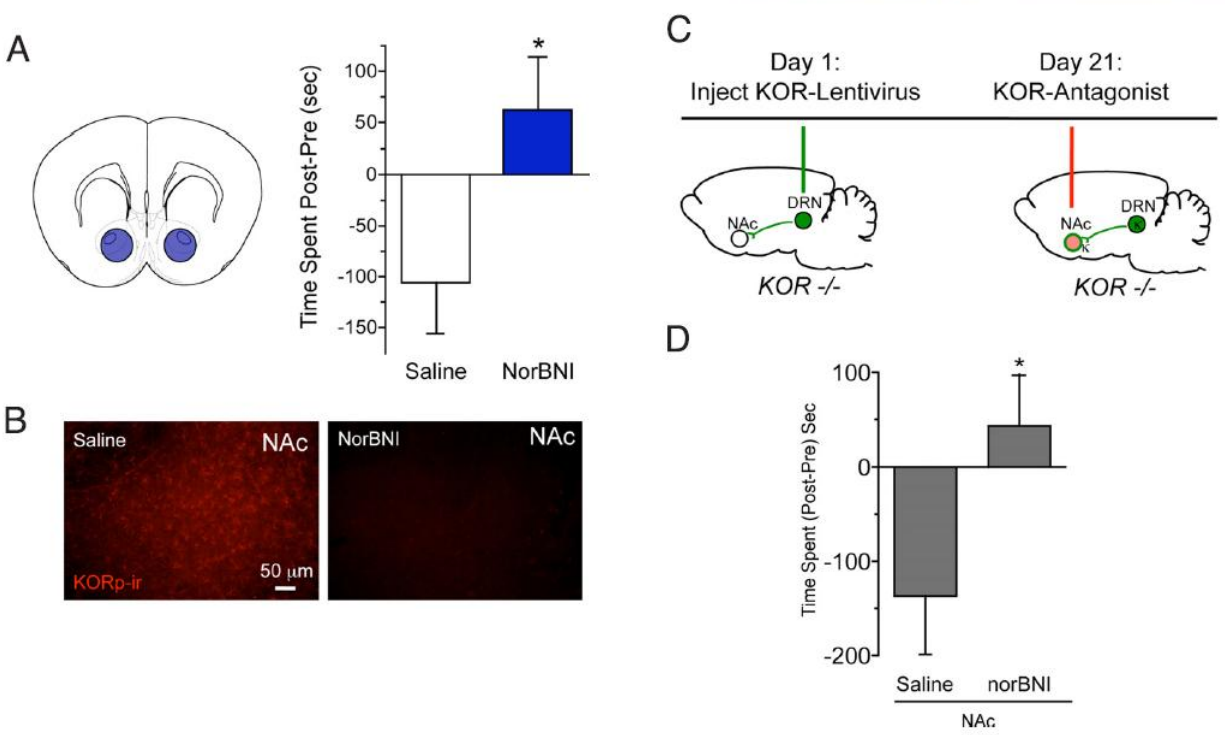


Figure 3.6. A DRN-NAc circuit regulates KOR CPA. (A, left) Coronal section of the NAc highlighting saline and norBNI injection areas (+1.45 mm from bregma); (right) U50,488-induced CPA scores for NAc-injected animals (n = 10; *, P < 0.05 t-test compared to saline). (B) Representative photomicrographs of KORp-immunoreactivity in the NAc after saline or norBNI injection, perfused 30 min after U50,488 injection. (C) Schematic of double-injection schedule for DRN and NAc place aversion study. (D) U50,488-induced CPA scores for lenti-KOR-DRN, saline/norBNI-NAc animals (n = 7–11; *, P < 0.05 t-test compared to saline). Bars represent mean ± SEM.

Discussion

New insights in opioid receptor signaling

This thesis adds to the body of research demonstrating that opioid receptors in general and the kappa opioid receptor in particular have their effects through multiple signaling cascades which are pharmacologically distinguishable. Classically, opioid receptors are phosphorylated within their C tail by GRKs, terminating receptor signaling. MOR is phosphorylated in response to agonist stimulation at multiple residues within the C tail which were shown in the third chapter of this thesis to include the Thr370 and Ser375 residues, while KOR is phosphorylated at a single residue. Phosphorylation of the C tail of opioid receptors results not only in desensitization of G protein-dependent signaling, but the initiation of a separate arrestin-mediated set of signal events.

In the case of KOR, these signaling events include activation of the p38 and ERK1/2 MAPKs. Experiments in the third chapter of this thesis demonstrate that a single GRK3 phosphorylation site, Ser369, within the C tail of rodent KOR is required in vivo not only for p38 phosphorylation, but some of the hallmark behavioral effects of KOR activation. This phosphorylation site is required for the development of aversion in response to KOR agonists, but not analgesia. These studies are in agreement with prior studies showing that GRK3 and p38 are required for KOR-mediated aversion, and add to the body of research suggesting that a KOR agonist which activated G protein signaling but not receptor phosphorylation and arrestin recruitment would have clinical utility as an analgesic. Increasing evidence exists that not all agonists necessarily activate all signaling cascades equally and that it is possible to develop ligands with biased signaling properties which preferentially activate specific pathways.

The second chapter of this thesis adds promise to this idea by showing that activation of human KOR also induces phosphorylation of p38 through a receptor phosphorylation and arrestin-dependent mechanism, despite species differences in receptor GRK phosphorylation sites. Therefore KOR agonists which do not efficiently promote phosphorylation of human KOR would also be unlikely to induce p38

activation which is implicated in the aversive response to KOR agonists, and therefore may produce less aversive responses in people. This chapter also brings up some concerns however regarding the development of a KOR analgesic using this approach. Some KOR agonists, such as U50,488, have comparable ability to activate arrestin-dependent signaling via both human and rodent KOR. Other agonists however, such as pentazocine, have similar potency for G-protein mediated signaling through both receptors, but markedly different potencies for arrestin-mediated signaling (i.e. p38 activation) via human and rodent KOR. This suggests that the predictive power of rodent models for the effects of particular KOR ligands in vivo may be limited.

Work in the third chapter of this thesis has also added to our understanding of the neurocircuitry of KOR-mediated aversion and analgesia through the development of lentiviral expression vectors as tools to selectively express KOR in specific brain regions of mice in which KOR has been genetically deleted. The warm water tail flick response, which is thought to be spinally-mediated, is likely modulated by KOR on descending neuronal projects from the dorsal raphe. The aversive effects of KOR are mediated in part by projects from the dorsal raphe to the nucleus accumbens. As the dorsal raphe is the major serotonergic nucleus within the brain, this also strongly implicates the serotonergic system in regulation of aversion and analgesia. This technique can be used in the future to extend our understanding of the neurocircuitry of other behaviors, such as KOR-mediated anxiety, and the role of other brain regions in the effects of KOR activation.

Finally, work in the third chapter of this thesis assisted in identification of a novel JNK-dependent mechanism of opioid receptor regulation underlying the unusually long lasting antagonist effect of norBNI and norBNI-like KOR antagonists. Radioligand binding was used to measure the drug binding sites of KOR from brain tissue of mice which had been injected with norBNI one week prior. At this time, the analgesic response of these mice to KOR agonists was still blunted, but no changes were observed for agonist binding of KOR either in number of drug binding sites or in drug affinity. The lack of change in the drug binding site of KOR established that the cause of norBNI's long duration of action

was not metabolic. Rather than being biotransformed into a metabolite which irreversibly bound, norBNI uncouples the receptor through activation of JNK, although the mechanism by which JNK uncouples the receptor is still unknown.

Significance for development of KOR therapeutics

This work has several significant implications for the development as KOR ligands as therapeutics. First, the similarity in mechanisms underlying MAPK signaling between rodent and human KOR, particularly that activation of p38 requires receptor phosphorylation and arrestin, suggests that KOR agonists which preferentially activate G protein-mediated pathways without activating arrestin-mediated pathways including p38 would have clinical use as analgesics with a decreased side effect profile. One of the initial steps in developing such a drug would be screening compounds for activity at KOR for the desired subset of signaling cascades. Functional high-throughput screening of drug libraries for agonist activity at receptors is already commonplace. However, functional screening of ligands often assumes classical receptor pharmacology principles in which a drug will be an antagonist, partial agonist, or agonist universally for all possible signaling pathways via a particular receptor. As a result, to optimize the efficiency of high throughput screening, a single output is measured. Therefore the screen will only identify the ability of a ligand to activate a specific pathway via the receptor of interest. For many ligands, this information accurately reflects all signaling pathways, but for those with ligand directed signaling properties, important information will be lost by not screening multiple pathways. This is a particularly important concern for KOR ligand screening since drugs with ligand directed signaling properties may be the most clinically useful.

Furthermore, many commonly used high throughput ligand screens for GPCRs are arrestin-dependent assays. These include automated internalization assays and numerous high-throughput arrestin recruitment assays, such as Bioluminescence Resonance Energy Transfer, PathHunter, and Tango. These assays have several advantages from a high-throughput screening perspective, including being independent of the particular subclass of G-protein which a GPCR acts through and requiring minimal

knowledge of a particular GPCR's signaling pathways. However, if arrestin-dependent assays are used as the initial screen for agonist activity at a GPCR, ligands which preferentially activate G-protein signaling but fail to effectively promoter receptor phosphorylation and arrestin recruitment will be falsely identified as having low partial agonist activity or even no agonist activity for the receptor. In the case of KOR, the most promising drugs – those which promote G-protein but not arrestin-dependent signaling – would be missed if arrestin-dependent assays are the first high-throughput screen used. In contrast, arrestin-dependent assays would be a valuable secondary screen for compounds which had previously been identified as KOR agonists in G protein-dependent assays. Ideally, KOR ligand screening should involve parallel or multiplex assays to identify compounds with agonist activity in G protein but not arrestin-dependent assays.

A second important implication of this work is that high-throughput screening of drug libraries based on rodent KOR systems may provide false positives or false negatives with regard to drugs with ligand directed signaling through human KOR. While it is unknown how common it is likely to be that KOR ligands have different activities for particular pathways via human or rodent KOR, this thesis demonstrates that this is possible with the example of pentazocine. While the example of dissociation between effects of a KOR agonist in humans and rodents in this thesis involved less aversive effects in mice than those reported in people, and a decreased potency for p38 activation via the rodent receptor as compared to the human receptor, the opposite may occur as well.

Agonist activity for human and rodent KOR is not necessarily equivalent and therefore screening of compounds should use the human KOR. Additionally, caution should be exercised regarding extrapolating the possible effects of KOR agonists in people based on behavioral studies in rats and mice. Agonists which appear to not cause p38 activation or aversion in rats and mice may not lack aversive properties in humans. Likewise, while not reported in this thesis, it may be possible for an agonist to be aversive (and activate arrestin-dependent p38 phosphorylation) in rodents but not humans. This poses a

particular problem for pre-clinical research for specific agonists in following in-vitro characterization of KOR agonists, as mice and rats are common early pre-clinical models.

Antagonists of KOR have also been considered for clinical therapeutic use. Because of the dysphoric, anxiogenic, and pro-depressive effects of KOR activation, and the anxiolytic and anti-depressive-like effects of KOR antagonists in rodent models, KOR antagonists are being considered as potential anxiolytics and antidepressants (Carlezon 2009, Chavkin 2011). One concern has been the long duration of action of typical KOR antagonists such as norBNI. Our current understanding that this is a result of selective activation of JNK by these antagonists has two important implications for the clinical use of KOR antagonists. The first is that KOR antagonists which do not activate JNK should function as standard competitive antagonists with a normal duration of action. The second implication is that JNK activation by norBNI may initiate signaling events in addition to KOR desensitization, and it will be important in the future to understand if these play a role in the anxiolytic and anti-depressive effects of norBNI in addition to KOR antagonism.

Future directions

JNK-dependent inactivation of GPCRs is not unique to KOR. Tolerance to the MOR agonist morphine is JNK dependent rather than GRK3 dependent (Melief 2010). The mechanism of JNK inactivation of KOR and MOR is not known, but we propose that JNK phosphorylates a site within the receptor signaling complex, which allows an arrestin-like protein to bind to the receptor and prevent receptor signaling. Stable association of a protein at the phosphorylation site would prevent dephosphorylation and may account for the long duration of antagonism of norBNI-like antagonists. The JNK phosphorylation site within the receptor signaling complex and arrestin-like protein are currently unidentified and their existence remains hypothetical. Whether JNK-dependent inactivation occurs for other GPCRs is not known, but may represent a common mechanism of receptor desensitization, distinct from the more characterized GRK/arrestin-dependent mechanism. Therefore future research into the

mechanism of JNK-mediated KOR inactivation may also provide insight more general insight into the molecular regulation of opioid receptor signaling.

One proposed JNK substrate is KOR itself. The second intracellular loop of KOR contains a potential MAPK phosphorylation site, with the consensus sequence of a serine or threonine followed by a proline (Tibbles 1999). This site is conserved between MOR (Thr180) and KOR (Thr171). MOR and KOR also both contain multiple potential JNK-binding motifs (consensus sequences R/KX0-2R/KX0-4L/I/VXL/I/V) (Tibbles 1999). Mutation of the Thr180 site of MOR has been previously shown to block MOR desensitization (Celver 2001, Lowe 2002, Celver 2004). Proposed future studies include determining if JNK phosphorylates this site, and determining if mutation of this site prevents JNK-mediated receptor inactivation.

In order to understand the JNK-mediated changes that occur in the KOR and MOR signalosomes, it will be beneficial to develop readily-manipulated model systems and molecular tools with which to study these changes. Transfected cells can be powerful tools for studying the mechanisms of GPCR signaling, and much of our understanding of GPCRs has come from experiments using transfected cell cultures as model systems. Because they are quick to replicate, readily grown in large scale, easy to transfect, and highly amenable to pharmacological manipulation, cell cultures would provide ideal model systems with which to study changes in the KOR signalosome following norBNI treatment and probe the molecular signaling underlying these changes. Previously all studies observing the long-lasting antagonist properties of norBNI have involved treatment with norBNI in-vivo, but norBNI has been shown to activate JNK in cell culture as well (Bruchas 2007, Melief 2011). Identifying cell culture models in which norBNI has a long duration of effect which is JNK-mediated will be beneficial in studying the mechanism of JNK-mediated receptor inactivation.

Lentiviral expression systems allow for the stable expression of KOR in discreet regions of the central nervous system. Furthermore, viral expression of KOR also enables the expression of point

mutants, allowing the study of receptor mutations in vivo without the expense or time required for transgenic mouse development. This was demonstrated with the KOR(Ser369Ala), but can be extended to other mutations as well. Development of a lentivirus expressing KOR(Thr171Ala) is underway. This lentivirus will enable us to determine the effect of mutation of this site in vivo and test if phosphorylation of this site is a mediator of norBNI desensitization of KOR.

By combining lentiviral expression with lox/cre technology, this technique can also be used to study the role of subpopulations of cells within a specific brain region without the cost and time consumption required to develop transgenic mouse lines. For this purpose I have developed a lentivirus which expresses a floxed KOR antiparrallel to the promoter; the lox sites which contain mutations rendering recombination unidirectional (Albert 1995, Oberdoerffer 2003) recombine to allow expression of KOR when cre is also expressed. By co-injecting a retrogradely transported cre-expressing CAV with this inverted-KOR lentivirus, we will be able to express KOR specifically in cell projecting from one discreet nuclei to another discreet nuclei. Similarly, KOR can be expressed in a specific cell type within a region, by injection of the inverted-KOR lentivirus in transgenic mice lacking KOR and expressing cre under a cell-type specific promoter. One example of this is specific expression of KOR in serotonergic cells within the dorsal raphe by using mice expressing cre under the Pet1 promoter, which is selective for serotonergic lineage cells.

Finally, although it has been established that GPCRs including the opioid receptors activate through several signaling cascades and ligands may preferentially activate a subset of signaling events, it is unclear to what extent ligand directed signaling occurs naturally. The chemokine CCL21 exhibits ligand directed signaling, preferentially activating Gq-mediated but nor arrestin-mediated cascades through activation of the CCR7 chemokine receptor (Kohout 2004), but few other examples of endogenous ligands with ligand directed signaling have been identified. The propeptides which the endogenous opioid are derived from can be cleaved into many different peptides. For prodynorphin alone these include DynA(1-17) and (1-8), big-Dyn (1-26), big-Dyn(6-26), α- and β-neo-endorphin, and

DynB(20-32) (Schwarzer 2009). The potencies and efficacies of the endogenous opioid peptides have historically been calculated from GTPγS and other G protein-dependent assays. The endogenous KOR ligands have not been fully characterized in their ability to activate the different signaling cascades to which KOR couples (G protein-dependent, arrestin-dependent, and JNK-dependent). An interesting but untested hypothesis is that a subset of prodynorphin-derived peptides have ligand directed signaling properties. The cleavage pattern of the different endogenous peptides may regulate cellular signaling not only through receptor selectivity and overall efficacy at each opioid receptor, but also through preferential activation of a subset of signaling cascades. If this hypothesis is true, it would add to the complexity of endogenous opioid receptor function.

References

Albert H, Dale EC, Lee E, Ow DW. (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. *Plant J.* **7**(4):649-659.

Akil H, Watson SJ, Young E, Lewis ME, Khachaturian H, Walker JM. (1984) Endogenous opioids: biology and function. *Annu Rev Neurosci*. **7**:223-55.

Amat J, Baratta MV, Paul E, Bland ST, Watkins LR, Maier SF. (2005) Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. *Nat Neurosci*. **8**(3):365-371.

Angers S, Li T, Yi X, MacCoss MJ, Moon RT, Zheng N. (2006) Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. *Nature*. **443:** 590-593.

Appleyard SM, Celver J, Pineda V, Kovoor A, Wayman GA, Chavkin C. (1999) Agonist-dependent desensitization of the kappa opioid receptor by G protein receptor kinase and beta-arrestin. J *Biol Chem*. **274:** 23802-23807.

Attali B, Saya D, Nah SY, Vogel Z. (1989) Kappa opiate agonists inhibit Ca2+ influx in rat spinal cord-dorsal root ganglion cocultures. Involvement of a GTP-binding protein. *J Biol Chem*. **264**(1):347-353.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, Piñeyro G. (2003) Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci U S A*. **100**(20):11406-11411.

Baamonde A, Daugé V, Ruiz-Gayo M, Fulga IG, Turcaud S, Fournié-Zaluski MC, Roques BP. (1992) Antidepressant-type effects of endogenous enkephalins protected by systemic RB 101 are mediated by opioid delta and dopamine D1 receptor stimulation. *Eur J Pharmacol*. **216**(2):157-166.

Baker JG, Hall IP, Hill SJ. (2003) Agonist and inverse agonist actions of beta-blockers at the human beta 2-adrenoceptor provide evidence for agonist-directed signaling. *Mol Pharmacol*. **64**(6):1357-1369.

Beaver WT, Wallenstein SL, Houde RW, Rogers A. (1966) A comparison of the analgesic effects of pentazocine and morphine in patients with cancer. *Clin Pharmacol Ther*. **7**(6):740-751.

Belcheva MM, Clark AL, Haas PD, Serna JS, Hahn JW, Kiss A, Coscia CJ. (2005) Mu and kappa opioid receptors activate ERK/MAPK via different protein kinase C isoforms and secondary messengers in astrocytes. *J Biol Chem*. **280**(30):27662-27669.

Belcheva MM, Vogel Z, Ignatova E, Avidor-Reiss T, Zippel R, Levy R, Young EC, Barg J, Coscia CJ. (1998) Opioid modulation of extracellular signal-regulated protein kinase activity is ras-dependent and involves Gbetagamma subunits. *J Neurochem*. **70**(2):635-645.

Berg KA, Cropper JD, Niswender CM, Sanders-Bush E, Emeson RB, Clarke WP. (2001) RNA-editing of the 5-HT(2C) receptor alters agonist-receptor-effector coupling specificity. *Br J Pharmacol*. **134**(2):386-392.

Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P, Clarke WP. (1998) Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol*. **54**(1):94-104.

Black JW, Leff P. (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci*. **220**(1219):141-162.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature*. **408**(6813):720-723.

Bohn LM, Raehal KM. Opioid receptor signaling: relevance for gastrointestinal therapy. (2006) *Curr Opin Pharmacol*. **6**(6):559-563.

Bonhaus DW, Chang LK, Kwan J, Martin GR. (1998) Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *J Pharmacol Exp Ther*. **287**(3):884-888.

Broadbear, J. H., Negus, S. S., Butelman, E. R., de Costa, B. R., and Woods, J. H. (1994) *Psychopharmacology (Berl*) **115**: 311–319

Brownstein MJ. (1993) A brief history of opiates, opioid peptides, and opioid receptors. *Proc Natl Acad Sci U S A*. **90**(12):5391-5393.

Bruchas MR, Land BB, Aita M, Xu M, Barot SK, Li S, Chavkin C. (2007) Stress-induced p38 mitogen-activated protein kinase activation mediates kappa-opioid-dependent dysphoria. *J Neurosci*. **27**(43):11614-11623.

Bruchas MR, Yang T, Schreiber S, Defino M, Kwan SC, Li S, Chavkin C. (2007) Long-acting kappa opioid antagonists disrupt receptor signaling and produce noncompetitive effects by activating c-Jun N-terminal kinase. *J Biol Chem*. **282**(41): 29803-29811.

Bruchas MR, Macey TA, Lowe JD, Chavkin C. (2006) Kappa opioid receptor activation of p38 MAPK is GRK3- and arrestin-dependent in neurons and astrocytes. *J Biol Chem*. **281**(26):18081-18089.

Bruchas MR, Schindler AG, Shankar H, Messinger DI, Miyatake M, Land BB, Lemos JC, Hagan CE, Neumaier JF, Quintana A, Palmiter RD, Chavkin C. (2011) Selective p38α MAPK deletion in serotonergic neurons produces stress resilience in models of depression and addiction. *Neuron*. **71**(3):498-511.

Butelman, E. R., Negus, S. S., Ai, Y., deCosta, B. R., and Woods, J. H. (1993) Kappa opioid antagonist effects of systemically administered nor-binaltorphimine in a thermal antinociception assay in rhesus monkeys. *J. Pharmacol. Exp. Ther*. **267:** 1269–1276

Carlezon WA Jr, Béguin C, DiNieri JA, Baumann MH, Richards MR, Todtenkopf MS, Rothman RB, Ma Z, Lee DY, Cohen BM. (2006) Depressive-like effects of the kappa-opioid receptor agonist salvinorin A on behavior and neurochemistry in rats. *J Pharmacol Exp Ther*. **316**(1):440-447.

Carlezon WA Jr, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ. (1998) Regulation of cocaine reward by CREB. *Science*. **282**(5397):2272-2275.

Carlezon WA Jr, Béguin C, Knoll AT, Cohen BM. (2009) Kappa-opioid ligands in the study and treatment of mood disorders. *Pharmacol Ther*. **123**(3):334-343.

Carroll, I., Thomas, J. B., Dykstra, L. A., Granger, A. L., Allen, R. M., Howard, J. L., Pollard, G. T., Aceto, M. D., and Harris, L. S. (2004) Pharmacological properties of JDTic: a novel kappa-opioid receptor antagonist. *Eur J Pharmacol*. **501**: 111–119

Celver, J., Lowe, J., Kovoor, A., Gurevich, V.V., and Chavkin, C. (2001) Threonine 180 is required for GRK3 and arr3 mediated desensitization of the Mu opioid receptor in Xenopus oocytes. *Journal of Biological Chemistry*, **276**: 4894-4900.

Celver, J., Vishnivetskiy, S.A., Chavkin, C., and Gurevich, V.V. (2002) Conservation of the phosphate-sensitive elements in the arrestin family of proteins. *J. Biol. Chem*. **277**, 9043-9048.

Celver, J.P., Jin, W., Xu, M., Lowe, J. and Chavkin, C. (2004) Distinct domains of the mu opioid receptor control uncoupling and internalization. *Molecular Pharmacology* **65:** 58-537.

Chavkin, C., James, JF., and Goldstein, A. (1982) Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science* **215:**413–415

Chavkin C. (2011) The therapeutic potential of κ-opioids for treatment of pain and addiction. *Neuropsychopharmacology*. **36**(1):369-370.

Chien CC, Pasternak GW. (1995) (-)-Pentazocine analgesia in mice: interactions with a sigma receptor system. *Eur J Pharmacol*. **294**(1):303-308.

Childers SR, Snyder SH. (1978) Guanine nucleotides differentiate agonist and antagonist interactions with opiate receptors. *Life Sci*. **23**(7):759-761.

Chu J, Zheng H, Loh HH, Law PY. (2008) Morphine-induced mu-opioid receptor rapid desensitization is independent of receptor phosphorylation and beta-arrestins. *Cell Signal*. **20**(9):1616-1624.

Clarke S, Czyzyk T, Ansonoff M, Nitsche JF, Hsu MS, Nilsson L, Larsson K,Borsodi A, Toth G, Hill R, Kitchen I, Pintar JE. (2002) Autoradiography of opioid and ORL1 ligands in opioid receptor triple knockout mice. *Eur J Neurosci*. **16**(9):1705-1712.

Clayton CC, Xu M, Chavkin C. (2009) Tyrosine phosphorylation of Kir3 following kappa-opioid receptor activation of p38 MAPK causes heterologous desensitization. *J Biol Chem*. **284**(46):31872-31881.

Cussac D, Newman-Tancredi A, Duqueyroix D, Pasteau V, Millan MJ. (2002) Differential activation of Gq/11 and Gi(3) proteins at 5-hydroxytryptamine(2C) receptors revealed by antibody capture assays: influence of receptor reserve and relationship to agonist-directed trafficking. *Mol Pharmacol*. **62**(3):578-589.

Daaka Y, Pitcher JA, Richardson M, Stoffel RH, Robishaw JD, Lefkowitz RJ. (1997) Receptor and G betagamma isoform-specific interactions with G protein-coupled receptor kinases. *Proc Natl Acad Sci U S A*. **94**(6):2180-2185.

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. dbSNP accession: ss43313677, (dbSNP Build ID: 126/135). Available from: http://www.ncbi.nlm.nih.gov/SNP/

De Lean A, Stadel JM, Lefkowitz RJ. (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J Biol Chem*. **255**(15):7108-7117.

Drake MT, Violin JD, Whalen EJ, Wisler JW, Shenoy SK, Lefkowitz RJ. (2008) Beta-arrestin-biased agonism at the beta2-adrenergic receptor. *J Biol Chem*. **283**(9):5669-5676.

El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY, Loh HH. (2001) Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. *J Biol Chem*. **276**(16):12774-12780.

Emmerson PJ, Clark MJ, Mansour A, Akil H, Woods JH, Medzihradsky F. (1996) Characterization of opioid agonist efficacy in a C6 glioma cell line expressing the mu opioid receptor. *J Pharmacol Exp Ther*. **278**(3):1121-1127.

Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [May, 2012].

Field MJ, Carnell AJ, Gonzalez MI, McCleary S, Oles RJ, Smith R, Hughes J, Singh L. (1999) Enadoline, a selective kappa-opioid receptor agonist shows potent antihyperalgesic and antiallodynic actions in a rat model of surgical pain. *Pain.* **80**(1-2):383-389.

Fraser HF, Rosenberg DE. (1964) Studies on the human addiction liability of 2'-hydroxy-5-9-dimethyl-2-(3,3-dimethylally)-6,7-benzomorphan (WIN 20,228): a weak narcotic antagonist. *J Pharmacol Exp Ther*. **143**:149-156.

Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB. (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol*. **63**(6):1256-1272.

Fukuda K, Kato S, Morikawa H, Shoda T, Mori K. (1996) Functional coupling of the delta-, mu-, and kappa-opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. *J Neurochem*. **67**(3):1309-1316.

Galandrin S, Bouvier M. (2006) Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol*. **70**(5):1575-1584.

Gay EA, Urban JD, Nichols DE, Oxford GS, Mailman RB. (2004) Functional selectivity of D2 receptor ligands in a Chinese hamster ovary hD2L cell line: evidence forinduction of ligand-specific receptor states. *Mol Pharmacol*. **66**(1):97-105.

Glass M, Northup JK. (1999) Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. *Mol Pharmacol*. **56**(6):1362-1369.

Gross RA, Macdonald RL. (1987) Dynorphin A selectively reduces a large transient (N-type) calcium current of mouse dorsal root ganglion neurons in cell culture. *Proc Natl Acad Sci U S A*. 1987 **84**(15):5469-5473.
Gurevich VV, Benovic JL. (1993) Visual arrestin interaction with rhodopsin. Sequential multisite binding ensures strict selectivity toward light-activated phosphorylated rhodopsin. *J Biol Chem*. **268**(16):11628-11638.
Gurevich VV, Benovic JL. (1995) Visual arrestin binding to rhodopsin. Diverse functional roles of positively charged residues within the phosphorylation-recognition region of arrestin. *J Biol Chem*. **270**(11):6010-6016.
Gurevich VV, Gurevich EV. (2003) The new face of active receptor bound arrestin attracts new partners. *Structure*. **11**(9):1037-1042.
Gurevich VV, Gurevich EV. (2006) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol Ther*. **110**(3):465-502.
Gurevich EV, Tesmer JJ, Mushegian A, Gurevich VV. (2012) G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. *Pharmacol Ther*. **133**(1):40-69.
Hamilton RC, Dundee JW, Clarke RS, Loan WB, Morrison JD. (1967) Studies of drugs given before anaesthesia. 13. Pentazocine and other opiate antagonists. *Br J Anaesth*. **39**(8):647-656.
Hiramatsu M, Hoshino T. (2005) Improvement of memory impairment by (+)- and (-)-pentazocine via sigma, but not kappa opioid receptors. *Brain Res*. **1057**(1-2):72-80.
Hirsch JA, Schubert C, Gurevich VV, Sigler PB. (1999) The 2.8 A crystal structure of visual arrestin: a model for arrestin's regulation. *Cell*. **97**(2):257-269.
Horan, P., Taylor, J., Yamamura, H. I., and Porreca, F. (1992) Extremely long-lasting antagonistic actions of nor-binaltorphimine (nor-BNI) in the mouse tail-flick test. *J. Pharmacol. Exp. Ther*. **260:** 1237–1243
Hough LB, Nalwalk JW, Chen Y, Schuller A, Zhu Y, Zhang J, Menge WM, Leurs R, Timmerman H, Pintar JE. (2000) Improgan, a cimetidine analog, induces morphine-like antinociception in opioid receptor-knockout mice. *Brain Res*. **880**(1-2):102-108.
Hnasko TS, Sotak BN, Palmiter RD. (2005) Morphine reward in dopamine-deficient mice. Nature. **438**(7069):854-857.
Hnasko TS, Sotak BN, Palmiter RD. (2007) Cocaine-conditioned place preference by dopamine-deficient mice is mediated by serotonin. *J Neurosci*. **27**(46):12484-12488.
Hsia JA, Moss J, Hewlett EL, Vaughan M. (1984) ADP-ribosylation of adenylate cyclase by pertussis toxin. Effects on inhibitory agonist binding. *J Biol Chem*. **259**(2):1086-1090.
Hubbell WL, Altenbach C, Hubbell CM, Khorana HG. (2003) Rhodopsin structure, dynamics, and activation: a perspective from crystallography, site-directed spin labeling, sulfhydryl reactivity, and disulfide cross-linking. *Adv Protein Chem*. **63**:243-290.
Hunton DL, Barnes WG, Kim J, Ren XR, Violin JD, Reiter E, Milligan G, Patel DD, Lefkowitz RJ. (2005) Beta-arrestin 2-dependent angiotensin II type 1A receptor-mediated pathway of chemotaxis. *Mol Pharmacol*. **67**(4):1229-1236.
Ide S, Minami M, Uhl GR, Satoh M, Sora I, Ikeda K. (2011) (-)-Pentazocine induces visceral chemical antinociception, but not thermal, mechanical, or somatic chemical antinociception, in μ-opioid receptor knockout mice. *Mol Pain*. **7**:23.
Iwamoto ET. Place-conditioning properties of mu, kappa, and sigma opioid agonists. (1985) Alcohol Drug Res. **6**(5):327-339.
Jasinski DR, Martin WR, Hoeldtke RD. (1970) Effects of short- and long-term administration of pentazocine in man. *Clin Pharmacol Ther*. **11**(3):385-403.
Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ, Mundell SJ, Bailey CP, Kelly E, Henderson G. (2006) Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. *Mol Pharmacol*. **70**(2):676-685.
Johnston CA, Siderovski DP. (2007) Receptor-mediated activation of heterotrimeric G-proteins: current structural insights. *Mol Pharmacol*. **72**(2):219-230.

Jones, D. N. C., and Holtzman, S. G. (1992) Long term kappa-opioid receptor blockade following nor-binaltorphimine. *Eur. J. Pharmacol*. **215:** 345-348.
Jones RM, Portoghese PS. (2000) 5'-Guanidinonaltrindole, a highly selective and potent kappa-opioid receptor antagonist. *Eur J Pharmacol*. **396**(1):49-52.
Jordan BA, Cvejic S, Devi LA. (2000) Kappa opioid receptor endocytosis by dynorphin peptides. *DNA Cell Biol*. **19**(1):19-27.
Jutkiewicz EM. (2006) The antidepressant -like effects of delta-opioid receptor agonists. *Mol Interv*. **6**(3):162-169.
Jutkiewicz EM, Baladi MG, Folk JE, Rice KC, Woods JH. (2006) The convulsive and electroencephalographic changes produced by nonpeptidic delta-opioid agonists in rats: comparison with entylenetetrazol. *J Pharmacol Exp Ther*. **317**(3):1337-1348.
Kam AY, Chan AS, Wong YH. (2004) Kappa-opioid receptor signals through Src and focal adhesion kinase to stimulate c-Jun N-terminal kinases in transfected COS-7 cells and human monocytic THP-1 cells. *J Pharmacol Exp Ther*. **310**(1):301-310.
Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, von Zastrow M. (1996) Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem*. **271**(32):19021-19024.
Kenakin T. (2003) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci*. **24**(7):346-354.
Kenakin T. (2007) Functional selectivity through protean and biased agonism: who steers the ship? *Mol Pharmacol*. **72**(6):1393-1401.
Kilts JD, Connery HS, Arrington EG, Lewis MM, Lawler CP, Oxford GS, O'Malley KL, Todd RD, Blake BL, Nichols DE, Mailman RB. (2002) Functional selectivity of dopamine receptor agonists. II. Actions of dihydrexidine in D2L receptor-transfected MN9D cells and pituitary lactotrophs. *J Pharmacol Exp Ther*. **301**(3):1179-1189.
Kim OJ, Gardner BR, Williams DB, Marinec PS, Cabrera DM, Peters JD, Mak CC, Kim KM, Sibley DR. (2004) The role of phosphorylation in D1 dopamine receptor desensitization: evidence for a novel mechanism of arrestin association. *J Biol Chem*. **279**(9):7999-8010.
Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ. (2005) Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci U S A*. **102**(5):1442-1447.
Kivell B, Prisinzano TE. (2010) Kappa opioids and the modulation of pain. *Psychopharmacology (Berl).* **210**(2):109-119.
Knoll AT, Meloni EG, Thomas JB, Carroll FI, Carlezon WA Jr. (2007) Anxiolytic-like effects of kappa-opioid receptor antagonists in models of unlearned and learned fear in rats. *J Pharmacol Exp Ther*. **323**(3):838-845.
Kohout TA, Nicholas SL, Perry SJ, Reinhart G, Junger S, Struthers RS. (2004) Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *J Biol Chem.* **279**(22):23214-23222.
Konkoy CS, Childers SR. (1989) Dynorphin-selective inhibition of adenylyl cyclase in guinea pig cerebellum membranes. *Mol Pharmacol*. **36**(4):627-633.
Kovoor A, Celver JP, Wu A, Chavkin C. (1998) Agonist induced homologous desensitization of mu-opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. *Mol Pharmacol*. **54**(4):704-711.
Kovoor, A., Celver, J., Abdryashitov, R.I., Chavkin, C., and Gurevich, V.V. (1999) Targeted construction of phosphorylation-independent β-arrestin mutants with constitutive activity in cells. *J.Biol.Chem*. **274**, 6831-6834.
Krupnick JG, Gurevich VV, Benovic JL. (1997) Mechanism of quenching of phototransduction. Binding competition between arrestin and transducin for phosphorhodopsin. *J Biol Chem*. **272**(29):18125-18131.

Land BB, Bruchas MR, Lemos JC, Xu M, Melief EJ, Chavkin C. (2008) The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *J Neurosci*. **28**(2):407-414.

Land BB, Bruchas MR, Schattauer S, Giardino WJ, Aita M, Messinger D, Hnasko TS, Palmiter RD, Chavkin C. (2009) Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. *Proc Natl Acad Sci U S A.* **106**(45):19168-19173.

Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, Barak LS. (1999) The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci U S A*. **96**(7):3712-3717.

Lefkowitz RJ, Shenoy SK. (2005) Transduction of receptor signals by beta-arrestins. *Science*. **308**(5721):512-517.

Levine JD, Gordon NC, Taiwo YO, Coderre TJ. (1988) Potentiation of pentazocine analgesia by low-dose naloxone. *J Clin Invest*. **82**(5):1574-1577.

Li J, Li JG, Chen C, Zhang F, Liu-Chen LY. (2002) Molecular basis of differences in (-)(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide-induced desensitization and phosphorylation between human and rat kappa-opioid receptors expressed in Chinese hamster ovary cells. *Mol Pharmacol*. **61**(1):73-84.

Li JG, Luo LY, Krupnick JG, Benovic JL, Liu-Chen LY. (1999) U50,488H-induced internalization of the human kappa opioid receptor involves a beta-arrestin- and dynamin-dependent mechanism. Kappa receptor internalization is not required for mitogen-activated protein kinase activation. *J Biol Chem*. **274**(17):12087-12094.

Livingston RJ, von Niederhausern A, Jegga AG, Crawford DC, Carlson CS, Rieder MJ, Gowrisankar S, Aronow BJ, Weiss RB, Nickerson DA. (2004) Pattern of sequence variation across 213 environmental response genes. *Genome Res*. **14**(10A):1821-1831.

Lowe, J.D., Celver, J.P., Gurevich, V.V. and Chavkin, C. (2002) Mu opioid receptors desensitize less rapidly than delta opioid receptors due to less efficient activation of arrestin. *Journal of Biological Chemistry*, **277:** 15729–15735.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science*. **283**(5402):655-661.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ. (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A*. **98**(5):2449-2454.

Luttrell LM, Lefkowitz RJ. (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci*. **115**(Pt3):455-65.

Luttrell DK, Luttrell LM.(2003) Signaling in time and space: G protein-coupled receptors and mitogen-activated protein kinases. *Assay Drug Dev Technol*. **1**(2):327-338.

Macdonald RL, Werz MA. (1986) Dynorphin A decreases voltage-dependent calcium conductance of mouse dorsal root ganglion neurones. *J Physiol*. **377**:237-249.

Mague SD, Pliakas AM, Todtenkopf MS, Tomasiewicz HC, Zhang Y, Stevens WC Jr, Jones RM, Portoghese PS, Carlezon WA Jr. (2003) Antidepressant-like effects of kappa-opioid receptor antagonists in the forced swim test in rats. *J Pharmacol Exp Ther*. **305**(1):323-330.

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ. (1987) Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. *J Neurosci*. **7**(8):2445-2264.

Mansour A, Burke S, Pavlic RJ, Akil H, Watson SJ. (1996) Immunohistochemical localization of the cloned kappa 1 receptor in the rat CNS and pituitary. *Neuroscience*. **71**(3):671-690.

Martin WR, Fraser HF, Gorodetzky CW, Rosenberg DE. (1965) Studies of the dependence-producing potential of the narcotic antagonist 2-cyclopropylmethyl-2'-hydroxy-5,9-dimethyl-6,7-benzomorphan (cyclazocine, WIN-20,740, ARC II-c-3*). J Pharmacol Exp Ther*. **150**(3):426-436.

Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE. (1976) The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. *J Pharmacol Exp Ther*. **197**(3):517-532.

Martin WR. (1983) Pharmacology of opioids. *Pharmacol Rev*. **35**(4):283-323.

Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, Evrard A, Cazala P, Cormier A, Mameli-Engvall M, Dufour N, Cloëz-Tayarani I, Bemelmans AP, Mallet J, Gardier AM, David V, Faure P, Granon S, Changeux JP. (2005) Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. *Nature*. **436**(7047):103-107.

McDonald PH, Cote NL, Lin FT, Premont RT, Pitcher JA, Lefkowitz RJ. (1999) Identification of NSF as a beta-arrestin1-binding protein. Implications for beta2-adrenergic receptor regulation. *J Biol Chem*. **274**(16):10677-10680.

McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ. (2000) Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science*. **290**(5496):1574-1577.

McLaughlin JP, Marton-Popovici M, Chavkin C. (2003) Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J Neurosci*. **23**(13):5674-5683.

McLaughlin JP, Xu M, Mackie K, Chavkin C. (2003) Phosphorylation of a carboxyl-terminal serine within the kappa-opioid receptor produces desensitization and internalization. *J Biol Chem*. **278**(36):34631-34640.

McLaughlin JP, Myers LC, Zarek PE, Caron MG, Lefkowitz RJ, Czyzyk TA, Pintar JE, Chavkin C. (2004) Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. *J Biol Chem*. **279**: 1810-1818.

McLennan GP, Kiss A, Miyatake M, Belcheva MM, Chambers KT, Pozek JJ, Mohabbat Y, Moyer RA, Bohn LM, Coscia CJ. (2008) Kappa opioids promote the proliferation of astrocytes via Gbetagamma and beta-arrestin 2-dependent MAPK-mediated pathways. *J Neurochem*. **107**(6):1753-1765.

Melief EJ, Miyatake M, Bruchas MR, Chavkin C. (2010) Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. *Proc Natl Acad Sci U S A*. **107**(25):11608-11613.

Melief EJ, Miyatake M, Carroll FI, Béguin C, Carlezon WA Jr, Cohen BM, Grimwood S, Mitch CH, Rorick-Kehn L, Chavkin C. (2011) Duration of action of a broad range of selective κ-opioid receptor antagonists is positively correlated with c-Jun N-terminal kinase-1 activation. *Mol Pharmacol*. **80**(5):920-929.

Mendez A, Burns ME, Roca A, Lem J, Wu LW, Simon MI, Baylor DA, Chen J. (2000) Rapid and reproducible deactivation of rhodopsin requires multiple phosphorylation sites. *Neuron*. **28**(1):153-164.

Millan MJ. (1990) Kappa-opioid receptors and analgesia. *Trends Pharmacol Sci*. **11**(2):70-76.

Mori T, Yoshizawa K, Nomura M, Isotani K, Torigoe K, Tsukiyama Y, Narita M, Suzuki T. (2011) Sigma-1 receptor function is critical for both the discriminative stimulus and aversive effects of the kappa-opioid receptor agonist U-50488H. *Addict Biol*. Mar 11.

Mucha RF, Herz A. (1985) Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. *Psychopharmacology (Berl)*. **86**(3):274-280.

Mukherjee S, Palczewski K, Gurevich V, Benovic JL, Banga JP, Hunzicker-Dunn M. (1999) A direct role for arrestins in desensitization of the luteinizing hormone/choriogonadotropin receptor in porcine ovarian follicular membranes. *Proc Natl Acad Sci U S A*. **96**(2):493-498.

Mukhopadhyay S, Howlett AC. (2005) Chemically distinct ligands promote differential CB1 cannabinoid receptor-Gi protein interactions. *Mol Pharmacol*. **67**(6):2016-2024.

Oberdoerffer P, Otipoby KL, Maruyama M, Rajewsky K. (2003) Unidirectional Cre-mediated genetic inversion in mice using the mutant loxP pair lox66/lox71. *Nucleic Acids Res*. **31**(22):e140.

Oldham WM, Hamm HE. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol*. **9**(1):60-71.

Overington JP, Al-Lazikani B, Hopkins AL. (2006) How many drug targets are there? *Nat Rev Drug Discov*. **5**(12):993-996.

Negus, S. S., Mello, N. K., Linsenmayer, D. C., Jones, R. M., and Portoghese, P. S. (2002) Kappa opioid antagonist effects of the novel kappa antagonist 5'-guanidinonaltrindole (GNTI) in an assay of schedule-controlled behavior in rhesus monkeys. *Psychopharmacology (Berl.)* **163**: 412–419.

North RA, Williams JT, Surprenant A, Christie MJ. (1987) Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proc Natl Acad Sci U S A*. **84**(15):5487-5491.

North RA, Williams JT, Surprenant A, Christie MJ. (1987) Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proc Natl Acad Sci U S A*. **84**(15):5487-5491.

Pan ZZ. (2003) Kappa-opioid receptor-mediated enhancement of the hyperpolarization-activated current (I(h)) through mobilization of intracellular calcium in rat nucleus raphe magnus. *J Physiol*. **548**(Pt 3):765-775.

Peckys D, Landwehrmeyer GB. (1999) Expression of mu, kappa, and delta opioid receptor messenger RNA in the human CNS: a 33P in situ hybridization study. *Neuroscience*. **88**(4):1093-1135.

Petraschka, M., Li, S., Gilbert T.L., Westenbroek, R.E., Bruchas, M.R., Schreiber, S., Lowe, J.D., Low, M., Pintar, J.E., Chavkin, C. (2007) The absence of endogenous beta-endorphin selectively blocks phosphorylation and desensitization of mu opioid receptors following partial sciatic nerve ligation. *Neuroscience*, **146:**1795-1807.

Pfeiffer A, Brantl V, Herz A, Emrich HM. (1986) Psychotomimesis mediated by kappa opiate receptors. *Science*. **233**(4765):774-776.

Pittenger C, Duman RS. (2008) Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology*. **33**(1):88-109.

Pizarro JM, Lumley LA, Medina W, Robison CL, Chang WE, Alagappan A, Bah MJ, Dawood MY, Shah JD, Mark B, Kendall N, Smith MA, Saviolakis GA, Meyerhoff JL. (2004) Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. *Brain Res*. **1025**(1-2):10-20.

Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA Jr. (2001) Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *J Neurosci*. **21**(18):7397-7403.

Porreca F, Filla A, Burks TF. (1983) Studies in vivo with dynorphin-(1-9): analgesia but not gastrointestinal effects following intrathecal administration to mice. *Eur J Pharmacol*. **91**(2-3):291-294.

Porreca F, Mosberg HI, Hurst R, Hruby VJ, Burks TF. (1984) Roles of mu, delta and kappa opioid receptors in spinal and supraspinal mediation of gastrointestinal transit effects and hot-plate analgesia in the mouse. *J Pharmacol Exp Ther*. **230**(2):341-348.

Portoghese, PS, Lipkowski, AW, and Takemori, AE (1987) Bimorphinans as highly selective, potent kappa opioid receptor antagonists. *J. Med. Chem*. **30:** 238–239

Portoghese, PS, Lipkowski, AW., and Takemori, AE. (1987) Binaltorphimine and nor binaltorphimine, potent and selective kappa-opioid receptor antagonists. *Life Sci.* **40:** 1287–1292

Preston KL, Bigelow GE. (1993) Differential naltrexone antagonism of hydromorphone and pentazocine effects in human volunteers. *J Pharmacol Exp Ther*. **264**(2):813-823.

Qian H, Pipolo L, Thomas WG. (2001) Association of beta-Arrestin 1 with the type 1A angiotensin II receptor involves phosphorylation of the receptor carboxyl terminus and correlates with receptor internalization. *Mol Endocrinol*. **15**(10):1706-1719.

Rau KK, Caudle RM, Cooper BY, Johnson RD. (2005) Diverse immunocytochemical expression of opioid receptors in electrophysiologically defined cells of rat dorsal root ganglia. *J Chem Neuroanat*. **29**(4):255-264.

Remmers AE, Clark MJ, Mansour A, Akil H, Woods JH, Medzihradsky F. (1999) Opioid efficacy in a C6 glioma cell line stably expressing the human kappa opioid receptor. *J Pharmacol Exp Ther*. **288**(2):827-833.

Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ. (2005) Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci U S A*. **102**(5):1448-1453.

Richardson MD, Balius AM, Yamaguchi K, Freilich ER, Barak LS, Kwatra MM. (2003) Human substance P receptor lacking the C-terminal domain remains competent to desensitize and internalize. *J Neurochem*. **84**(4):854-863.

Rimoy GH, Wright DM, Bhaskar NK, Rubin PC. (1994) The cardiovascular and central nervous system effects in the human of U-62066E. A selective opioid receptor agonist. *Eur J Clin Pharmacol*. **46**(3):203-207.

Rosow CE. (1987) The clinical usefulness of agonist-antagonist analgesics in acute pain. *Drug Alcohol Depend*. **20**(4):329-337.

Sadja R, Alagem N, Reuveny E. (2002) Graded contribution of the Gbeta gamma binding domains to GIRK channel activation. *Proc Natl Acad Sci U S A*. **99**(16):10783-10788.

Satoh M, Minami M. (1995) Molecular pharmacology of the opioid receptors. *Pharmacol Ther*. **68**(3):343-364.

Schuller AG, King MA, Zhang J, Bolan E, Pan YX, Morgan DJ, Chang A, Czick ME, Unterwald EM, Pasternak GW, Pintar JE. (1999) Retention of heroin and morphine-6 beta-glucuronide analgesia in a new line of mice lacking exon 1 of MOR-1. *Nat Neurosci*. **2**(2):151-156.

Schulz S, Mayer D, Pfeiffer M, Stumm R, Koch T, Höllt V. (2004) Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. *EMBO J*. **23**(16):3282-3289.

Schwarzer C. (2009) 30 years of dynorphins--new insights on their functions in neuropsychiatric diseases. *Pharmacol Ther*. **123**(3):353-370.

Sheline YI, Gado MH, Kraemer HC. (2003) Untreated depression and hippocampal volume loss. *Am J Psychiatry*. **160**(8):1516-1518.

Shippenberg TS, Herz A. (1986) Differential effects of mu and kappa opioid systems on motivational processes. *NIDA Res Monogr.* **75**:563-566.

Shu H, Hayashida M, Arita H, Huang W, Zhang H, An K, Wu G, Hanaoka K. (2011) Pentazocine-induced antinociception is mediated mainly by μ-opioid receptors and compromised by κ-opioid receptors in mice. *J Pharmacol Exp Ther*. **338**(2):579-587.

Sirven A, Ravet E, Charneau P, Zennou V, Coulombel L, Guétard D, Pflumio F, Dubart-Kupperschmitt A. (2001) Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors. *Mol Ther*. **3**(4):438-448.

Smith, C. B., Medzihradsky, F., Hollingsworth, P. J., DeCosta, B., Rice, K. C., and Woods, J. H. (1990) Nor-binaltorphimine is a reversible, noncompetitive opioid antagonist in the mouse vas deferens with high affinity for kappa receptors in monkey brain membranes. *Prog. Clin. Biol. Res*. **328:** 65–68

Song X, Coffa S, Fu H, Gurevich VV. (2009) How does arrestin assemble MAPKs into a signaling complex? *J Biol Chem*. **284**(1):685-695.

Spencer RJ, Jin W, Thayer SA, Chakrabarti S, Law PY, Loh HH. (1997) Mobilization of Ca2+ from intracellular stores in transfected neuro2a cells by activation of multiple opioid receptor subtypes. *Biochem Pharmacol*. **54**(7):809-818.

Stephens M, Sloan JS, Robertson PD, Scheet P, Nickerson DA. (2006) Automating sequence-based detection and genotyping of SNPs from diploid samples. *Nat Genet*. **38**(3):375-381.

Stephenson RP. (1956) A modification of receptor theory. *Br J Pharmacol Chemother*. **11**(4):379-393.

Stout BD, Clarke WP, Berg KA. (2002) Rapid desensitization of the serotonin(2C) receptor system: effector pathway and agonist dependence. *J Pharmacol Exp Ther*. **302**(3):957-962.

Suzuki T, Narita M, Misawa M, Nagase H. (1991) Pentazocine-induced biphasic analgesia in mice. *Life Sci*. **48**(19):1827-1835.

Takemori AE, Ho BY, Naeseth JS, Portoghese PS. (1988) Nor-binaltorphimine, a highly selective kappa-opioid antagonist in analgesic and receptor binding assays. *J Pharmacol Exp Ther*. **246**(1):255-258.

Tibbles LA, Woodgett JR. (1999) The stress-activated protein kinase pathways. *Cell Mol Life Sci*. **55:** 1230-1254.

Todtenkopf MS, Marcus JF, Portoghese PS, Carlezon WA Jr. (2004) Effects of kappa-opioid receptor ligands on intracranial self-stimulation in rats. *Psychopharmacology (Berl)*. **172**(4):463-470.

Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. (2002) beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J Biol Chem*. **277**(11):9429-9436.

Tohgo A, Choy EW, Gesty-Palmer D, Pierce KL, Laporte S, Oakley RH, Caron MG, Lefkowitz RJ, Luttrell LM. (2003) The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation. *J Biol Chem*. **278**(8):6258-6267.

Tortella FC, Robles LE, Holaday JW, Cowan A. (1983) A selective role for delta-receptors in the regulation of opioid-induced changes in seizure threshold. *Life Sci*. **33 Suppl 1**:603-606.

Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ. (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci*. **9**(4):519-525.

Unterwald E, Sasson S, Kornetsky C. (1987) Evaluation of the supraspinal analgesic activity and abuse liability of ethylketocyclazocine. *Eur J Pharmacol*. **133**(3):275-281.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, Mailman RB. (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther*. **320**(1):1-13.

Vanderah TW. (2010) Delta and kappa opioid receptors as suitable drug targets for pain. *Clin J Pain*. **26 Suppl 10**:S10-5.

Vishnivetskiy SA, Paz CL, Schubert C, Hirsch JA, Sigler PB, Gurevich VV. (1999) How does arrestin respond to the phosphorylated state of rhodopsin? *J Biol Chem*. **274**(17):11451-11454.

Vishnivetskiy SA, Schubert C, Climaco GC, Gurevich YV, Velez MG, Gurevich VV. (2000) An additional phosphate-binding element in arrestin molecule. Implications for the mechanism of arrestin activation. *J Biol Chem*. **275**(52):41049-41057.

Vishnivetskiy SA, Hosey MM, Benovic JL, Gurevich VV. (2004) Mapping the arrestin-receptor interface. Structural elements responsible for receptor specificity of arrestin proteins. *J Biol Chem*. **279**(2):1262-1268.

von Zastrow M, Svingos A, Haberstock-Debic H, Evans C. (2003) Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. *Curr Opin Neurobiol.* **13**(3):348-353.

Wadenberg ML. (2003) A review of the properties of spiradoline: a potent and selective kappa-opioid receptor agonist. *CNS Drug Rev*. **9**(2):187-198.

Walsh SL, Strain EC, Abreu ME, Bigelow GE. (2001) Enadoline, a selective kappa opioid agonist: comparison with butorphanol and hydromorphone in humans. *Psychopharmacology (Berl).* **157**(2):151-162.

Walther, C., Nagel, S., Gimenez, L.E., Morl, K., Gurevich, V.V., Beck-Sickinger, A.G. (2010) Ligand induced internalization and recycling of the human neuropeptide Y2 receptor is regulated by its C-terminal tail. *J Biol Chem* **285**, 41578-41590.

Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M. (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron*. **23**(4):737-746.

Whistler JL, Tsao P, von Zastrow M. (2001) A phosphorylation-regulated brake mechanism controls the initial endocytosis of opioid receptors but is not required for post-endocytic sorting to lysosomes. *J Biol Chem*. **276**(36):34331-34338.

Wise RA. (2004) Dopamine, learning and motivation. *Nat Rev Neurosci*. **5**(6):483-494.
Xu M, Bruchas MR, Ippolito DL, Gendron L, Chavkin C. (2007) Sciatic nerve ligation-induced proliferation of spinal cord astrocytes is mediated by kappa opioid activation of p38 mitogen-activated protein kinase. *J Neurosci* **27**(10):2570-2581.
Zacny JP, Hill JL, Black ML, Sadeghi P. (1998) Comparing the subjective, psychomotor and physiological effects of intravenous pentazocine and morphine in normal volunteers. *J Pharmacol Exp Ther*. **286**(3):1197-1207.
Zennou V, Serguera C, Sarkis C, Colin P, Perret E, Mallet J, Charneau P. (2001) The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. *Nat Biotechnol*. **19**(5):446-450
Zhang F, Li J, Li JG, Liu-Chen LY. (2002) (-)U50,488H [(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide] induces internalization and down-regulation of the human, but not the rat, kappa-opioid receptor: structural basis for the differential regulation. *J Pharmacol Exp Ther*. **302**(3):1184-1192.
Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY, Caron MG. (1998) Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proc Natl Acad Sci U S A*. **95**(12):7157-7162.
Zhao ZQ, Gao YJ, Sun YG, Zhao CS, Gereau RW 4th, Chen ZF. (2007) Central serotonergic neurons are differentially required for opioid analgesia but not for morphine tolerance or morphine reward. *Proc Natl Acad Sci U S A*. **104**(36):14519-14524.
Zhu J, Luo LY, Li JG, Chen C, Liu-Chen LY. (1997) Activation of the cloned human kappa opioid receptor by agonists enhances [35S]GTPgammaS binding to membranes: determination of potencies and efficacies of ligands. *J Pharmacol Exp Ther*. **282**(2):676-684.
Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol*. **72**(12):9873-9880.

Appendix I Unpublished studies on KOR-dependent regulation of BDNF by acute social defeat stress

Considerable evidence suggests that stress promotes depression in susceptible individuals, and changes in the hippocampus have been implicated in this process (Pittenger 2008). Depressed patients have decreased hippocampal volume (Sheline 2003), and animal models of stress and depression show changes in cellular morphology in hippocampus (Magarinos 1996). Hippocampal brain derived neurotrophic factor (BDNF) mRNA and protein levels are also decreased following both acute and chronic animal models of stress (Pizarro 2004). Decreases in BDNF have been correlated with depression-like behavior, whereas infusion of BDNF into the hippocampus has anti-depressant effects and antidepressants increase BDNF mRNA in hippocampus (Pliakas 2001, Tsankova 2006). The mechanisms involved in stress regulation of BDNF are poorly understood. It has been previously shown that the kappa opioid receptor (KOR) is activated by stress and has been shown to play a role in immobility induced by forced swim stress and social defeat stress (Pliakas 2001, McLaughlin 2003, McLaughlin 2006). We set out to test whether stress induced changes in BDNF message are KOR dependent and to further characterize the mechanism by which KOR might regulate BDNF.

Methods

Acute Social Defeat Stress. Acute social defeat stress (SDS) was performed as previously described (Pizarro 2004). Intruder mice were placed in the cage of a resident aggressor and subjected to SDS for 2 minutes. The intruder was then placed in a permeable protective barrier inside the resident cage for 2 minutes. Each intruder was subjected to a total of 3 SDS sessions lasting 10 minutes. Control undefeated mice were housed in parallel but did not undergo SDS.

qRT-PCR. Mice were killed by cervical dislocation 24 hours after drug injection or acute SDS, and brains removed. The hippocampus was dissected and immediately frozen in liquid nitrogen and stored at -80°C until processing. RNA was harvested by Qiashredder columns and the Rneasy miniprep kit (Qiagen).

One step quantitative PCR was performed on an Mx3000 using Brilliant Sybr Green and Stratascript reverse transcriptase (Stratagene). Total BDNF, BDNF transcript I, BDNF transcript II, BDNF transcript III, BDNF transcript IV, and BDNF transcript V were amplified in separate reactions and each sample run in triplicate. Sample RNA was calculated based on a standard curve for each primer set and normalized to the housekeeping gene ARBP.

Promoter analysis. GenBank accession AY057907 was used for the DNA sequence of BDNF. The DNA sequence 250 base pairs upstream and downstream of the transcription start site of exon III were used for promoter analysis. Potential transcription factor sites were identified using the Zlab Possum tool.

Results

To measure the effects of an acute social defeat stress on BDNF message levels, an intruder mouse was placed in a cage sequentially with three different resident aggressor mice for a total of ten minutes. RNA was extracted from the intruder mouse 24 hours after the social defeat stress, and BDNF message levels were measured by qRT-PCR and normalized to ARBP message. To test if KOR was required for the decrease of BDNF mRNA following social defeat stress, BDNF mRNA was measured in hippocampus of wild type and KOR knockout mice 24 hours after an acute social defeat stress. A significant 40% decrease was seen in wild type mice following social defeat stress. KOR-/- mice expressed BDNF message at a lower level than wild type mice under non-stressed control conditions, but no change in BDNF expression was observed in KOR-/- mice following social defeat stress. (Fig I.1a) These results indicate that KOR is required for the defeat stress-induced decrease in BDNF mRNA. They also indicate that either KOR regulates BDNF expression under controlled conditions, or that a lack of KOR in during development results in compensatory changes which include changes in BDNF expression.

We next tested the effect of pharmacological disruption of KOR on social defeat stress-induced changes in BDNF expression. Mice were treated with the saline vehicle or the KOR antagonist norBNI

(10mg/kg) 3 hours prior to social defeat stress. No decrease in BDNF expression was measured in socially defeated mice pretreated with norBNI (Fig I.1b). In combination with the KOR-/- data, these results suggest that the decrease in BDNF message following an acute social defeat requires KOR activation.

To determine if activation of KOR is sufficient to decrease hippocampal expression of BDNF mRNA, BDNF message in hippocampus was measured 24 hours after treatment with saline vehicle or the KOR agonist U50,488 (5mg/kg). Treatment with U50,488 resulted in a significant decrease in BDNF message, similar in magnitude to the change observed after an acute social defeat stress (Fig I.2). These data show that KOR activation results in a decrease in BDNF message after 24 hours.

The BDNF gene structure is composed of a single coding exon which can be spliced to the 3' end of one of five differentially regulated non-coding exons with distinct promoters. To determine which promoter may be regulated by social defeat stress-induced KOR activation, qRT-PCR was used to measure the levels of each transcript following an acute social defeat stress. Social defeat stress was found to specifically decrease the level of transcript III mRNA (Fig I.3). Transcript III message was significantly decreased by 40% following social defeat stress, but no significant changes were found in transcripts I, II, IV, or V. This indicates the promoter for this transcript is the primary target of regulation by an acute social defeat stress. The regulation of BDNF transcript III has not been well characterized, but promoter analysis of the DNA sequence 250 base pairs upstream and downstream of the transcription start site of exon III using the Zlab possum tool reveals consensus sequences for several transcription factors (Fig I.4). These include consensus sequences for regulation by ETS, AP-1, and Sp1 family transcription factors in regions of the promoter which are conserved between mouse and man.

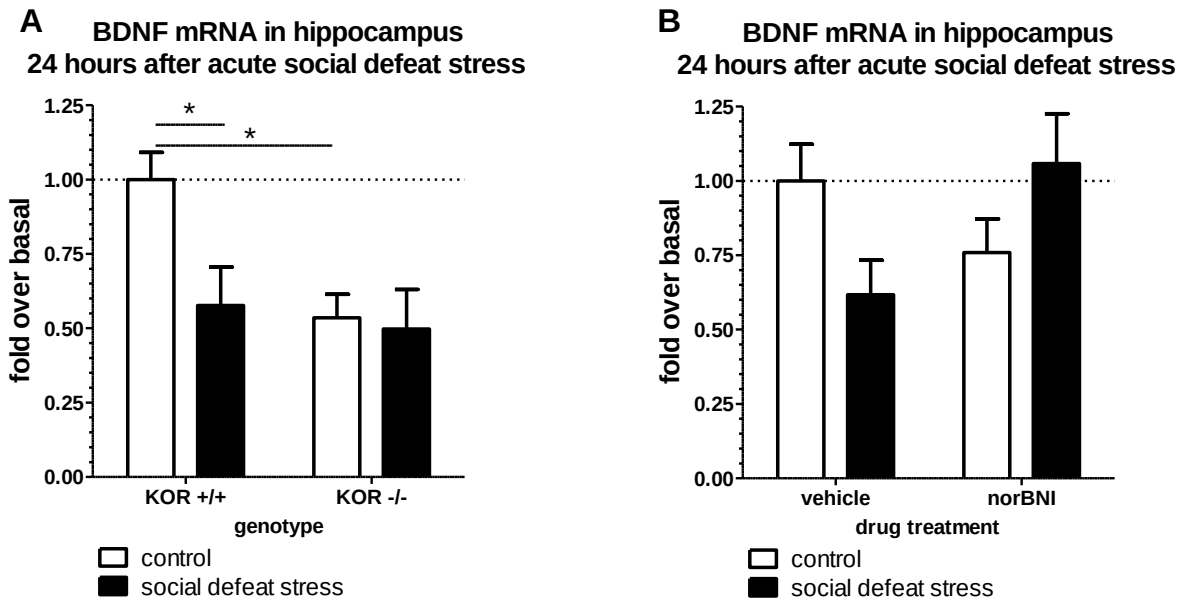


Figure I.1 Total BDNF RNA was is decreased in hippocampus 24 hours after an acute SDS. (A) Hippocampal BDNF mRNA was quantified in KOR-/- and wild type mice. Significant effect of stress ($p<0.05$) and genotype ($p<0.05$) was revealed by two-way ANOVA (n=7-8). Significance of the differences, according to Bonferroni-Dunn post-hoc test, is indicated by * ($p<0.05$). (B) Hippocampal BDNF mRNA was quantified in wild type animal injected with saline or norBNI (10mg/kg) 3 hours prior to SDS. Significant interaction effect ($p<0.05$) was revealed by two-way ANOVA (n=8-10).

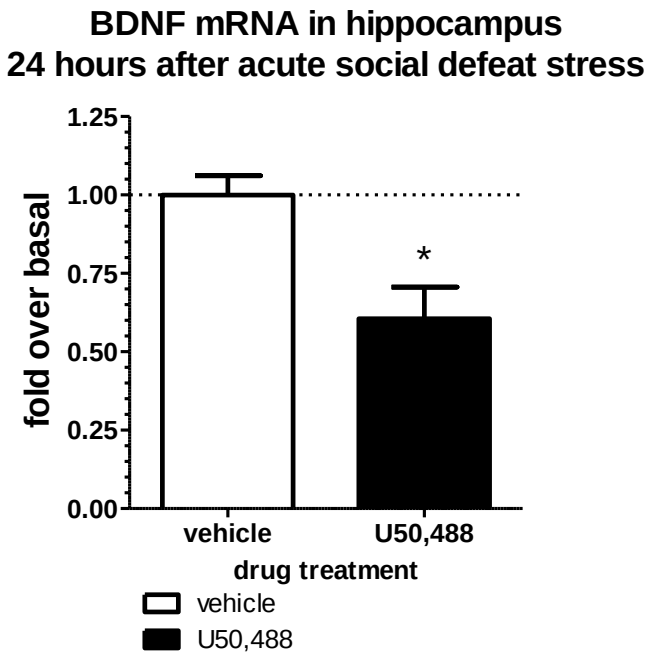


Figure I.2 BDNF RNA in hippocampus was is decreased 24 hours after U50,488 (5mg/kg, i.p.). decrease in BDNF is indicated by *, student's t-test ($p<0.01$, $n=7$).

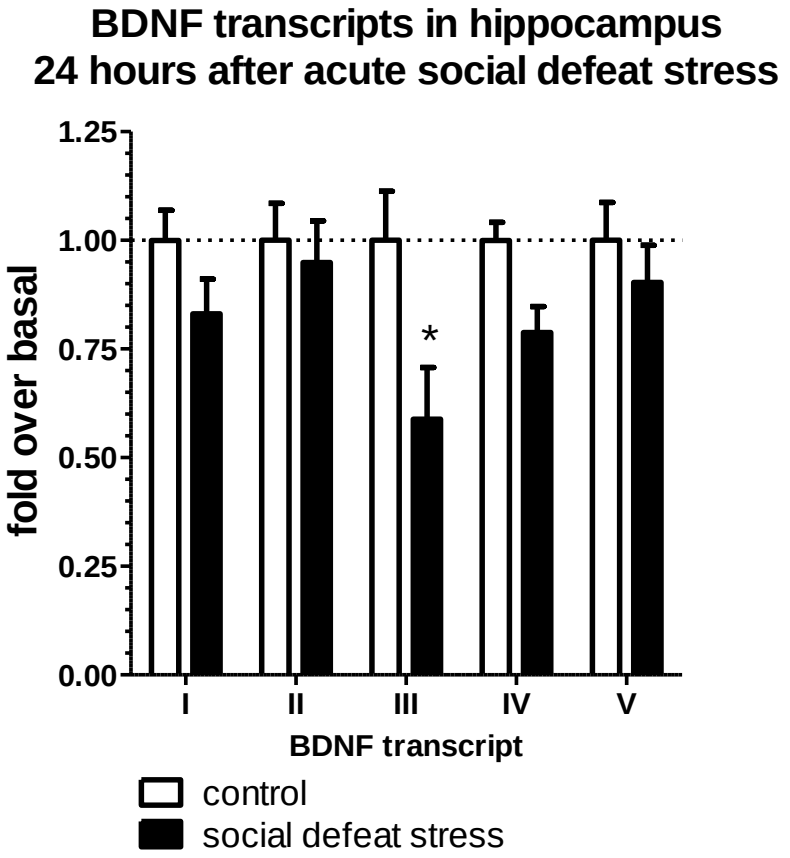


Figure I.3 BDNF transcripts I-V were quantified by qRT-PCR 24 hours after SDS. Significant effect of stress ($p<0.001$) was revealed by two-way ANOVA (n=5-8). Significance of the differences, according to Bonferroni-Dunn post-hoc test, is indicated by * ($p<0.01$).

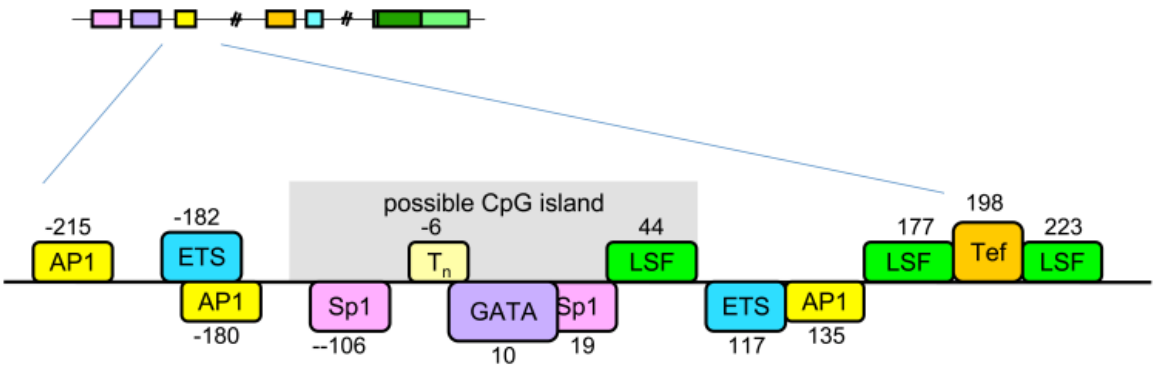


Figure I.4 Putative regulatory elements within the promoter of BDNF transcript III. The DNA sequence 250 base pairs upstream and downstream of the transcription start site for exon III was analyzed for consensus sequences for common transcription factors. The potential ETS and AP1 binding sites and the SP1 site at -106 are conserved between mouse and human.

Appendix II: Unpublished studies on the development of model systems and molecular tools to study JNK-mediated regulation of opioid receptors

Although classically considered competitive antagonists, norBNI and several related kappa opioid receptor (KOR) selective antagonists exhibit long lasting antagonism of KOR (Horan 1992, Jones 1992, Butelman 1993, Negus 2002). This antagonism of KOR by norBNI lasts over two weeks in vivo with no change in receptor binding. Furthermore, norBNI exhibits collateral agonist activity, activating JNK, and JNK activation is required for the long lasting antagonist activity of norBNI (Bruchas 2007). Similar results have been found for the KOR antagonists JDTic and GNTI, and a recent analysis of 12 KOR antagonists from Pfizer, AstraZeneca, RTI, McLean, and Eli Lilly found a strong correlation between the duration of antagonist effect in vivo and the magnitude of induced JNK phosphorylation (Melief 2011). Therefore JNK-mediated regulation of KOR by antagonists with long durations of action is not unique to norBNI. The mu opioid receptor (MOR) agonist morphine also shows atypical properties. Unlike fentanyl tolerance, morphine tolerance is not GRK3 dependent and morphine does not induce MOR internalization. However, tolerance to morphine but not fentanyl is JNK dependent (Melief 2010).

We propose that norBNI and morphine both lead to receptor inactivation through a similar mechanism and that JNK activation by norBNI or morphine results in association of a protein with the cytoplasmic face of the receptor, sterically occluding G protein and arrestin signaling. This mechanism of JNK regulation of opioid receptors would be analogous to receptor regulation by GRK and arrestin. Because the duration of action of norBNI is comparable to that of the covalently binding non-competitive antagonist βCNA, suggesting new receptor synthesis is required for signaling, this protein association is expected to be very stable in the case of KOR. In order to understand the changes that occur in the KOR and MOR signalosomes following norBNI or morphine treatment and to identify the mechanism of JNK

mediated regulation, it will be beneficial to develop readily-manipulated model systems and molecular tools with which to study these changes.

Results

HEK293 and NG108 cell culture as in-vitro models for studying duration of norBNI effect

The duration of action of norBNI in KOR expressing NG108-15 and HEK293 cells was tested by agonist stimulated ERK1/2 phosphorylation following antagonist pretreatment or 24 hours following wash-off of antagonist treatment. Cells were treated with 1μM norBNI or 10μM naloxone for 24 hours, and then immediately treated with 10μM U50,488 (T0), or washed to remove antagonist 24 or 48 hours prior to U50,488 treatment (T24 and T48). In HEK293 and differentiated NG108-15, pretreatment with norBNI and naloxone blocked U50,488-stimulated phosphorylation ERK1/2 at T0 (in the presence of antagonist), as expected. In both cell lines, U50,488 stimulated ERK1/2 phosphorylation was still inhibited by norBNI pretreatment 24 hours after norBNI had been washed off (T24) (Fig II.1). In NG108, but not HEK293 cells, this inhibition of ERK1/2 stimulation was still observed at 48 hours after wash-off of norBNI. As a control, the duration of action of naloxone, which does not activate JNK and has a short duration of action in vivo, was tested. In contrast to norBNI, no antagonism of the ERK1/2 response by naloxone was observed after antagonist wash-off in either cell line. The selective antagonism of KOR in HEK293 and NG108-15 cell cultures by norBNI after wash-out indicates that norBNI has a long duration of action in these cells.

If the antagonism of the ERK1/2 response by norBNI at T24 occurs via a similar mechanism to that underlying the long-lasting effects of norBNI in vivo, inhibition of JNK should accelerate recovery of the agonist-induced ERK1/2 response following norBNI treatment. To test this hypothesis, KOR expressing HEK293 cells were treated with the JNK inhibitor SP610025 (100nM) 30 minutes prior to norBNI treatment. SP610025 had no significant effect on norBNI inhibition of ERK1/2 phosphorylation at T0 when drug was still on board. In contrast, SP610025 pretreatment significantly reduced the

inhibition of ERK1/2 phosphorylation by norBNI 24 hours after wash-out (Fig II.2). These data show that the long-lasting inhibition by norBNI is JNK-dependent in HEK293, similar to in vivo, and validate these cells as model to study JNK-mediated uncoupling of KOR by norBNI.

The validated cell culture model will provide a valuable functional assay for studying the mechanism of norBNI's long duration of action. These cell culture models will allow for a wide range of studies, including site-directed receptor mutagenesis, large-scale receptor purification for analysis by mass spectrometry, and manipulation of candidate interactors. Utilizing two different cell culture models, NG108-15 and HEK293, and focusing on data which is consistent between these cell lines will provide greater confidence in conclusions drawn.

Tandem affinity purification tagged KOR

To identify novel opioid receptor-interacting proteins whose association was changed by norBNI treatment we adopted a discovery based approach. We generated a tandem affinity purification (TAP) tagged KOR with an amino terminal domain containing streptavidin binding peptide (SBP), hemagglutinin (HA), and calmodulin binding peptide (CBP) as previously described (Angers 2006). A similar TAP-tagged MOR cell line has also been developed. TAPKOR-associated proteins can be affinity-purified by streptavidin and calmodulin crosslinked agarose, and analyzed by mass spectrometry. This approach has previously been used to identify α-adrenergic receptor binding partners (Lyssand 2010).

Lentiviral expression of TAPKOR

The TAPKOR sequence has been inserted into a lentiviral vector for expression in vivo (Fig II.3a). Injection of lenti-KOR into the dorsal raphe has previously been shown to restore the analgesic and aversive responses to U50,488 (Land 2009). HEK293 transduced with lenti-TAPKOR express functional receptor (Fig II.3b,c). Injection of lenti-TAPKOR in the dorsal raphe of KOR-/- mice restored the analgesic response to U50,488, which was blocked by norBNI (Fig II.4). Furthermore, norBNI

antagonism was found to be long lasting. Lenti-TAPKOR will allow for purification of TAPKOR-associated proteins from mouse brain and in-vivo validation of candidate proteins.

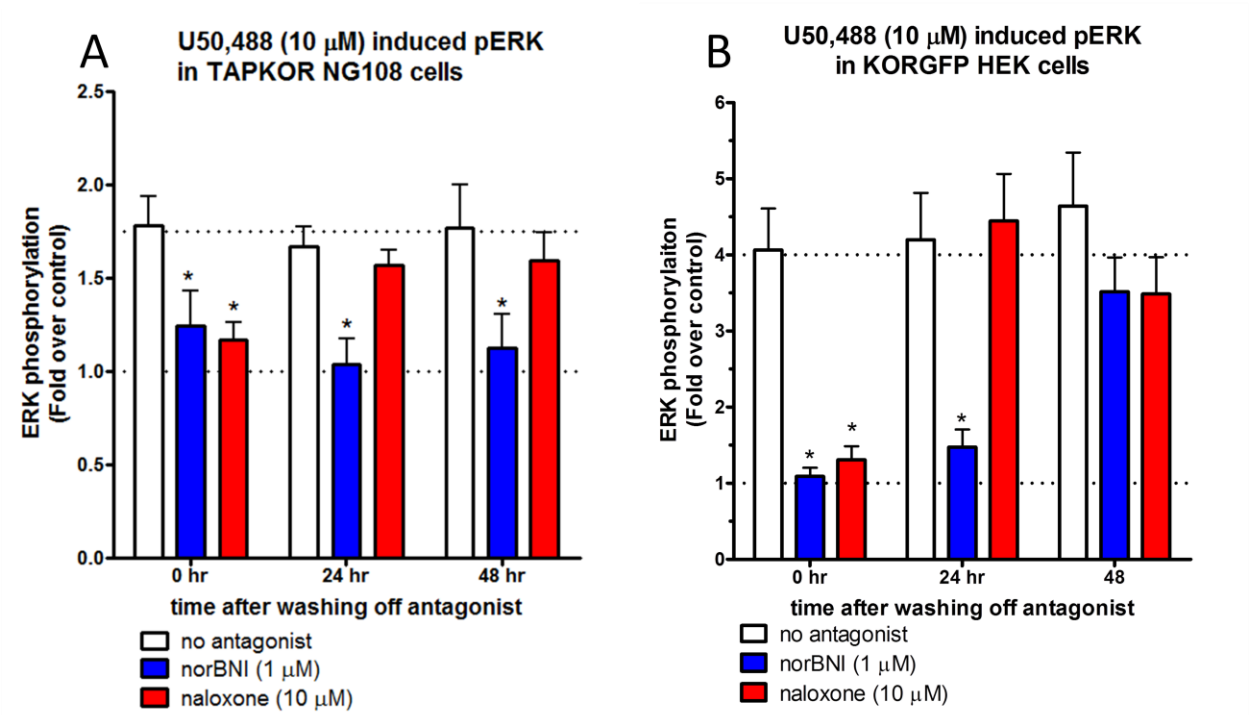


Figure II.1 NorBNI, but not naloxone, treatment has long-lasting effects in HEK293 and NG108-15. A. The kinetics of antagonist reversal was measured by the increase in ERK1/2 phosphorylation stimulated by 10μM U50,488 of KORGFP expressing HEK cells in the presence of antagonist (0 hr) or at 24 or 28 hr after antagonist was washed off. Significant effect of antagonist ($p<0.001$), recovery time ($p<0.001$) and interaction effect ($p<0.01$) was revealed by two-way ANOVA (n=8-23). Significance of the differences as compared to no antagonist, according to Bonferroni-Dunn post-hoc test, is indicated by * ($p<0.05$). B. The kinetics of antagonist reversal was measured by the increase in ERK1/2 phosphorylation stimulated by 10μM U50,488 of TAPKOR expressing NG108-15 cells in the presence of antagonist (0 hr) or at 24 or 28 hr after antagonist was washed off. Significant effect of antagonist ($p<0.001$) was revealed by two-way ANOVA (n=8-15). Significance of the differences as compared to no antagonist, according to Bonferroni-Dunn post-hoc test, is indicated by * ($p<0.05$).

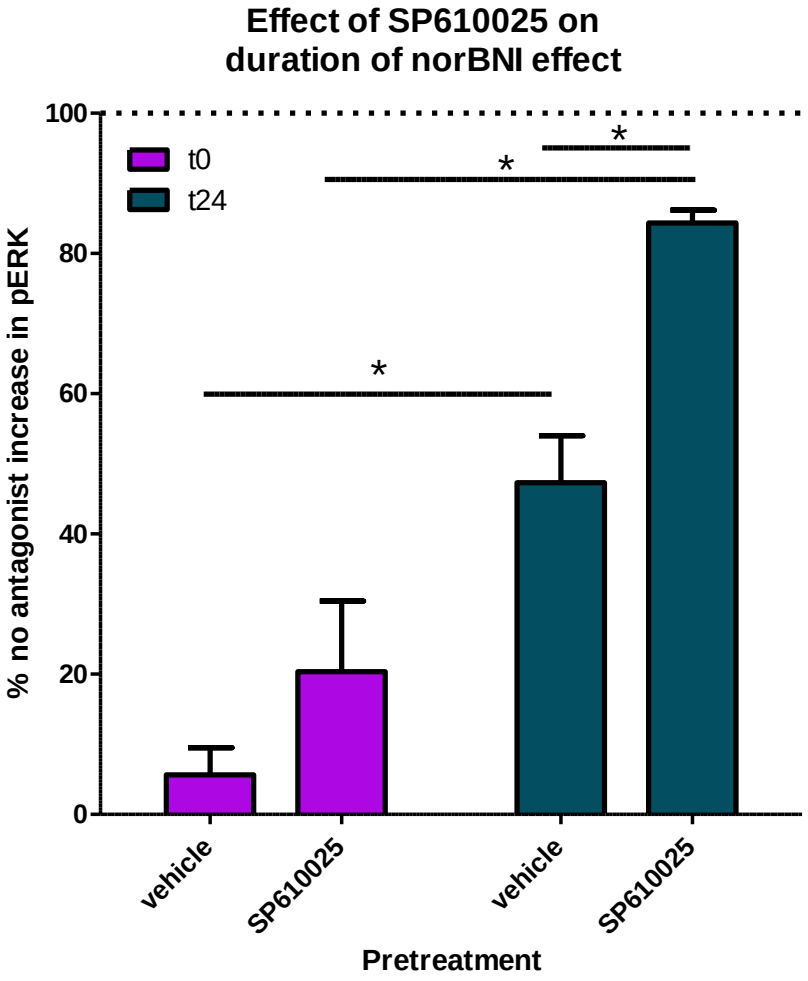


Figure II.2 JNK is required for the long-lasting effects of norBNI in HEK293. Pretreatment with the JNK inhibitor SP610025 (100nM) accelerates reversal of norBNI antagonism. Y-axis is expressed as percent of the U50,588 stimulated increase in phospho-ERK in the absence of antagonist. Significant effect of antagonist ($p<0.001$) and recovery time ($p<0.001$) was revealed by two-way ANOVA (n=3). Significance of the differences, according to Bonferroni-Dunn post-hoc test, is indicated by * ($p<0.05$).

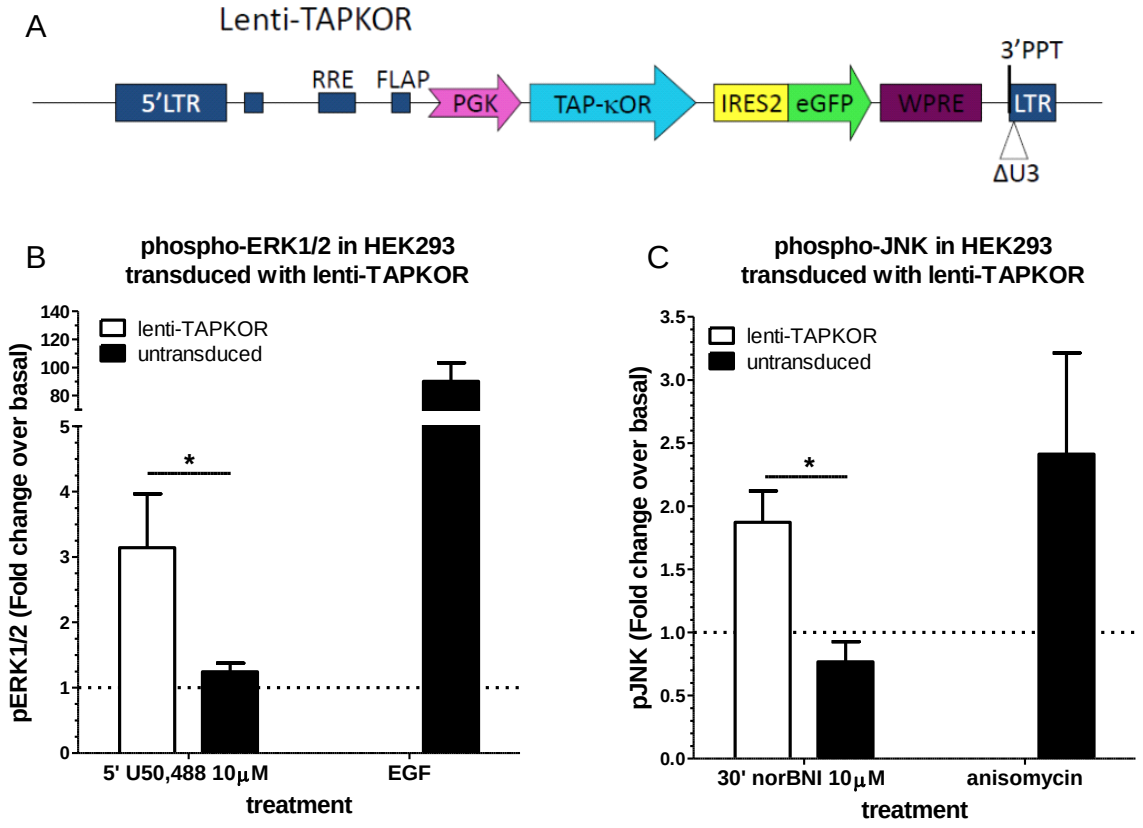


Figure II.3. Transduction of HEK293 with lenti-TAPKOR results in function receptor. A. Schematic of the lentiviral TAPKOR construct. B. Treatment with 10μM U50,488 (5 min) results in a significant increase in ERK1/2 phosphorylation in HEK293 transduced with lenti-TAPKOR, but not in untransduced HEK293 ($p<0.05$, students t-test). C. Treatment with 10μM norBNI (30 min) results in a significant increase in JNK phosphorylation in HEK293 transduced with lenti-TAPKOR, but not in untransduced HEK293 ($p<0.05$, students t-test).

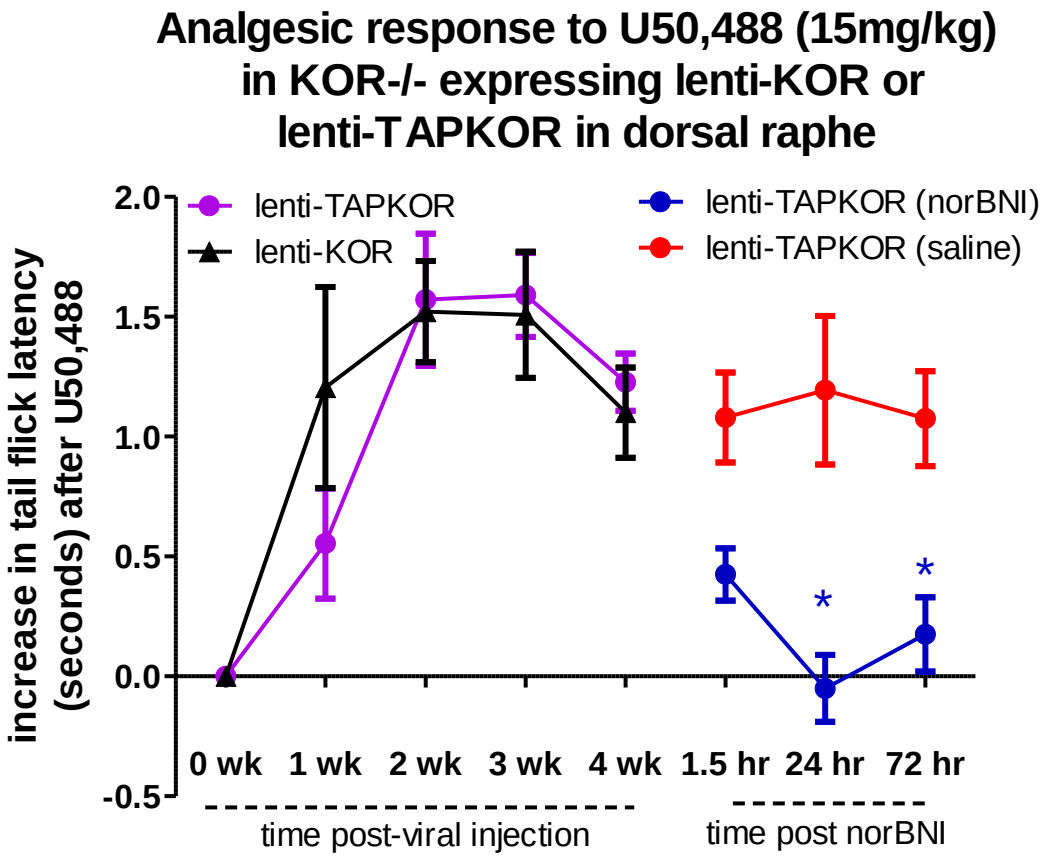


Figure II.4. The duration of norBNI antagonism is reconstituted by lenti-TAPKOR expression in DRN. The warm water tail withdrawal latency following U50,488 injection was used to assess the recovery of the analgesic response following Lenti-TAP-KOR injection into dorsal raphe of KOR-/- mice. Lenti-TAPKOR was as effective as lenti-KOR in recovering analgesic response. Significant effect of time after viral injection ($p<0.001$) was revealed by two-way ANOVA (n=6-11). Significant differences as compared to wk 0 were found for both viruses for weeks 1-4, according to Bonferroni-Dunn post-hoc test ($p<0.05$). NorBNI (10mg/kg, i.p.) was injected at wk 5. NorBNI, but not vehicle, blocked the analgesic response to U50,488 for at least 3 days, whereas vehicle injection was ineffective. Significant effect of norBNI treatment ($p<0.001$) was revealed by two-way ANOVA (n=4-6). Significance of the differences as compared to vehicle, according to Bonferroni-Dunn post-hoc test, is indicated by * ($p<0.05$).

Appendix III: Lentiviral expression vectors developed during thesis

Lentiviral backbone

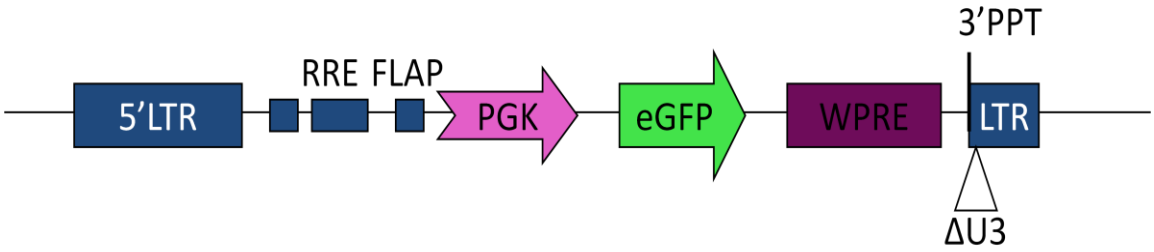
All lentiviral vectors were derived from the lentiviral construct expressing the β2 subunit of the nicotinic acetylcholine receptor under the mouse phosphoglycerol kinase (PGK) promoter, published by the Changeux group (Maskos 2006). The viral expression vectors use pUC18 as the backbone plasmid. Use of the PGK promoter rather than a viral promoter reduces gene silencing which results from DNA methylation of CpG repeats. The integrated virus is rendered replication incompetent by deletion of the U3 region of the 3' long terminal repeat. Sequences were incorporated to enhance RNA stability and transgene expression (WPRE) and infection of nondividing cells (99 base pair DNA "flap"). Production of viral particles requires cotransfection with plasmids expressing envelope proteins and lentiviral packaging proteins.

In the viral schematics below the following abbreviations are used:

WPRE = woodchuck hepatitis B virus post-transcriptional regulatory element. FLAP = 99 bp DNA "flap". PGK = phosphoglycerate kinase promoter. RRE = rev response element. ΔU3 = deletion of U3 element of 3'LTR. LTR = long terminal repeat

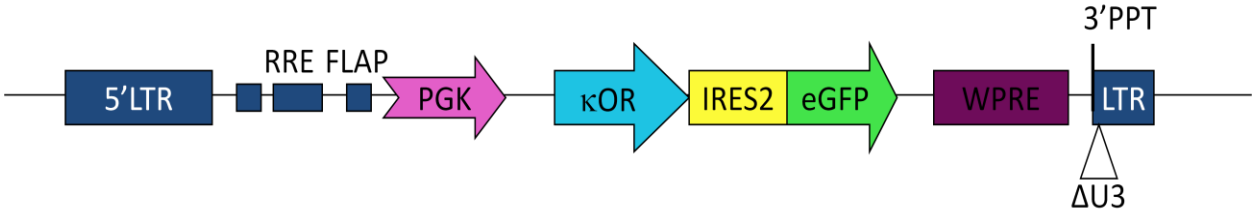
Lenti-GFP

GFP under the control of the PGK promoter



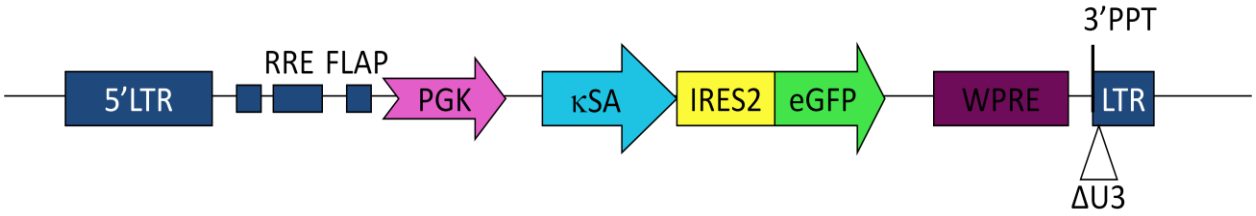
Lenti-KOR

Bicisctronic cassette using internal ribosomal entry sequences; rat sequence KOR and GFP under the control of the PGK promoter



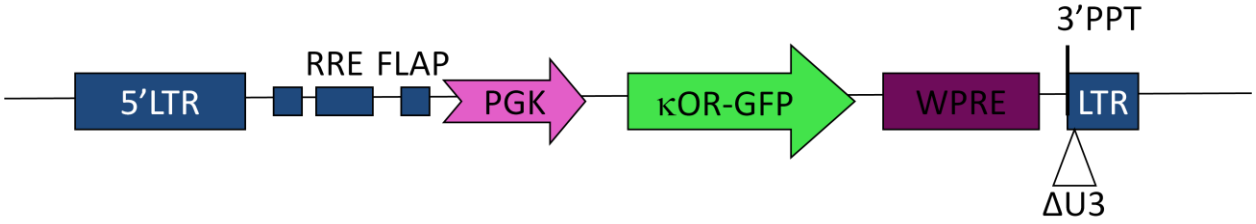
Lenti-KSA

Bicisctronic, using internal ribosomal entry sequences; rat sequence KOR(Ser369Ala) and GFP under the control of the PGK promoter



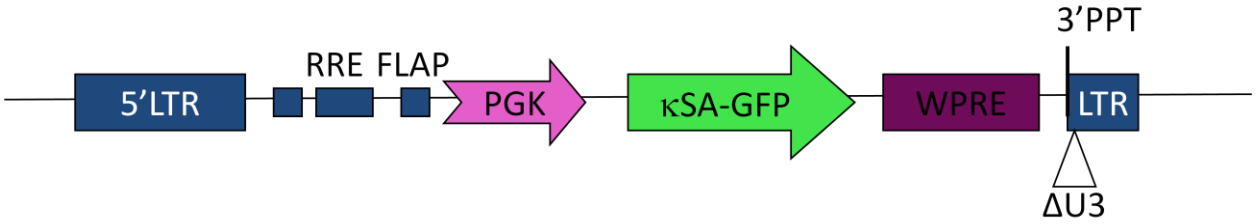
Lenti-KORGFP

C-terminal GFP-tagged rat sequence KOR under the control of the PGK promoter



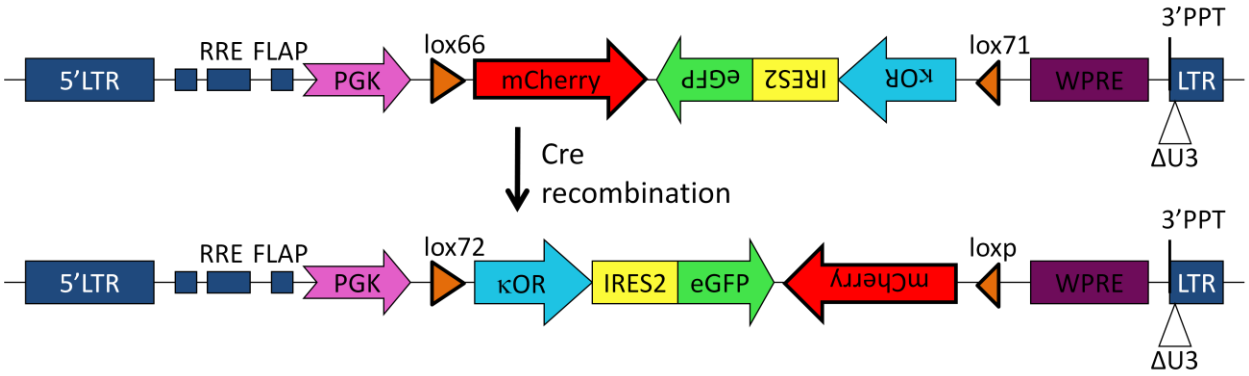
Lenti-KSAGFP

C-terminal GFP-tagged rat sequence KOR(Ser369Ala) under the control of the PGK promoter



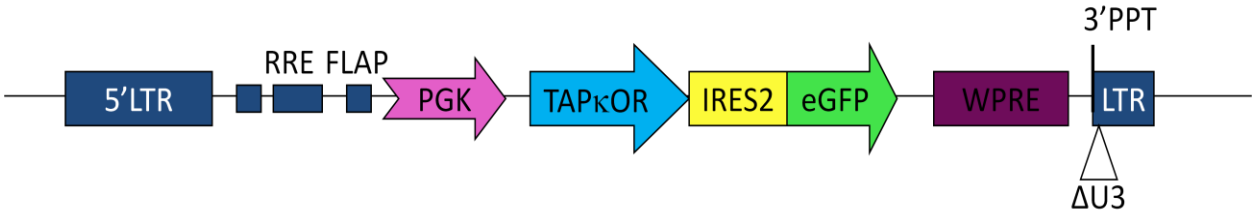
Lenti-ifKOR

Expresses mCherry and KOR/GFP in opposite orientations between two lox sites. Prior to cre-recombination, the mCherry is in the forward orientation from the PGK promoter, while KOR and eGFP are inverted and not expressed. Following cre-recombination, KOR and GFP are in the correct forward orientation relative to the promoter, while mCherry is inverted and not expressed. Mutations within the lox sites strongly favor the forward reaction, effectively preventing the reverse reaction (Albert 1995, Oberdoerffer 2003).



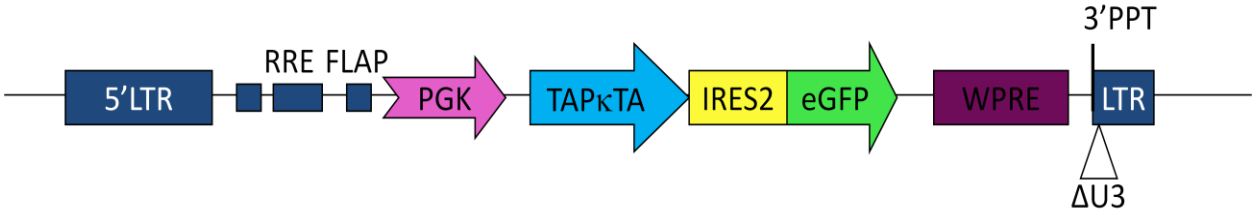
Lenti-TAPKOR

Bicisctronic cassette using internal ribosomal entry sequences; N-terminal tandem-affinity purification (TAP)-tagged rat sequence KOR and GFP under the control of the PGK promoter. The TAP tag is comprised of a streptavidin binding peptide, an HA tag, a TEV cleavage site, and a calmodulin binding peptide (Angers 2006).



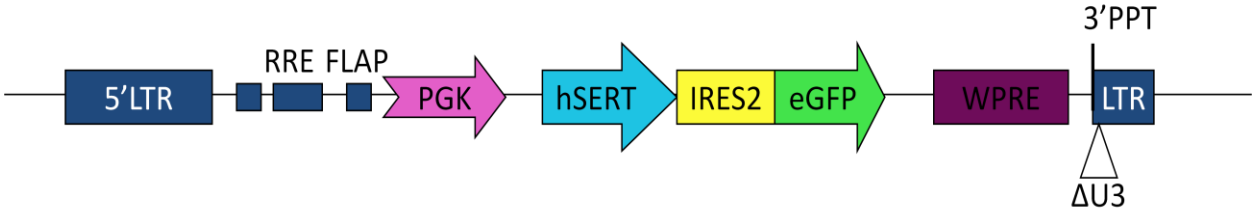
Lenti-TAPKTA

Bicisctronic cassette using internal ribosomal entry sequences; N-terminal tandem-affinity purification (TAP)-tagged rat sequence KOR(Thr171Ala) and GFP under the control of the PGK promoter. The TAP tag is comprised of a streptavidin binding peptide, an HA tag, a TEV cleavage site, and a calmodulin binding peptide (Angers 2006).



Lenti-hSERT

Bicisctronic cassette using internal ribosomal entry sequences; human sequence serotonin reuptake transporter and GFP under the control of the PGK promoter



Curriculum Vitae

Selena Schreiber Schattauer

Department of Pharmacology

EDUCATION

9/2004 – 6/2012	University of Washington Seattle, Seattle, Wa Ph.D., Pharmacology Charles Chavkin, Ph.D., mentor
8/2000 – 5/2004	University of Texas at Austin, Austin, Tx B.S. Biology, Molecular and Cellular Option

PAPERS IN SUBMISSION

Schattauer SS, Miyataki M, Shankar H, Zietz C, Levin JR, Liu-Chen LY, Gurevich VV, Rieder MJ, Chavkin C. Ligand directed signaling differences between rodent and human kappa opioid receptors. (Submitted to *Journal of Biological* Chemistry, 2012)

PUBLICATIONS

Land BB, Bruchas MR, **Schattauer S**, Giardino WJ, Aita M, Messinger D, Hnasko TS, Palmiter RD, Chavkin C. Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. *Proc Natl Acad Sci U S A*. 2009 Nov 10;106(45):19168-73.

Bruchas MR, Yang T, **Schreiber S**, Defino M, Kwan SC, Li S, Chavkin C. Long-Acting Kappa Opioid Antagonists Disrupt Receptor Signaling And Produce Noncompetitive Effects By Activating C-Jun N-Terminal Kinase. *J Biol Chem*. Oct 12;282(41):29803-11, 2007.

Petraschka M, Li S, Gilbert TL, Westenbroek RE, Bruchas MR, **Schreiber S**, Lowe J, Low MJ, Pintar JE, Chavkin C. The absence of endogenous beta-endorphin selectively blocks phosphorylation and desensitization of mu opioid receptors following partial sciatic nerve ligation. *Neuroscience*. Jun 8;146(4):1795-807, 2007.

Maldve RE, Zhang TL, Ferrani-Kile K , Lippmann M, **Schreiber SS**, Snyder GL, Fienberg AA, Leslie SW, Gonzales RA and Morrisett RA. The DARPP-32 cascade and regulation of the ethanol sensitivity of NMDA receptors in the nucleus accumbens. *Nat Neuroscience* 7:641-648, 2002.

MEETINGS ATTENDED

2002	25th Annual Scientific Meeting of the Research Society on Alcoholism, June 28 - July 3, 2002, San Francisco, CA.
2003	26th Annual Scientific Meeting of the Research Society on Alcoholism, June 21 - June 25, 2003, Fort Lauderdale, FL.
2004	27th Annual Scientific Meeting of the Research Society on Alcoholism, June 26 - June 30, 2003, Vancouver, BC Canada.
2009	International Narcotics Research Conference, July12-16, 2009, Portland, Or.
2011	Kappa Therapeutics, July 10-13, 2011, Seattle, Wa.

ABSTRACTS/POSTERS

S. Schattauer, H. Shankar, M. Tarabochia, C. Chavkin. Arrestin dependent p38 activation by the human kappa opioid receptor. *Kappa Therapeutics*, 2011, Seattle, Wa.

S. Schattauer, C. Chavkin. Acute social defeat stress decreases brain derived neurotrophic factor message in hippocampus by a kappa opioid receptor dependent mechanism. *International Narcotics Research Council*, 2009, Portland Or.

B.B. Land, M.R. Bruchas, W. Giardino, **S. Schattauer**, M. Aita and C. Chavkin. Dorsal raphe kappa opioid receptors (KOR are necessary for stress-induced reinstatement of cocaine CPP. *International Narcotics Research Council*, 2009, Portland Or.

Bruchas MR. Yang T, **Schreiber S**, Defino M, Kwan SC, Li S, Chavkin C. The anomalous, long lasting effects of kappa opioid antagonists are cause by JNK-induced disruption of the kappa opioid receptor signaling complex. Abstracts from the American College of Neuropsychopharmacology, 2007, Boca Raton, Fl.

Bruchas MR, Yang T, **Schreiber S**, Defino M, Kwan SC, Carroll I, Chavkin C. Uncovering the atypical pharmacology of kappa opioid receptor antagonists. Page 25. Abstracts of the 2007 International Narcotics Research Council Conference, July 8-13, 2007, Berlin, Germany.

Schreiber, SS, Reyna, G, Morrisett, RA, Maldve, RE. "Visualizing alterations in DARPP-32 phosphorylation using immunoblot analysis and confocal microscopy." *Alcoholism Clinical and Experimental Research (Supplement)* 28(5): 53, May 2004.

Schreiber, SS, Reyna, G, Morrisett, RA, Maldve, RE. "A biphasic response to ethanol of DARPP-32 phospho-isoforms in the nucleus accumbens." *Alcoholism Clinical and Experimental Research (Supplement)* 27(5): 52, May 2003.

Maldve, RE, **Schreiber, SS**, Morrisett, RA. "Ethanol induced changes in DARPP-32 phosphorylation via a Cyclin-Dependent Kinase-5 pathway." *Alcoholism Clinical and Experimental Research (Supplement)* 26(5): 32, May 2002.

Maldve, RE, **Schreiber, SS**, Steinwand, TV, Morrisett, RA. "Immunofluorescent localization of DARPP-32 and DARPP-32 phosphorylation in the extended amygdala." Washington, DC: Society for Neuroscience, 2002.

FELLOWSHIPS AND HONORS

2004	Recipient of Hurd Fellowship
2005-2008	Recipient of Pharmacological Sciences Training Grant
2009	International Narcotics Research Conference Travel Award