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Mechanisms of Opioid Receptor Desensitization

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Abstract

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Agonists targeting MOR are effective analgesics, but their clinical use is hindered by side effects, including tolerance and addiction. KOR agonists also produce analgesia, but clinical use of these compounds has remained minimal due to aversive properties in humans. The μ-opioid receptor (MOR) agonists, morphine and fentanyl, both activate c-Jun N-terminal kinase (JNK), which is required for spinally-mediated morphine acute analgesic tolerance, whereas acute analgesic tolerance to fentanyl is blocked by G protein-coupled receptor kinase 3 (GRK3) gene deletion. Similarly, the κ-opioid receptor (KOR) collateral antagonist, norBNI, stimulates phosphorylation of JNK, and JNK1 is specifically required for norBNI's long duration of antagonism. The durations of action of a broad range of KOR antagonists, including norBNI, positively correlate with the ability of the antagonist to activate JNK1 (Melief *et al.*, 2011), whereas there is no correlation between duration of antagonist action and drug clearance (Munro

et al., 2012). A better understanding of the mechanisms that contribute to opioid receptor desensitization is necessary for the development of improved therapeutics that avoid tolerance. This JNK-mediated mechanism of desensitization is likely to be more broadly applicable across the myriad of other GPCR systems. Additionally, elucidating the circuits responsible for p38-dependent, KOR-mediated aversion will assist with the development of KOR analgesics that avoid classic MOR side effects. Therefore, the goal of my thesis project has been to identify ligand-directed signaling mechanisms that contribute to JNK activation following morphine and fentanyl administration and better understand how JNK activation promotes MOR and KOR desensitization. Additionally, this thesis describes efforts to identify G biased KOR compounds with a reduced potential to cause dysphoria. Ultimately, this work provides a deeper understanding of opioid receptor signaling and can provide the basis for future therapeutic development targeting this receptor system.

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Chapter 1. INTRODUCTION AND BACKGROUND

The first isolation of opioids in the form of opium can be traced back to the 3rd century B.C. and by 1500 B.C. there is documentation of its medicinal use, both to prevent excessive crying and for the more commonly known use as a pain reliever, or analgesic. By the 8th century A.D., opium had spread throughout Asia and Europe and documentation of addiction and tolerance is found as early as the 16th century A.D. In 1806, Friedrich Wilhelm Adam Sertürner isolated the active ingredient in opium and named it morphine after Morpheus, the god of dreams. By the 1850s, morphine was routinely used to control surgical and postsurgical pain. However, morphine is highly addictive and therefore work has been dedicated to synthesizing a non-addictive opioid, which has led to the development of numerous opioid compounds (Brownstein, 1993). By the 1960s, it became clear that these compounds were acting on endogenous opiate receptors that had yet to be identified.

Opioids remain the gold standard for treatment of moderate to severe pain (Gutstein and Akil, 2001; McNally and Akil, 2002) with opioid prescriptions reaching 240.9 million and generating sales of $8.34 billion in 2012 (Meier and Marsh, 2013). However, one of the greatest barriers to effective pain management with opiates is the development of tolerance to the analgesic, but not the respiratory depressive, effects of the drug, which can result in death (Ling *et al.*, 1989; Paronis and Woods, 1997; JM White and Irvine, 1999). Additionally, the development of opioid tolerance leads to an escalation of drug consumption, which can ultimately increase the risk of addiction (Whistler, 2012). Although targeting the κ-opioid receptor remains an attractive strategy for analgesic development, drugs that have been used clinically produce unwanted dysphonia in humans (Kumor *et al.*, 1986). A better understanding

of the molecular signaling pathways that lead to these unwanted side effects will enhance our knowledge of opioid receptor signaling and enhance drug development both at the opioid receptors and more broadly at G protein-coupled receptors (GPCR), which as a receptor family are the largest target of currently approved drugs (Overington *et al.*, 2006).

THE ENDOGENOUS OPIOID RECEPTOR SYSTEM

The opioid receptors (μ, κ, and δ) are activated both by endogenous peptides and exogenous compounds. The receptors are part of the class A (Rhodopsin) family of $G_{i/o}$ protein-coupled receptors. After extensive development of synthetic opiate compounds, it became clear that an endogenous opioid system must exist (Martin, 1979). This hypothesis was substantiated through a series of experiments using tritiated ligands to identify the location of these novel receptors (Pert *et al.*, 1973; Terenius, 1973; Simon *et al.*, 1973) and it was shown that these receptors were concentrated in specific regions of the nervous system (Hiller *et al.*, 1973; Kuhar *et al.*, 1973). It was also hypothesized that endogenous opioid ligands must target these receptors and the enkephalins, methionine-enkephalin and leucine-enkephalin were the first to be identified (Hughes *et al.*, 1975), followed by β-endorphin (CH Li and D Chung, 1976; CH Li *et al.*, 1976; Loh *et al.*, 1976), and the dynorphins (Cox *et al.*, 1975; Goldstein *et al.*, 1981). It was shown that footshock stress or direct brain stimulation could produce naloxone-sensitive analgesia, suggesting that these novel endogenous ligands could be endogenously released by behavioral stress *in vivo* (Akil *et al.*, 1976).

Each endogenous opioid ligand is derived from a larger precursor protein. The enkephalins are derived from proenkephalin (Noda *et al.*, 1982), the β-endorphins from proopiomelanocortin (Nakanishi *et al.*, 1979), and the dynorphins from prodynorphin (Kakidani *et al.*, 1982). Distinct genes produce each of these larger precursor proteins and 20 known

biologically active peptides are derived from these three genes. The hypothesis that three opioid receptors existed was substantiated through a series of behavioral experiments using multiple opiate agonists and antagonists (Goldstein *et al.*, 1971; Pert *et al.*, 1973; Martin *et al.*, 1976) and led to the naming of the μ-opioid receptor (MOR) after morphine and the κ-opioid receptor (KOR) after ketocyclazocine. The δ-opioid receptor (DOR) was later documented and named after the vas deferens, where it was identified (Lord *et al.*, 1977). Enkephalins were shown to be selective for both DOR and KOR, the β-endorphins for MOR (Grossman and Clement-Jones, 1983), and the dynorphins for KOR (Chavkin *et al.*, 1982). It would be another decade before the opioid receptors were each cloned (Kieffer *et al.*, 1992; Evans *et al.*, 1992; Yasuda *et al.*, 1993; Chen *et al.*, 1993). The sequences of MOR, KOR, and DOR are roughly 58% homologous with the highest similarity in the transmembrane (73-76%) and intracellular (63-66%) portions of the receptor, and the most divergence in the extracellular domains (34-40%) (Satoh and Minami, 1995). Activation of all three opioid receptors results in analgesia in addition to modulation of mood, regulation of hedonic state, and regulation of the stress response. Additionally, opioid receptors are involved in respiration, gastrointestinal motility, and immune function.

The opioid receptors are widely distributed throughout the nervous system. MOR mRNA has been detected in a variety of brain regions (Delfs *et al.*, 1994; Mansour, Fox, Thompson, *et al.*, 1994; Minami *et al.*, 1994). MOR expression is highest in areas responsible for the analgesic effect of MOR agonists, including regions of the lower brain stem that activate descending pain inhibition (Basbaum and Fields, 1984), the periaqueductal gray (PAG) (Bennett and Mayer, 1979; Satoh *et al.*, 1983), and in laminae I and II of the spinal dorsal horn (Maekawa *et al.*, 1994), where nociceptive C and Aδ fibers terminate (Light and Perl, 1977). MOR expression is also high in regions associated with common side effects of opioids, including reward centers,

respiratory control regions (Vibert *et al.*, 1976), and the locus coeruleus, which modulates opiate withdrawal (Werling *et al.*, 1987). DOR is widely distributed throughout the brain (Mansour *et al.*, 1993; Bzdega *et al.*, 1993; Le Moine *et al.*, 1994) in reward centers and spinal sites. The same is true of KOR (Minami *et al.*, 1993; Mansour, Fox, Meng, *et al.*, 1994; DePaoli *et al.*, 1994; Maekawa *et al.*, 1994). All three opioid receptors are highly expressed in dorsal root ganglia (DRG), where the cell bodies of primary afferent neurons are located (Maekawa *et al.*, 1994; Minami *et al.*, 1995). Roughly 55% of DRG neurons are positive for MOR mRNA, 20% for DOR, and 18% for KOR.

Opioid Receptor Signaling

All three opioid receptors are $G_{i/o}$ coupled GPCRs. GPCRs are characterized by seven transmembrane domains with an extracellular N-terminus and an intracellular C-terminus. GPCRs are coded by almost 800 different genes and are divided into five families based on sequence identity (Fredriksson *et al.*, 2003). The rhodopsin family of GPCRs, which includes MOR, KOR, and DOR, is the largest. GPCRs are involved in a wide variety of physiological processes and their diversity results in a range of stimuli that they can respond to as well as numerous intracellular signaling pathways that they can modulate.

Canonically, agonist binding to a GPCR results in a conformational shift that leads to G protein activation. There are at least 21 Gα subunits, 6 Gβ subunits, and 12 Gγ subunits (Oldham and Hamm, 2006). Activated GPCRs cause GDP release from the Gα subunit followed by rapid binding of GTP, due to a large concentration gradient within the cell. Activated Gα dissociates from the receptor and Gβγ subunits, allowing for modulation of downstream effectors by both subunits. Gα subunits also contain a GTPase domain that hydrolyzes GTP, which terminates signaling. Freed Gβγ also recruits G protein-coupled receptor kinase 2 (GRK2) and GRK3 to the

membrane (K Haga and T Haga, 1992; Pitcher *et al.*, 1992; Koch *et al.*, 1993; Pitcher *et al.*, 1995; Daaka *et al.*, 1997; J Li *et al.*, 2003), which in part mediates GRK2 and GRK3's ability to phosphorylate only the active form of GPCRs.

Opioid receptor activation results in an overall reduction in cellular excitability. This occurs through modulation of multiple canonical cellular processes. Activation of MOR, KOR, and DOR leads to decreased adenylate cyclase activity in several brain regions (Collier and Roy, 1974; Fedynyshyn and Lee, 1989; Konkoy and Childers, 1989; Attali *et al.*, 1989; Childers, 1991), inhibition of voltage-dependent Ca^{2+} channels, as demonstrated in numerous heterologous expression systems and tissue types (Macdonald and Werz, 1986; Gross and Macdonald, 1987; Surprenant *et al.*, 1990; Shen and Surprenant, 1990; Seward *et al.*, 1991; Schroeder *et al.*, 1991; Tallent *et al.*, 1994), and an increase in K^+ conductance through the activation of G protein-coupled inwardly-rectifying K^+ channels (GIRK) (North *et al.*, 1987; Wimpey and Chavkin, 1991; Grudt and Williams, 1993).

GPCR Desensitization and Arrestin-Mediated Signaling

The canonical two-step GPCR desensitization mechanism occurs via GPCR phosphorylation by GRK, followed by arrestin binding to phosphorylated receptors, which are in the active state. Arrestin binding uncouples G protein signaling from GPCRs by sterically blocking further G protein binding and subsequent activation by the GPCR (Krupnick *et al.*, 1997). Arrestin binding also promotes receptor internalization by coupling the receptor to endocytic machinery, including clathrin (Goodman *et al.*, 1996), clathrin adaptor protein-2 (Laporte *et al.*, 1999), and N-ethylmaleimide-sensitive fusion protein (McDonald *et al.*, 1999). Receptor endocytosis results in either receptor degradation and downregulation or receptor resensitization and recycling back to the surface (Luttrell and Lefkowitz, 2002). Although this

two-step mechanism of desensitization involving GRK and arrestin has been well characterized, in some cases GRK is able to mediate phosphorylation- and arrestin-independent GPCR desensitization (Ferguson, 2007). It has been hypothesized that this occurs though GRK functioning as a scavenger for Gβγ and sequestering it away from the receptor (Raveh *et al.*, 2010).

GRK1 was the first GRK identified as the protein responsible for phosphorylating activated rhodopsin (Shichi and Somers, 1978) and GRK2 was first to be cloned (Benovic *et al.*, 1989). It was demonstrated that this novel kinase was a subclass of AGC serine/threonine kinases. Subsequently GRK3 (Benovic *et al.*, 1991), GRK4 (Ambrose *et al.*, 1992), GRK5 (Kunapuli and Benovic, 1993), GRK6 (Benovic and Gomez, 1993), and GRK7 (Hisatomi *et al.*, 1998; Weiss *et al.*, 1998) were cloned. The general structure of the GRK family of proteins consists of an N-terminal 25 amino acid region specific to the GRK family, a regulator of G protein (RGS) homology domain (Siderovski *et al.*, 1996), an AGC kinase family serine/threonine kinase domain, and a domain that regulates membrane association. Unlike most AGC kinase proteins, the GRK kinase domain is open and disordered (Lodowski *et al.*, 2006) and it is thought that this domain is rearranged to form an active kinase domain upon binding to active GPCRs. The seven GRKs are organized into three subfamilies based on sequence similarity: GRK1 subfamily comprising GRK1 and GRK7 with expression largely limited to the visual system (Somers and Klein, 1984; Zhao *et al.*, 1998; 1999; Kawakami and Kawamura, 2014), GRK2 subfamily comprising GRK2 and GRK3, and GRK4 subfamily comprising GRK4, GRK5, and GRK6 (Premont *et al.*, 1996). Each subfamily uses a distinct mechanism to recruit and retain the kinase at the cell membrane. GRK2 and GRK3 are recruited by active Gβγ (K Haga and T Haga, 1992; Pitcher *et al.*, 1992; Koch *et al.*, 1993; Pitcher *et al.*, 1995; Daaka *et al.*,

1997; J Li *et al.*, 2003). GRK4 and GRK6 rely on palmitoylation sites as well as lipid-binding elements (Premont *et al.*, 1996; Stoffel *et al.*, 1998; Jiang *et al.*, 2007), whereas GRK5 relies on lipid binding elements alone (Kunapuli *et al.*, 1994; Pitcher *et al.*, 1996).

Phosphorylation of the C-tail or other intracellular GPCR moieties is critical for receptor/arrestin interactions (Gurevich and Benovic, 1993; Gurevich *et al.*, 1995; Mendez *et al.*, 2000; Cen, Xiong, *et al.*, 2001). The non-visual arrestins were first identified by their ability to desensitize the β2 adrenergic receptor (Lohse *et al.*, 1990; Attramadal *et al.*, 1992). The requirement of receptor phosphorylation for arrestin recruitment was then demonstrated using the visual arrestin/rhodopsin model (Gurevich and Benovic, 1993), although some receptors can bind arrestin in the absence of phosphorylation. A series of mutation studies identified Arg^{175} as a key phosphate sensor that guides arrestin binding to phosphorylated GPCRs (Gurevich and Benovic, 1995; 1997) ultimately resulting in the structural rearrangement and activation of arrestin (Gurevich and Gurevich, 2006). Despite similarities in phosphate sensing between arrestin isoforms, the arrestins display obvious differences in their selectivity (Gurevich *et al.*, 1993; 1995; Kovoor *et al.*, 1999; Celver *et al.*, 2002; Sutton *et al.*, 2005). The relative flexibility of arrestin (Hirsch *et al.*, 1999; McDowell *et al.*, 1999; Han *et al.*, 2001; Vishnivetskiy *et al.*, 1999; 2000; 2002) allows for accommodation of hundreds of GPCRs as well as distinct downstream modulation of receptor desensitization and receptor signaling.

The non-visual arrestins (arrestin-2 and -3) also function as scaffolds for a variety of kinase signaling cascades, including non-receptor tyrosine kinases and components of the mitogen-activated protein kinase (MAPK) cascades (McDonald *et al.*, 2000; Maudsley *et al.*, 2000; Luttrell *et al.*, 2001; Miller and Lefkowitz, 2001). By serving as adaptors linking these cascades to GPCRs, arrestins serve as signal transducers for GPCR-mediated signaling pathways

distinct from those initiated by heterotrimeric G proteins. The same receptor-arrestin complex may be capable of initiating different signaling events, depending on the arrestin conformation induced. The unique receptor/arrestin confirmation is based in part on the specific pattern of receptor phosphorylation induced by GRK and other kinases. 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 by GRK5 and GRK6 is required for arrestin-dependent extracellular-signal-regulated kinase (ERK1/2) activation (J Kim *et al.*, 2005; Ren *et al.*, 2005). The particular mechanism by which a GPCR activates a kinase cascade affects the substrates of that cascade. The process by which different ligands are capable of promoting unique receptor confirmations that lead to distinct signaling events is known as ligand-directed signaling, functional selectivity, or biased agonism and will be discussed with respect to the opioid receptors throughout this thesis (Berg *et al.*, 1998; Urban *et al.*, 2007; Kenakin, 2007; Drake *et al.*, 2008; Galandrin *et al.*, 2008; Kenakin *et al.*, 2012).

Mechanisms of Opioid Receptor Desensitization and Arrestin-Mediated Signaling

δ-Opioid Receptor

GRK phosphorylation and subsequent arrestin recruitment has been described for each of the opioid receptors. GRK2 specifically phosphorylates residues on DOR that are clustered in the C-tail (Thr^{358}, Thr^{361}, Ser^{363}; Guo *et al.*, 2000) and it has also been shown that mutation of Thr^{161}, which is homologous to MOR Thr^{180} (Figure 1.2), slows the normally rapid rate of GRK2-mediated receptor internalization (Lowe *et al.*, 2002). Additionally, DOR can be desensitized in the absence of GRK2, either by introduction of constitutively active arrestin mutants (Kovoor *et*

al., 1997) or by arrestin-3 which can internalize DOR independent of receptor phosphorylation (X Zhang *et al.*, 2005).

Others have investigated the role of arrestin in DOR-mediated tolerance and receptor recycling more specifically (Qiu *et al.*, 2007; X Zhang *et al.*, 2008; Audet *et al.*, 2012). Although phosphorylation of the C-tail is known to be critical for the DOR and arrestin interaction (Cen, Xiong, *et al.*, 2001; Cen, Q Yu, *et al.*, 2001), little work has been performed to explore the contribution of GRK to arrestin recruitment and subsequent signaling and trafficking of DOR. The remainder of this thesis will focus on MOR and KOR.

μ-Opioid Receptor

Mutation studies first identified the critical role of serine and threonine residues in the third intracellular loop and C-tail of MOR for G protein coupling and receptor trafficking (Capeyrou *et al.*, 1997). Many studies had observed that different MOR agonists varied in their ability to desensitize MOR (Blake *et al.*, 1997; Y Yu *et al.*, 1997). It was also shown that the ability of an agonist to induce MOR internalization varied where agonists such as etorphine induced rapid desensitization and internalization, whereas morphine failed to do so (Arden *et al.*, 1995; Keith *et al.*, 1996; Sternini *et al.*, 1996; Capeyrou *et al.*, 1997; Segredo *et al.*, 1997). The finding that chronic morphine led to increased expression of GRK2 and arrestin in the rat locus coeruleus (Terwilliger *et al.*, 1994) led to the hypothesis that differences in GRK recruitment might explain the ability of certain MOR agonists to induce internalization more effectively than others.

Using *Xenopus* oocytes expressing MOR, GRK3, and arrestin-3, it was shown that high efficacy agonists, including [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), fentanyl, and sufentanil, could induce rapid GRK3-dependent desensitization (Kovoor *et al.*, 1998). However,

when co-expressed with GRK3 and arrestin-3, morphine was unable to desensitize the receptor. Additionally, when GRK5 was expressed instead of GRK3, DAMGO and fentanyl retained the ability to rapidly desensitize the receptor, but morphine was still unable to do so. This suggested that the ability of different agonists to induce MOR desensitization and internalization was dependent on recruitment of GRK to the receptor and the relative levels of GRK expression.

Multiple MOR residues have been identified as GRK phosphorylation sites and different agonists are differentially capable of promoting phosphorylation of specific key residues. Thr^{354}, Ser^{355}, Ser^{356}, and Thr^{357} in the C-tail were identified as key GRK phosphorylation sites responsible for receptor desensitization following DAMGO treatment and dominant-negative GRK2 blocked this phosphorylation-mediated desensitization (Figure 1.2; H Wang, 2000). Thr^{394} and Ser^{363} in the C-tail were also identified as critical residues mediating GRK-dependent, DAMGO-mediated MOR desensitization (Pak *et al.*, 1997; 1999; Burd *et al.*, 1998; Wolf *et al.*, 1999; Deng *et al.*, 2000). Additionally, mutations in the third intracellular loop (Thr^{180}) blocked GRK3-dependent, DAMGO-mediated desensitization (Celver *et al.*, 2001)**,** but did not alter the ability of a dominant-positive arrestin to desensitize the receptor. This suggested that Thr^{180} is a critical GRK3 phosphorylation site required for arrestin recruitment, but does not alter the ability of arrestin to bind the receptor. Further work demonstrated that in AtT20 cells, Thr^{180} was specifically responsible for uncoupling MOR in response to DAMGO, but this residue did not alter DAMGO-mediated internalization (Celver *et al.*, 2004). Together this work suggests that the C-tail is more tightly linked to GRK-dependent MOR internalization, whereas Thr^{180} specifically regulates GRK-dependent receptor uncoupling. These studies also highlight the complexity of GRK regulation of MOR. Recent work addresses GRK specificty at MOR. In HEK293, DAMGO promoted phosphorylation of Thr^{370} and Ser^{375} through a GRK2- and GRK3-

dependent mechanism, wheras morphine preferentially led to Ser^{375} phosphorylation through a GRK5-dependent mechanism (Figure 1.2; Petraschka *et al.*, 2007; Doll *et al.*, 2012). Similar results were found *in vivo,* where etonitazene and fentanyl produced a similar phosphorylation profile to DAMGO but morphine treament only promoted Ser^{375} phosphorylaton through a GRK5- and GRK3-dependent mechanism (Glück *et al.*, 2014). These different patterns of C-tail phosphorylation could explain differences in receptor internalization and ligand-directed initiation of arrestin-dependent MAPK cascade activation.

The generation of GRK3 (Peppel *et al.*, 1997)**,** GRK5 (Gainetdinov *et al.*, 1999), and GRK6 (Fong *et al.*, 2002) knockout mice allowed for a better understanding of the physiological consequenses of MOR phosphorylation by GRK. GRK3 is necessary for the development of acute and chronic fentanyl tolerance (Terman *et al.*, 2004; Melief *et al.*, 2010; Glück *et al.*, 2014) in both the spinally-mediated tail-flick analgesia assay (Irwin *et al.*, 1951; Le Bars *et al.*, 2001) and the centrally-mediated hotplate analgesia assay (Tsou and Jang, 1964; Yaksh *et al.*, 1976; Le Bars *et al.*, 2001; Tortorici *et al.*, 2001). However, GRK3 knockout has no effect on morphine induced tolerance or opiate withdrawal (Terman *et al.*, 2004; Melief *et al.*, 2010) and instead tolerance to morphine is c-Jun N-terminal kinase 2 (JNK2)-dependent (Melief *et al.*, 2010). It is also important to note that GRK3 knockout mice do not show changes in basal analgesia. In contrast, reduced basal analgesia is observed in GRK5 knockout mice but GRK5 does not appear to mediate the development of morphine tolerance at MOR (Glück *et al.*, 2014). GRK6 knockout has no effect on analgesia, tolerance, or reward, but presensitizes the hyperlocomotor response to morphine (Raehal *et al.*, 2009). These studies indicate that GRK3 mediates MOR analgesic tolerance induced by fentanyl-, but not morphine-like ligands, GRK5 mediates MOR agonist withdrawal and reward, and GRK6 regulates the locomotor response to MOR agonists.

It is clear that morphine-like agonists behave differently than DAMGO-like agonists in the context of MOR desensitization. Many alternative mechanisms of MOR desensitization have been described for morphine, including protein kinase C (PKC) and JNK. Using cultured neurons, it was shown that PKC could mediate morphine-induced MOR desensitization (Bailey, Oldfield, *et al.*, 2009). This finding was replicated in HEK293 cells (E Johnson, 2006) and *in vivo* with respect to the development of morphine tolerance (Hull *et al.*, 2010). It has also been demonstrated in HEK293 cells that morphine stimulated PKC can phosphorylate both the C-tail of MOR (Feng *et al.*, 2011) in addition to $G\alpha_{i2}$ (Chu *et al.*, 2010). Melief *et al.* (2010) identified JNK2 as a critical player in morphine-mediated MOR desensitization and found that PKC was necessary for activation of JNK, suggesting PKC and JNK work in series to result in MOR desensitization. This finding was replicated in DRG neurons (Mittal *et al.*, 2012) and will be discussed further in Chapter 2.

κ-Opioid Receptor

Treatment with KOR agonists, including U50,488, stimulate receptor phosphorylation both in slice preparations and in tolerant animals (Appleyard *et al.*, 1997). GRK was shown to be required for desensitization in *Xenopus* oocyte expression systems and internalization in HEK293 expression systems (Appleyard *et al*., 1999; McLaughlin, Xu, *et al.*, 2003). Further, Ser^{369} was identified as a key GRK phosphorylation site in the C-tail of KOR, mediating receptor desensitization and internalization, with no effect on desensitization seen with mutations in the third intracellular loop (Appleyard *et al.*, 1997; McLaughlin, Xu, *et al.*, 2003). Together these results established that GRK phosphorylation of Ser^{369} leads to rapid KOR desensitization following agonist treatment (Figure 1.2). Ser^{369}, however, is specific for rodent KOR (rKOR) and is not conserved in human KOR (hKOR). Instead, hKOR is phosphorylated by GRK at

Ser^{358} (J Li *et al.*, 2002; Schattauer *et al.*, 2012; Chapter 4.1, Figure 1.2). Agonist stimulation of hKOR also induces GRK3 phosphorylation of KOR and arrestin-dependent receptor desensitization and internalization (J Li *et al.*, 1999; 2002) similar to rKOR, but the efficacy and potency of agonists for inducing desensitization and internalization is not consistent between rKOR and hKOR (J Li *et al.*, 1999; Jordan *et al.*, 2000; F Zhang *et al.*, 2002). This differential phosphorylation site has implications for preclinical assessment of KOR compounds in GRK-mediated behaviors, since the results in rodents might not be directly translatable to human studies and this concept is further explored and discussed in Chapter 4.1.

As with MOR, the generation of GRK3 knockout animals (Peppel *et al.*, 1997) allowed for a clearer understanding of the physiological role of GRK3 on KOR signaling. GRK3 knockout mice show reduced analgesic tolerance to the KOR agonist U50,488, as measured by the tail-flick analgesia assay, and a reduction in Ser^{369} phosphorylation in the brain following prolonged agonist treatment (McLaughlin *et al.*, 2004). In addition to exogenous ligands, activation of KOR by the endogenous agonist dynorphin using partial sciatic nerve ligation (Wagner *et al.*, 1993; Z Wang *et al.*, 2001) also led to an increase in Ser^{369} phosphorylation in the mouse spinal cord, which is GRK3 dependent (Xu *et al.*, 2004).

Similar to other GPCRs, including MOR, KOR 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 that requires phosphoinositide 3-kinase (PI3-kinase) and PKCʒ (Fukuda *et al.*, 1996; Belcheva *et al.*, 1998; 2005; Bruchas *et al.*, 2006). ERK1/2 activation via KOR is biphasic and includes a later phase, which requires arrestin-3 (McLennan *et al.*, 2008; Schattauer *et al.*, 2012). KOR-dependent ERK1/2 activation has also been observed *in vivo* (Bruchas *et al.*, 2008; Potter *et al.*, 2011). KOR activation of ERK1/2 is known to

regulate embryonic stem cell differentiation (E Kim *et al.*, 2006), KOR inhibition of GABA (C Li *et al.*, 2012), astrocyte proliferation (McLennan *et al.*, 2008; C Li *et al.*, 2012), and cAMP response element-binding protein (CREB) activation (Bruchas *et al.*, 2008). However, 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 studied. The functional difference in ERK1/2 signaling via activation by these two mechanisms is unclear. Stimulation of KOR has been shown to result in activation of p38 MAPK through a mechanism requiring GRK3 phosphorylation of KOR at Ser^{369} followed by arrestin-3 recruitment (Bruchas *et al.*, 2006). Unlike in studies focusing on analgesic tolerance, the GRK3-dependent p38 signaling pathway is not required for the analgesic effects of KOR agonists. Instead, p38 activation mediates several therapeutically undesirable KOR induced changes, described below and in Chapter 4.

KOR induced p38 activation is required for the increase in glial fibrillary acidic protein immunoreactivity and astrocyte proliferation in the dorsal horn of the spinal cord following partial sciatic nerve ligation, which both prevents the reestablishment of neuronal pathways and contributes to chronic pain (Xu *et al.*, 2007). Activation of p38 via KOR and GRK3 also results in a Src-dependent phosphorylation of Tyr^{12} on GIRK, resulting in GIRK desensitization, and reduced K^+ conductance through the channel (Clayton *et al.*, 2009). This phosphorylation event is observed following sciatic nerve ligation, and may result in increased cellular excitability leading to chronic pain.

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, Land, *et al.*, 2007). Although, lentiviral

reexpression of KOR or mutant KOR (Ser^{369}Ala; KSA) specifically in the dorsal raphe of KOR knockout mice is sufficient to recover thermal analgesia in the tail-flick assay, the mutant KSA does not recover KOR conditioned place aversion. These experiments confirm that phosphorylation of KOR at Ser^{369}, which results in GRK3-dependent p38 activation, is required for the aversive but not analgesic properties of KOR agonists (Land *et al.*, 2009). p38 has been shown to modulate the serotonin transporter (SERT) *in vitro* (C-B Zhu *et al.*, 2004; Samuvel *et al.*, 2005) and a similar KOR- and p38-dependent mechanism of SERT regulation has been characterized *in vivo* (Schindler *et al.*, 2012) providing a physiological mechanism of p38-dependent aversion resulting from decreased synaptic serotonin as a result of increased surface expression of SERT. Chapter 4 of this thesis aims to identify G biased analgesic compounds that target KOR with reduced dysphoric side effects.

c-Jun N-Terminal Kinase Signaling at μ- and κ-Opioid Receptors

JNK1, JNK2, and JNK3 were first identified as stress-activated protein kinases (also called SAPK-γ, SAPK-α, and SAPK-β) and the kinase responsible for phosphorylation and activation of the transcription factor c-Jun (Adler *et al.*, 1992; Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994). JNK is part of the MAPK family of serine/threonine protein kinases (Davis, 1994). JNK1, JNK2, and JNK3 are alternatively spliced, resulting in 10 splice variants: JNK1α1, JNK1α2, JNK1β1, JNK1β2, JNK2α1, JNK2α2, JNK2β1, JNK2β2, JNK3α1, and JNK3α2 (Gupta et al., 1996). JNK1α1, JNK1β1, JNK2α1, and JNK2β1 have a shorter C-terminus, resulting in a 46 kDa protein, JNK1α2, JNK1β2, JNK2α2, and JNK2β2 have a longer C-terminus giving rise to a 55 kDa protein, and JNK3α1 and JNK3α2 are slightly larger, resulting in a 48 and 57 kDa protein,

respectively. The α and β splice variants contain different sequences within their protein kinase domains. JNK is activated by dual phosphorylation of a Thr^{183} and Tyr^{185} in the conserved Thr-Pro-Tyr (TPY) motif in the kinase domain by MKK7 and MKK4, respectively (Gupta *et al.*, 1996; Lawler *et al.*, 1998). Numerous upstream modulators of MKK7 and MKK4 allow JNK to respond to a diversity of stimuli. Additionally, JNK1 and JNK2 are widely distributed throughout the body, while JNK3 expression is limited to the brain and testis (Carboni *et al.*, 1998; Kumagae *et al.*, 1999). JNK is canonically involved in regulating apoptosis (Xia *et al.*, 1995; Park *et al.*, 1997; Tournier *et al.*, 2000), cell motility, and immune responses (Davis, 1999) and is known to be activated by ultraviolet irradiation (Pulverer *et al.*, 1991; Dérijard *et al.*, 1994), heatshock (Adler *et al.*, 1995), interleukins (Ng *et al.*, 2001; Kendrick *et al.*, 2004), and growth factors (Assefa *et al.*, 1999).

Although these canonical functions of JNK have been well characterized, less is known about JNK regulation of GPCRs. The KOR antagonist norBNI was shown to have a long duration of action (up to 21 days) *in vivo* (Horan *et al.*, 1992; Butelman *et al.*, 1993) although norBNI does not covalently bind to KOR (Smith *et al.*, 1990). It was later demonstrated that norBNI results in a KOR-dependent activation of JNK and inhibition of JNK blocks its long duration of action (Bruchas, T Yang, *et al.*, 2007; Melief *et al.*, 2010). Additionally, the duration of action of twelve KOR antagonists positively correlates with their ability to activate JNK (Melief *et al.*, 2011). At MOR, tolerance to morphine, as measured by warm-water tail-withdrawal, was blocked by JNK inhibition and in $JNK2^{-/-}$ mice, suggesting a similar mechanism of JNK-mediated MOR inactivation (Melief *et al.*, 2010). These results were also replicated in DRG neurons (Mittal *et al.*, 2012). Although the canonical GRK3/β-arrestin pathway is necessary for the development of acute analgesic tolerance to fentanyl and U50,488, both MOR

and KOR can be inactivated by a JNK2- and JNK1-dependent mechanism, respectively (Bruchas, T Yang, *et al.*, 2007; Melief *et al.*, 2010; 2011). How JNK activation causes MOR and KOR inactivation is not yet understood and this thesis aims to address that question. Furthermore, this JNK-dependent mechanism of opioid receptor inactivation may be a more general mechanism of GPCR regulation.

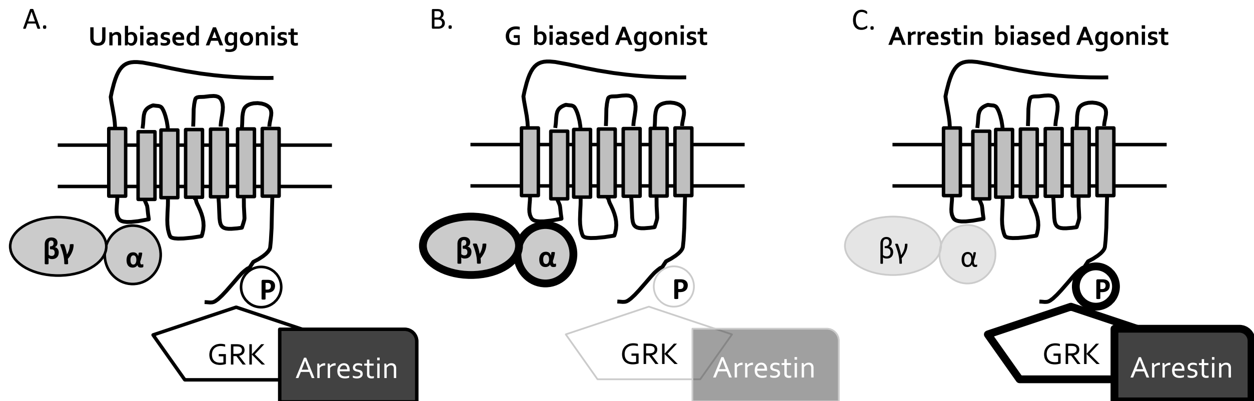

Figure 1.1 - Schematic of Ligand-Directed Signaling.

Ligands at GPCRs can be *A,* unbiased, activating G protein and arrestin-mediated cascades with equal efficacy, *B,* G biased, activating G protein-mediated signaling cascades preferentially, or *C,* arrestin-biased, activating arrestin-mediated cascades and/or receptor internalization preferentially.

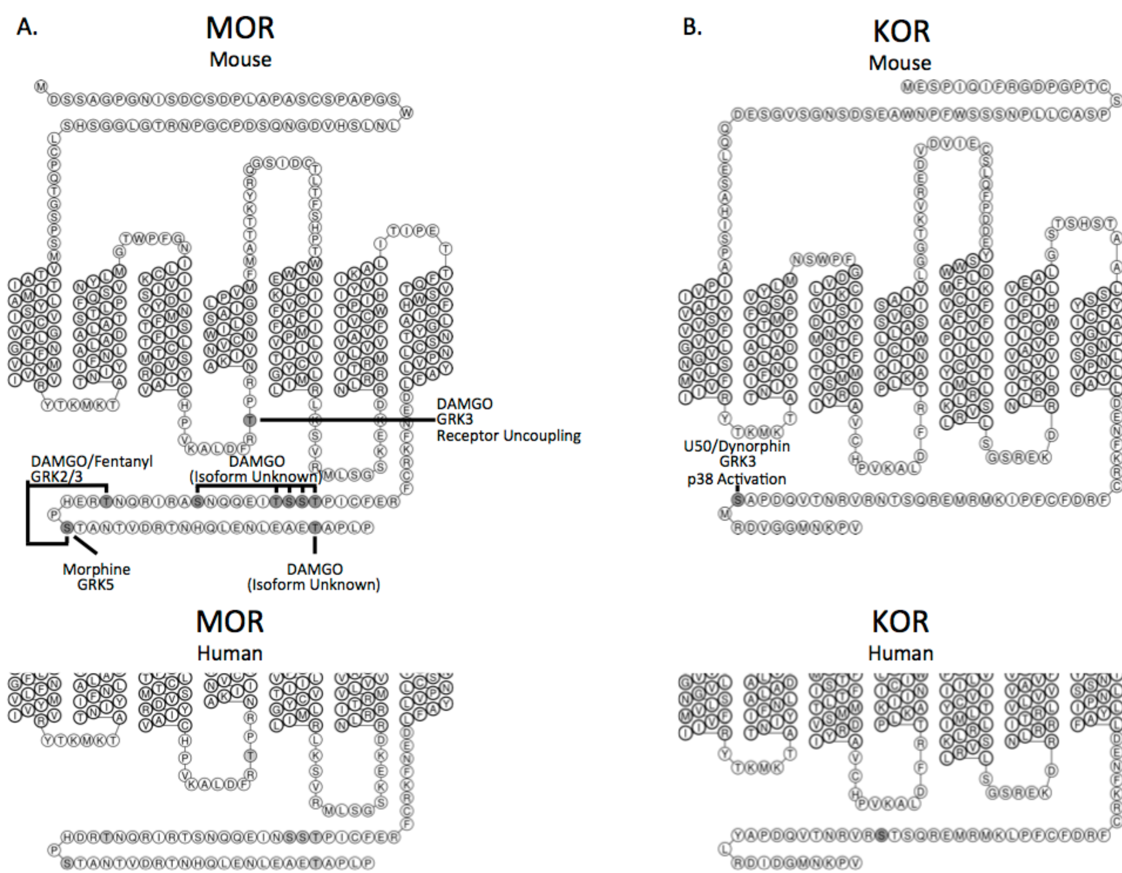

Figure 1.2 - Key GRK phosphorylation residues on MOR and KOR.

A, GRK phosphorylates a large number of residues on the C-terminal tail of MOR and Thr^{180} in the second intracellular loop. GRK specificity for numerous DAMGO induced GRK-phosphorylated residues is unknown. Thr^{370} and Thr^{370} and Ser^{375}, however, are specifically phosphorylated by GRK2/3 following DAMGO and fentanyl, whereas morphine induces GRK5-dependent phosphorylation of Ser^{375} only. Thr^{180} is a GRK3 target and phosphorylation of this residue specifically leads to receptor uncoupling. All GRK phosphorylation sites on rodent MOR are conserved in human MOR except Thr^{357}, suggesting similar GRK-mediated signaling between the two species. *B*, GRK phosphorylation of KOR is less complex, with a single GRK3 phosphorylation site that mediates p38 activation. However, this site is not conserved in human KOR, leading to potential differences in drug effects between the two species.

Chapter 2. μ-OPIOID RECEPTOR STIMULATION ACTIVATES C-JUN N-TERMINAL KINASE 2 BY DISTINCT ARRESTIN-DEPENDENT AND INDEPENDENT MECHANISMS

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2.1 INTRODUCTION

The concept of ligand-directed signaling, also known as biased agonism or functional selectivity, has become increasingly important for understanding the different signaling events that occur following GPCR ligand binding (Kenakin, 2007). While the canonical mechanism of GPCR desensitization is GRK/arrestin mediated (Lohse *et al.*, 1990; Krupnick and Benovic, 1998; Kovoor *et al.*, 1998; J Zhang *et al.*, 1998; Luttrell and Gesty-Palmer, 2010; Shenoy and Lefkowitz, 2011), alternative mechanisms of receptor desensitization have been documented. For instance, the development of spinally-mediated acute analgesic tolerance following treatment with the MOR agonist fentanyl requires the GRK3/arrestin pathway, whereas tolerance following administration of the MOR agonist morphine requires a distinctly different mechanism involving JNK2 (Melief *et al.*, 2010). This JNK-dependent mechanism of receptor inactivation has also been observed at KOR, and the durations of action of a broad range of KOR antagonists positively correlates with the ability of the antagonist to activate JNK in mouse spinal cord (Bruchas, Yang, *et al.*, 2007; Melief *et al.*, 2011). While JNK has been shown to be directly involved in morphine-induced MOR desensitization in the dorsal root ganglion (Mittal *et al.*, 2012), others have reported that JNK is not required for morphine-induced MOR desensitization

in the locus coeruleus (Levitt and Williams, 2012). Thus mechanisms of regulation of MOR signaling mechanisms may be neuronal pathway selective, and further characterization of the role of JNK in different pain circuits and brain regions is required.

An additional issue is that although morphine and fentanyl desensitize MOR by different mechanisms, both compounds activate JNK (Melief *et al.*, 2010). How these ligands activate JNK and why one inactivates MOR, but the other does not, is not known. JNK activation requires phosphorylation of a threonine and tyrosine residue in the activation loop and the canonical mitogen-activated protein kinase kinases (MAPKK) MKK4 and MKK7 have been shown to phosphorylate these residues directly (Gupta *et al.*, 1996; Lawler *et al.*, 1998). Additional kinases have been implicated in opioid receptor-mediated JNK activation upstream of the MAPKKs, including PKC and Src (Kam *et al.*, 2003; Kam *et al.*, 2004a; b; Melief *et al.*, 2010). In some instances, arrestin has been shown to act as a scaffold for JNK activation recruiting MKK4 and MKK7, in addition to ASK1, in close proximity with JNK (McDonald *et al.*, 2000; Zhan *et al.*, 2013; Kook *et al.*, 2013). It has also been demonstrated that arrestin can regulate the cellular localization of ERK and other signaling molecules (Tohgo *et al.*, 2003; Kobayashi *et al.*, 2005; Hanson *et al.*, 2007). Based on our *in vivo* tolerance studies, we hypothesized that JNK activation by morphine and fentanyl required distinct upstream mechanisms, which can help explain the differential behavioral effect. A better understanding of the ligand-directed mechanisms that contribute to MOR-mediated JNK activation and receptor desensitization will be necessary for the development of improved therapeutics that avoid tolerance or minimize arrestin-dependent responses. Additionally, this JNK-mediated mechanism of receptor regulation may be more generally involved in other GPCR systems.

In this study, we examined the role of JNK in centrally-mediated pain circuits and dissected the arrestin-dependent and arrestin-independent mechanisms of JNK activation by MOR in mouse spinal cord and MOR-GFP expressing HEK293 cells. Our study also suggests a role for additional kinases in JNK activation, including PKC and Src, which have been implicated in MOR desensitization following morphine (E Johnson, 2006; Hull *et al.*, 2010) and in [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO)-mediated JNK activation (Kam *et al.*, 2004b), respectively. These results further elucidate mechanisms of arrestin-dependent and arrestin-independent JNK activation in an effort to better understand ligand-directed mechanisms of MOR desensitization.

2.2 MATERIALS AND METHODS

Reagents. Morphine sulfate was provided by the NIDA Drug Supply (Bethesda, MD). Fentanyl citrate was purchased from Sigma (St Louis, MO). PP2 and Gö6976 were purchased from Calbiochem (Billerica, MA) and dissolved in DMSO. All compounds were diluted in 0.9% NaCl for animal studies and H_20 for cell based assays.

Animals. Male wild type (WT) mice or $GRK3^{-/-}$, $JNK1^{-/-}$, and $JNK2^{-/-}$ mice (20-30g) on a C57Bl/6 background were generated and genotyped as previously described (Melief *et al.*, 2010). $JNK3^{-/-}$ mice were generated by Dr. RA Flavell (Yale University; D Yang *et al.*, 1997) and provided by Dr. Zhengui Xia (UW Toxicology/Environmental Health). Mice 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 of the National Institutes of Health on the care and use of animals.

Analgesia. Nociceptive responses were measured using a 55°C hotplate (ICTC Life Sciences, model 39/336T) as previously described (Terman *et al.*, 2004). To make animals tolerant, mice were injected with morphine (20mg/kg, s.c.) or saline once per day in the morning for 3 days (Fig. 2.1). Baseline response latencies were assessed 4 hr post injection on Day 1 and Day 3, then again 30 min following a second dose of morphine (10mg/kg, s.c.) on that day. Mice were removed from the hot plate after a nociceptive response (hindpaw withdrawal or shake) or before 30 sec to avoid tissue damage. The investigator was blinded to pretreatment and genotype.

Spinal Cord Dissection and Immunoblot. Mice were injected with saline, morphine (10mg/kg s.c.), or fentanyl (0.3mg/kg s.c.) at a volume of 10 mL/kg. Mice were decapitated 30-60 min post injection, and the lumbar region of the spinal cords were removed and homogenized in lysis buffer (50mM Tris-HCl, 300mM NaCl, 1mM EDTA, 1mM Na3VO4, 1 mM NaF, 10% Glycerol, 1% Triton-X 100) with phosphatase inhibitor cocktail (1:100, Calbiochem, Billerica, MA) and protease inhibitor cocktail (1:100, Calbiochem, Billerica, MA). Lysates were spun at 14,000xg for 30 min at 4°C and protein concentration of the supernatant was determined by BCA assay (Pierce). Lysates (15-20 µg of protein) were heated at 100°C for 5 min and resolved on a 10% Bis-Tris polyacrylamide gel (Invitrogen Life Technologies, Grand City, NY) at 120V for 2 hr. Blots were transferred to nitrocellulose membranes (Whatman, Middlesex, UK) for 1.5–2 hr at 30V. Membranes were blocked with 5% BSA-TBST for 1 hr at 23°C and blotted for total-JNK (Cell Signaling Technology, Danvers, MA; 1:1000) or for phospho-JNK (Cell Signaling, Danvers, MA; 1:1000) and actin (Abcam, Cambridge, MA; 1:5000) immunoreactivities (-IR) in 5% BSA-TBST overnight at 4°C. Membranes were washed 3x5 min in TBST and incubated in IRdye secondary (Li-Cor Biosciences, Lincoln, NE; 1:10,000) in 1:1 5% milk-TBST and Odyssey buffer (Li-Cor Biosciences, Lincoln, NE) for 1 hr at 23°C. Membranes were washed for

3x5 min in TBST and scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences; Lincoln, NE). Relative fluorescent band intensity was measured using the Odyssey software and expressed as a ratio of total- or phospho-JNK-IR to actin-IR band intensity and then expressed as change over vehicle treatment. Both the 46 and 54kDa band were included for total- and phospho-JNK analysis. All westerns were run and analyzed blinded to treatment and genotype.

siRNA transfection. HEK293 cells stably expressing MOR-GFP were transiently transfected with siRNA against arrestin-2, arrestin-3, or a scrambled siRNA control (Dharmacon Research, Pittsburgh, PA, 50nM final concentration; 48 hr), by using Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY) according to manufacturer's recommendations. To assess expression knockdown, cells were homogenized in lysis buffer (50mM Tris-HCl, 300mM NaCl, 1mM EDTA, 1mM Na3VO4, 1mM NaF, 10% Glycerol, 1% Triton-X 100) with phosphatase inhibitor cocktail (1:100, Calbiochem, Billerica, MA) and protease inhibitor cocktail (1:100, Calbiochem, Billerica, MA). Lysates were spun at 14,000xg for 30 min at 4°C and protein concentration of the supernatant was determined by BCA assay (Pierce, Rockford, IL). Lysates (10μg of protein) were heated at 100°C for 5 min and resolved on a 10% Bis-Tris polyacrylamide gel as described above, then blotted for arrestin-2-IR or arrestin-3-IR (antibody provided by Dr. Jeffrey Benovic, Thomas Jefferson University, Philadelphia, PA) (1:3000) and actin (Abcam, Cambridge, MA; 1:5000) in 5% BSA-TBST overnight at 4°C. Relative fluorescent band intensity was measured using the Odyssey software and expressed as arrestin-2-IR or arrestin-3-IR intensity over actin-IR intensity and then expressed as change over scrambled siRNA treatment.

HEK293 Cell Treatments and Protein Extraction. Cells were incubated in serum-free media overnight and subsequently treated with opioid ligands and inhibitors as indicated.

Fentanyl and morphine were tested at 10µM for 30 min. To inhibit Src family kinases, MOR-GFP HEK293 cells were treated with 5µM PP2 (Hanke *et al.*, 1996) 30 min before opioid administration. To inhibit Ca^{2+} sensitive PKC, MOR-GFP HEK293 cells were treated with 5µM Gö6976 (Martiny-Baron *et al.*, 1993) 30 min before opioid; an equal volume of DMSO was employed as vehicle for PP2 and Gö6976.

HEK293 Immunoblots. To determine JNK phosphorylation levels in MOR-GFP expressing HEK293 cells, 40 µg protein of each lysate was loaded onto 10% Bis-Tris polyacrylamide gel (Life Technologies, Grand Island, NY) and western blot was performed as described above and blotted for phospho-JNK-IR (Cell Signaling Technology, Danvers, MA; 1:1000) and actin-IR (Abcam, Cambridge, MA; 1:5000). To determine Src phosphorylation levels in MOR-GFP expressing HEK293 cells, 30 µg of each lysate was resolved then blotted for phospho-Tyr416-Src-IR (Cell Signaling Technology, Danvers, MA; 1:1000) and actin-IR (Abcam, Cambridge, MA; 1:5000).

Analysis. Analysis of Variance, Student's t-tests, or one-sample t-tests were used to assess statistical significance of the data ($\alpha=0.05$). Outliers were distinguished by the Grubb's Test ($\alpha=0.05$). In the figures shown, statistical significance is indicated by: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$)

2.3 Results

JNK2$^{-/-}$ Mice Do Not Develop Centrally-Regulated Analgesic Tolerance to Morphine. To assess whether JNK2 mechanisms contribute to acute analgesic tolerance to MOR agonists in the nociceptive circuits controlling hotplate responses as they do in the tail flick analgesia assay (Melief *et al.*, 2010), wild-type (WT) and JNK2$^{-/-}$ (knockout) mice were treated with one

20mg/kg morphine injection every morning for three days or saline as a control (Fig. 2.1A). Tail flick analgesia is spinally-mediated (Irwin *et al.*, 1951; Le Bars *et al.*, 2001), whereas the opioid-sensitive nociception controlling the hotplate response is centrally-regulated in part by the periaqueductal gray and rostral ventromedial medulla (Tsou and Jang, 1964; Yaksh *et al.*, 1976; Le Bars *et al.*, 2001; Tortorici *et al.*, 2001). On Day 1 and Day 3, nociceptive responses were assessed on the hotplate before and 30 min after an injection of 10mg/kg morphine, which was given 4 hr after a pretreatment of 20mg/kg morphine or saline (Fig. 2.1A). Animals did not show acute analgesic tolerance to morphine on Day 1 (data not shown) whereas on Day 3, WT animals pretreated with morphine (Fig. 2.1A) showed a significantly shorter latency to withdraw from the hotplate after a test dose of morphine (10mg/kg) compared to mice pretreated with saline, indicative of analgesic tolerance (Fig. 2.1B; $p<0.01$). This robust analgesic tolerance to morphine was not evident in JNK2$^{-/-}$ mice, which had equivalent latencies to withdraw regardless of pretreatment (Fig. 2.1B; $p>0.05$). Both genotypes showed equivalent baseline responses prior to the test dose of morphine (WT Sal=5.47 (±0.595) sec, WT Morphine=5.55 (±0.489) sec, JNK2$^{-/-}$ Sal=6.61 (±0.770) sec, JNK2$^{-/-}$ Morphine=7.27 (±0.812) sec). These results mirror previous results using the warm-water tail-withdrawal assay (Melief *et al.*, 2010), suggesting that similar JNK2 mechanisms regulate morphine tolerance in both spinal and central pain circuits. However, multiple morphine injections were required over the course of three days to observe the development of tolerance (Fig. 2.1) on the hotplate, suggesting that the mechanisms regulating centrally-regulated analgesia differ temporally from those regulating spinal analgesia.

JNK2 is Specifically Activated by Morphine and Fentanyl In Vivo. Both morphine and fentanyl increase phospho-JNK-immunoreactivity (phospho-JNK-IR), but produce acute analgesic tolerance by different mechanisms (Melief *et al.*, 2010). To understand the basis for

this difference, we assessed the specific isoforms of JNK activated by morphine and fentanyl. $JNK1^{-/-}$, $JNK2^{-/-}$, and $JNK3^{-/-}$ male C57Bl/6 mice were treated with saline, morphine (10mg/kg s.c.), or fentanyl (0.3mg/kg s.c.) for 30 min followed by decapitation and dissection of spinal cords. This time point corresponds to the peak of the opioid analgesic response (Melief *et al.*, 2010). Although spinal cord contains a diversity of cell types that could respond to treatment either directly or indirectly, DAMGO stimulated [^{35}S]GTPγS binding to spinal cord membranes was reduced by morphine pretreatment in wild type, but not mice pretreated with JNK inhibitor (Melief *et al.*, 2010), suggesting that morphine activation of JNK does have direct effects on MOR signaling in spinal cord.

$JNK1^{-/-}$, and $JNK3^{-/-}$ animals showed robust phospho-JNK-IR following morphine treatment (Fig. 2.2A,B; $p<0.05$ $JNK1^{-/-}$, $p<0.01$ $JNK3^{-/-}$). In contrast phospho-JNK-IR was not increased in $JNK2^{-/-}$ mice, indicating that the JNK2 isoform was specifically activated by morphine (Fig. 2.2A,B; $p>0.05$ $JNK2^{-/-}$). Similarly, both $JNK1^{-/-}$ and $JNK3^{-/-}$ mice showed significant increases in phospho-JNK-IR in response to fentanyl, but this was absent in $JNK2^{-/-}$ mice (Fig. 2.2A,B; $p<0.01$ $JNK1^{-/-}$, $p>0.05$ $JNK2^{-/-}$, $p<0.01$ $JNK3^{-/-}$). Together these data indicate that both morphine and fentanyl specifically activate spinal JNK2, but not JNK1 or JNK3.

Because phospho-JNK-IR was not increased in $JNK2^{-/-}$ mice, we confirmed that these mice did not show a change in total-JNK-IR following morphine or fentanyl (Fig. 2.2C,D; $p>0.05$), indicating that the lack of JNK activation in these mice is not due to alterations in total protein levels.

GRK3 is Required for Fentanyl, but not Morphine, Activation of JNK in vivo. Prior studies have demonstrated that JNK can be activated by an arrestin scaffold (Zhan *et al.*, 2013; Kook *et al.*, 2013). To assess the role of arrestin in opioid-induced JNK activation, we measured

the effect of GRK3 gene deletion (GRK3$^{-/-}$) on phospho-JNK-IR *in vivo*. GRK3$^{+/+}$ (wildtype littermates) and GRK3$^{-/-}$ male C57Bl/6 mice were treated with saline, morphine (10mg/kg s.c.), or fentanyl (0.3mg/kg s.c.), then spinal cords were harvested 60 min later. GRK3$^{+/+}$ animals showed significant increases in phospho-JNK-IR following either morphine ($p<0.01$) or fentanyl ($p<0.01$; Fig. 2.3A). GRK3$^{-/-}$ animals treated with morphine also showed an increase in phospho-JNK-IR over baseline ($p<0.0001$), whereas fentanyl did not change phospho-JNK-IR in GRK3$^{-/-}$ mice ($p>0.05$; Fig. 2.3A,B). These results indicate that fentanyl and morphine activate JNK2 through distinct signaling cascades *in vivo*.

Additionally, GRK3$^{-/-}$ mice did not show a change in total-JNK-IR following morphine or fentanyl (Fig. 3C,D; $p>0.05$), indicating that the effects on phospho-JNK were not caused by alterations in total protein levels.

Arrestin-2 is Required for Fentanyl, but not Morphine, Activation of JNK. While the requirement of GRK3 for fentanyl activation of JNK *in vivo* implied an arrestin-dependent mechanism of JNK activation, to directly establish the role of arrestin in fentanyl activation of JNK, MOR-GFP expressing HEK293 cells were transfected with 50nM siRNA against either arrestin-2, arrestin-3, or a scrambled siRNA control for 48 hr. Cells were then lysed and analyzed for arrestin-2 and arrestin-3 expression using western blot. Arrestin-2 siRNA resulted in a 49 ± 5.6% reduction ($p<0.01$) in arrestin-2-IR without significantly affecting arrestin-3-IR compared to scrambled control ($p>0.05$; Fig. 2.4A). Similarly, arrestin-3 siRNA reduced arrestin-3-IR by 54 ± 7.8% ($p<0.01$) without altering arrestin-2-IR compared to scrambled control ($p>0.05$; Fig. 2.4B).

To assess the role of arrestin in JNK activation, MOR-expressing HEK293 cells were treated with vehicle, 10μM morphine, or 10μM fentanyl for 30 min, then lysed and processed for

phospho-JNK-IR. Morphine treatment significantly increased phospho-JNK-IR, and the increase was not affected by either arrestin-2 or arrestin-3 siRNA (Fig. 2.4C,D). Fentanyl treatment also increased phospho-JNK-IR in scrambled siRNA and arrestin-3 siRNA treated cells (Fig. 2.4C,D). In contrast, fentanyl failed to increase phospho-JNK-IR in arrestin-2 siRNA treated cells (Fig. 2.4C,D; $p>0.05$). These results suggest that fentanyl activation of JNK required a GRK3/arrestin-2 dependent mechanism whereas morphine activation of JNK was arrestin-independent. Similar to the *in vivo* experiments previously described, morphine and fentanyl did not induce changes in total-JNK-IR in HEK293 cells (Fig 2.4E).

Src is Required for Fentanyl and Morphine Activation of JNK. In addition to an arrestin-dependent mechanism, Src kinase has also been implicated in JNK activation by opioid receptors (Kam *et al.*, 2003; Kam, *et al.*, 2004a; b). To investigate the role of Src family kinases, MOR-GFP expressing HEK293 cells were treated with vehicle, 10μM morphine, or 10μM fentanyl for 30 min. Both opioids significantly increased phospho-Src-IR (Fig. 2.5A,D). To determine the role of Src in JNK activation, MOR-GFP expressing HEK293 cells were pretreated for 30 min with 5μM PP2 (Salazar and Rozengurt, 1999; Rey *et al.*, 2006; L Zhang *et al.*, 2009), which inhibits Src family kinases (Hanke *et al.*, 1996), or vehicle prior to treatment with vehicle, 10μM morphine, or 10μM fentanyl for 30 min. Both morphine and fentanyl treatment significantly increased phospho-JNK-IR, and these effects were blocked by PP2 (Fig. 2.5B-D). These results suggest that Src mediates JNK phosphorylation either directly when activated by morphine or indirectly when activated by fentanyl.

PKC is Required for Morphine, but not Fentanyl, Activation of JNK. Previous work has indicated that PKC is involved in morphine-mediated activation of JNK (Melief *et al.*, 2010) and morphine-mediated receptor desensitization (Hull *et al.*, 2010). Therefore, MOR-GFP expressing

HEK293 cells were treated with 5μM Gö6976, which inhibits PKC (Martiny-Baron *et al.*, 1993), or DMSO prior to treatment with vehicle, 10μM morphine, or 10μM fentanyl for 30 min. Both morphine and fentanyl significantly increased phospho-JNK-IR (Fig. 2.6A-C). Pretreatment with Gö6976 also increased phospho-JNK-IR. Morphine did not further increase phospho-JNK-IR in the presence of Gö6976, whereas even in the presence of Gö6976, fentanyl treatment still elicited an increase in phospho-JNK-IR (Fig. 2.6B,C). These results suggest that PKC was required for morphine but not fentanyl induced JNK activation.

2.4 DISCUSSION

The principal findings of this study are that morphine and fentanyl activate JNK through distinct arrestin-independent and arrestin-dependent mechanisms, respectively, and this underlying ligand-directed signaling might explain the differential mechanisms of opioid tolerance observed *in vivo*. The concept of ligand-directed signaling has been applied to uncover multiple mechanisms of MOR desensitization, including GRK2 and GRK3 (Kovoor *et al.*, 1998; J Zhang *et al.*, 1998), PKC (E Johnson, 2006; Bailey, Oldfield, *et al.*, 2009; Bailey, Llorente, *et al.*, 2009), JNK (Melief *et al.*, 2010; Mittal *et al.*, 2012), and ERK (Dang *et al.*, 2009). While GRK/arrestin mediated desensitization has been extensively characterized in multiple GPCR systems (Krupnick and Benovic, 1998), less is known about JNK signaling and its role in receptor desensitization, as only a few studies have directly tested the role of JNK on MOR desensitization directly using [^{35}S]GTPγS and slice recordings in dorsal root ganglion neurons (Melief *et al.*, 2010; Mittal *et al.*, 2012).

Previous studies have determined a role for JNK2 in spinally-mediated morphine acute analgesic tolerance (Melief *et al.*, 2010) and in this study we show that this mechanism of JNK2-

mediated tolerance exists in additional centrally-regulated pain circuits (Tsou and Jang, 1964; Yaksh *et al.*, 1976; Le Bars *et al.*, 2001; Tortorici *et al.*, 2001). From this data, we conclude that JNK2 regulation of MOR is a more broadly applicable mechanism of morphine-induced MOR desensitization. How JNK2 activation causes MOR desensitization is not yet known; presumably a JNK-phosphorylated arrestin-like substrate binds and interferes with MOR signaling, but this hypothetical substrate has not yet been identified. Both morphine and fentanyl are able to activate JNK, however, and it was necessary to determine the signaling events that contribute to JNK activation in order to uncover a molecular basis for the differential JNK-dependent regulation of MOR following morphine and fentanyl stimulation (Fig. 2.7). Fentanyl desensitization of MOR requires GRK3/arrestin, thus we presume that arrestin binding to MOR prevents the association of the putative JNK-phosphorylated substrate, but this hypothesis requires substantiation.

JNK was first characterized for its role in the stress response (Hibi *et al.*, 1993; Dérijard *et al.*, 1994). Three genes encode JNK1, JNK2, and JNK3, and among these isoforms, ten splice variants have been identified (Gupta *et al.*, 1996; D Yang *et al.*, 1997; Davis, 2000). In this study we determined that both morphine and fentanyl activate JNK2 specifically *in vivo* using knockout mice. While both compounds were able to activate the same isoform, which is involved in the development of morphine tolerance specifically, fentanyl activation of JNK requires GRK3, whereas morphine activation of JNK does not. This suggested arrestin-dependent and arrestin-independent mechanisms of JNK activation by MOR (Fig. 2.7).

It has been shown that among the four arrestin subtypes, arrestin-3 can scaffold all three JNK isoforms along with the upstream kinases responsible for JNK phosphorylation and activation (McDonald *et al.*, 2000; Zhan *et al.*, 2013; 2014). Surprisingly, we found that fentanyl

activation of JNK required arrestin-2 and not arrestin-3, although it is possible that greater arrestin-3 knockdown might have suggested a requirement for arrestin-3 as well. Regardless, this suggests a previously unidentified mechanism of arrestin-2-mediated JNK2 activation. It is possible that arrestin-2 acts as a scaffold for JNK2, similar to arrestin-3, and based on studies with arrestin-3, it is likely that arrestin-2 is capable of scaffolding all three JNK isoforms. Further work will be required to confirm this proposed scaffolding activity of arrestin-2. Together, our data suggests that fentanyl activation of JNK2 requires GRK3/arrestin-2, whereas morphine activation of JNK2 is arrestin-independent.

As opiate tolerance is a multidimensional and complex process, we sought to uncover additional signaling molecules involved in JNK activation. Both morphine and fentanyl activate Src, and this Src activation was required for activation of JNK by both compounds at the time point tested. It has been previously shown that Src is responsible for MOR-mediated JNK activation following DAMGO through a PI3K-, Cdc42-, and Sos-dependent mechanism (Kam *et al.*, 2004b) and our study indicates that morphine-like compounds also require Src for JNK activation, although it is possible that upstream mechanisms of Src activation are distinct (Fig. 2.7). Other studies would suggest that this is likely the case. For example, it has been shown that morphine-mediated Src activation is arrestin-independent (L Zhang *et al.*, 2009), whereas DAMGO-mediated Src activation is likely arrestin-dependent whereby arrestin plays a critical role in maintaining Src at the cell membrane in an organized “lattice-like” distribution, which is critical for the ability of DAMGO to stimulate Src phosphorylation (Walwyn *et al.*, 2007). These studies in combination with our results form the basis for our model, where fentanyl-mediated Src activation is downstream of arrestin recruitment whereas morphine-mediated Src activation is arrestin-independent (Fig. 2.7). While Src was not differentially involved in morphine and

fentanyl-mediated JNK activation, our study uncovered a differential role for PKC. These results indicate that PKC is specifically involved in morphine, but not fentanyl, activation of JNK at the time point tested. This demonstrates that although PKC has been shown to be involved in tolerance to both compounds *in vivo* (E Johnson, 2006; Hull *et al.*, 2010), it is differentially required for JNK activation in our studies. This study helps tie these previously separate findings together and provides the basis for future studies to further elucidate more comprehensive signaling mechanisms involved in MOR desensitization.

In conclusion, the present study further supports the role of JNK in morphine-induced MOR desensitization, and we propose distinct mechanisms of JNK activation by morphine and fentanyl (Summarized in Fig. 2.7). The arrestin-dependent and arrestin-independent mechanisms of JNK activation further our understanding of JNK regulation and provide an explanation for the differential mechanisms of tolerance observed *in vivo*. How morphine-activated JNK2 causes MOR desensitization is not clear. Further work will be required to confirm that arrestin-2 scaffolds JNK2 specifically and continue to explore the mechanism by which PKC and Src activate JNK. It still remains to be determined how this differential activation of JNK leads to distinct regulation of MOR downstream of JNK activation, although it is possible that differential upstream signaling cascades sequester JNK in distinct cellular compartments. This study provides the foundation for future work aimed at answering these questions and suggests that the arrestin-dependent and arrestin-independent mechanisms of JNK activation might be broadly applicable to additional GPCR systems and JNK signaling cascades.

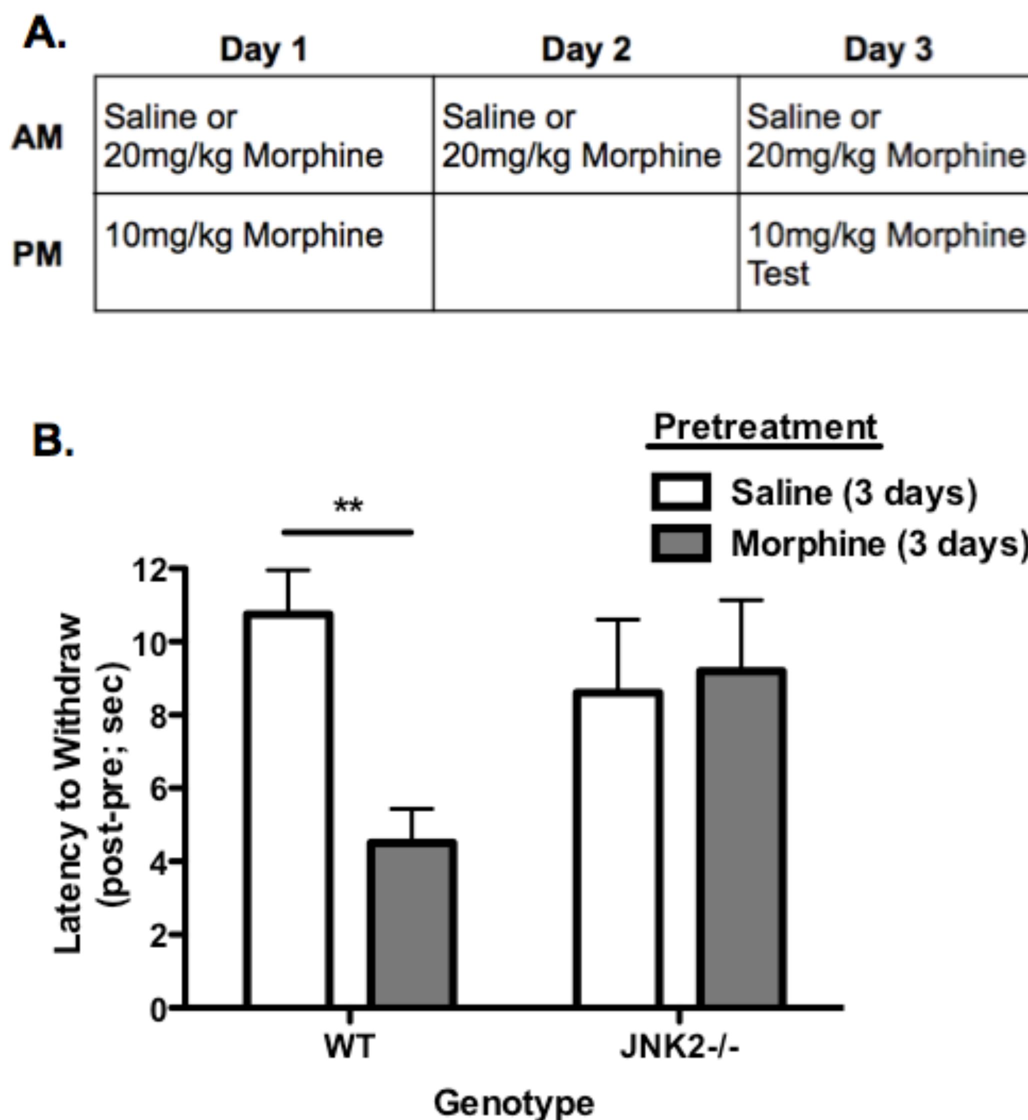

Figure 2.1 - JNK2 is Required for Hotplate Analgesic Tolerance to Morphine.

A, Animals were treated with 20mg/kg morphine or saline as a control for 3 days to induce tolerance. On Day 1 and Day 3 all mice were challenged with 10mg/kg morphine 4 hr after their morning dose and tested on a 55°C hot plate pre and post 10mg/kg injection to assess analgesia, which was measured as the latency for hind paw withdrawal/flicks. Animals did not show robust analgesic tolerance on Day 1 (data not shown). *B*, WT animals pretreated with saline show a robust analgesic response to morphine (10mg/kg) (as indicated by an increased latency to withdraw) on Day 3 whereas animals pretreated with morphine (20mg/kg) do not. $JNK2^{-/-}$ animals show robust analgesia, as indicated by an increased latency to withdraw, to morphine regardless of pretreatment with saline or morphine, demonstrating that JNK2 is required for morphine analgesic tolerance on the hotplate. Data presented were calculated as the response latency 30 min post drug administration minus baseline withdrawal latency resulting in the post-pre value presented. Baseline responses did not vary across groups (data summarized in text). n=10-18; Data analyzed by two-way ANOVA with Bonferroni posttest.

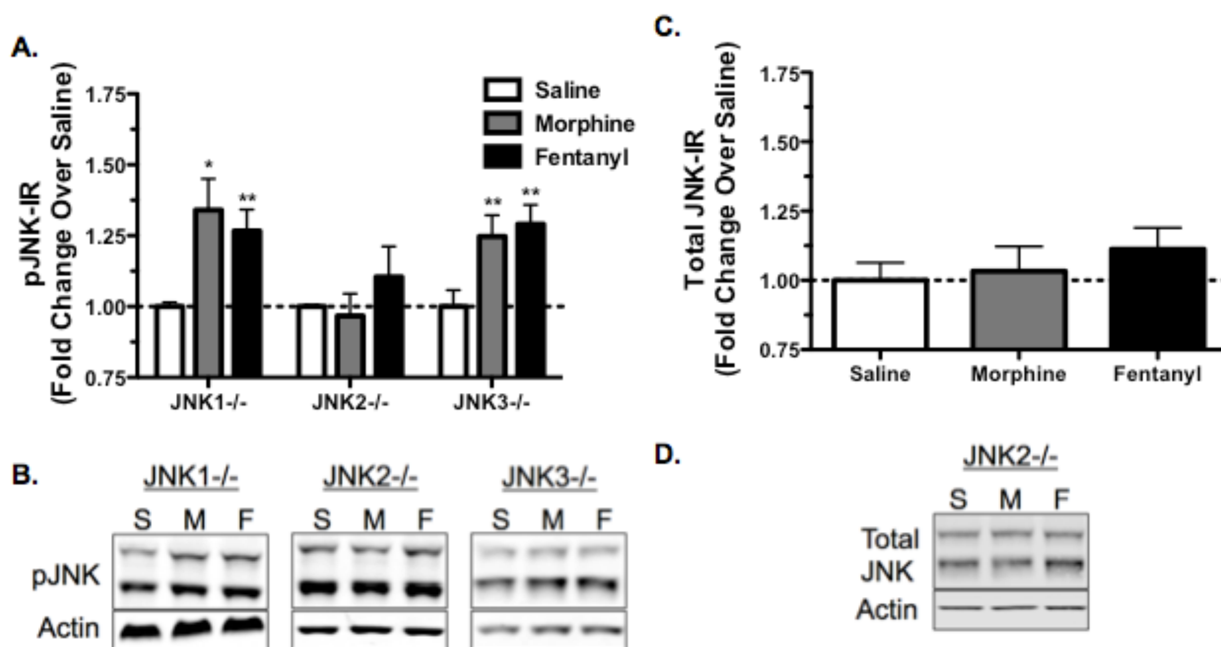

Figure 2.2 - Morphine and Fentanyl-Mediated Phospho-JNK Increase in the Spinal Cord is Selectively Abolished in JNK2$^{-/-}$ Mice, but not Significantly Affected by JNK1 or JNK3 Gene Knockout.

A, Animals were treated with saline, morphine (10mg/kg, s.c.), or fentanyl (0.3mg/kg, s.c.) and their spinal cords were harvested 30 min post-treatment for phospho-JNK-IR analysis. Both MOR agonists increase phospho-JNK-IR in JNK1$^{-/-}$ and JNK3$^{-/-}$ mice but failed to increase phospho-JNK-IR over baseline in JNK2$^{-/-}$ mice. *B*, Representative images are shown for each phospho-JNK data set. *C,* Total-JNK-IR was also not altered by morphine or fentanyl in JNK2$^{-/-}$ mice, indicating that the effects on phospho-JNK were not a result of alterations in total protein levels. *D,* Representative images are shown for the total-JNK data set. n=3-10; Data analyzed by one-sample t-test; statistical outliers were determined by the Grubb's Test with significance set to α=0.05.

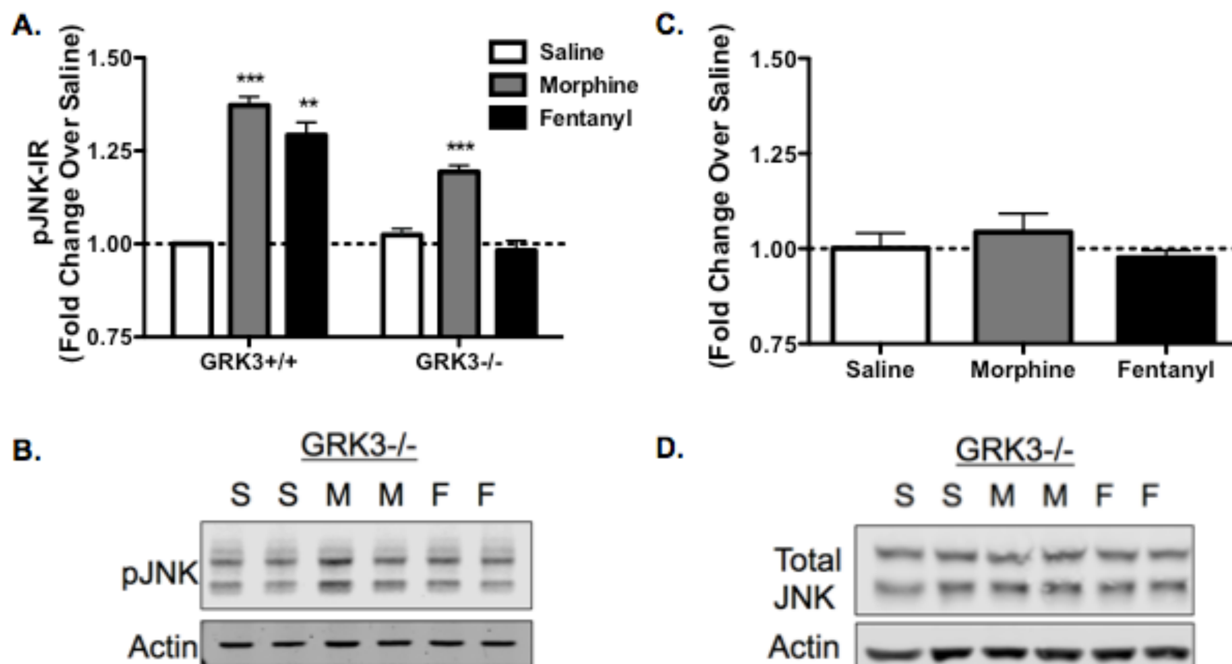

Figure 2.3 - Fentanyl, but not Morphine, Mediated Phospho-JNK Increase in the Spinal Cord is Selectively Abolished in GRK3$^{-/-}$ Mice.

A, JNK activation following fentanyl administration requires GRK3 whereas morphine activation of JNK does not. Animals were treated with saline, morphine (10mg/kg, s.c.), or fentanyl (0.3mg/kg, s.c.) and their spinal cords were harvested 60 min post-treatment for phospho-JNK-IR analysis. Both MOR agonists activate JNK in GRK3$^{+/+}$ mice. However, in GRK3$^{-/-}$ mice, morphine increased phospho-JNK-IR over baseline but fentanyl failed to do so. *B*, Representative images are shown for each data set. *C,* Total-JNK-IR was also not altered by morphine or fentanyl in GRK3$^{-/-}$ mice, indicating that the effects on phospho-JNK were not a result of alterations in total protein levels. *D,* Representative images are shown for the total-JNK data set. n=3-7; Data analyzed by one-sample t-test; statistical outliers were determined by the Grubb's Test with significance set to α=0.05.

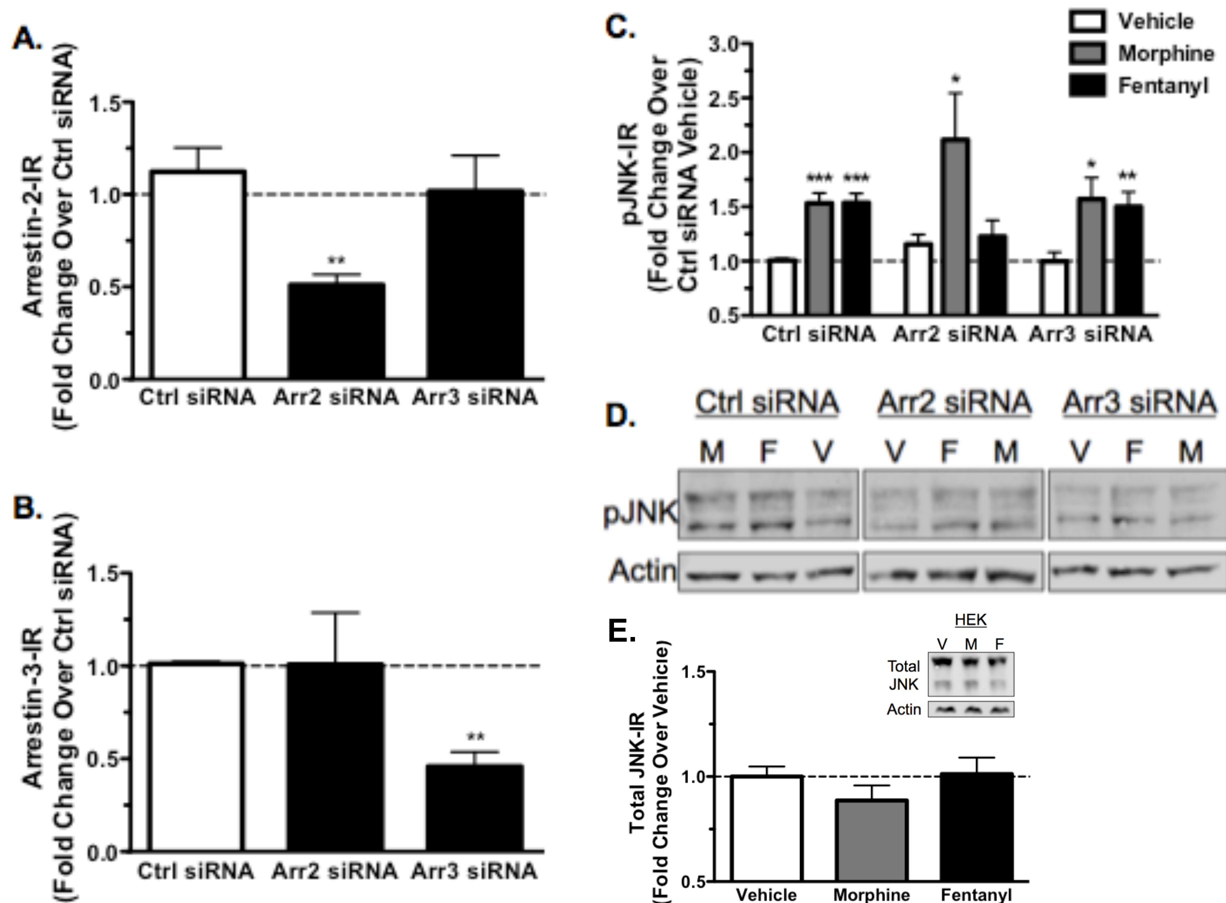

Figure 2.4 - Fentanyl Mediated Phospho-JNK Increase is Abolished Following Treatment with Arrestin-2 siRNA.

MOR-GFP expressing HEK293 cells were transfected with siRNA against arrestin-2, arrestin-3, or a scrambled siRNA control. Cells were subsequently lysed for arrestin-2 and -3-IR (A) or treated with vehicle (H_2O), 10μM morphine, or 10μM fentanyl for 30 min before lysis and analysis of phospho-JNK-IR for each sample, which was normalized as a percent of scrambled siRNA vehicle treated cells (C,D). *A,* Arrestin-2 siRNA reduced arrestin-2-IR by 49 ± 5.6%. Arrestin-3 siRNA did not significantly reduce arrestin-2-IR compared to a scrambled siRNA control (ctrl). *B,* Similarly, arrestin-2 siRNA did not significantly alter arrestin-3-IR compared to a scrambled siRNA control, whereas arrestin-3 siRNA reduced arrestin-3-IR by 54 ± 7.8%. *C*, Morphine treatment increased phospho-JNK-IR regardless of siRNA treatment, indicating that arrestin is not involved in morphine activation of JNK. Fentanyl, however, increased phospho-JNK-IR in control siRNA (ctrl) and arrestin-3 siRNA treated cells but this effect was abolished in arrestin-2 siRNA treated cells, indicating that arrestin-2 is required for fentanyl activation of JNK. *D*, Representative westerns are shown for each JNK dataset. *E,* Compared to vehicle, morphine and fentanyl did not alter total-JNK-IR. This confirms that changes in phospho-JNK-IR observed in subsequent figures were not a result of changes in total JNK levels. Representative images are shown for each total-JNK data set. n=10-13; Data analyzed by one-sample t-test (ctrl only) and student's t-test with Welch's correction when necessary.

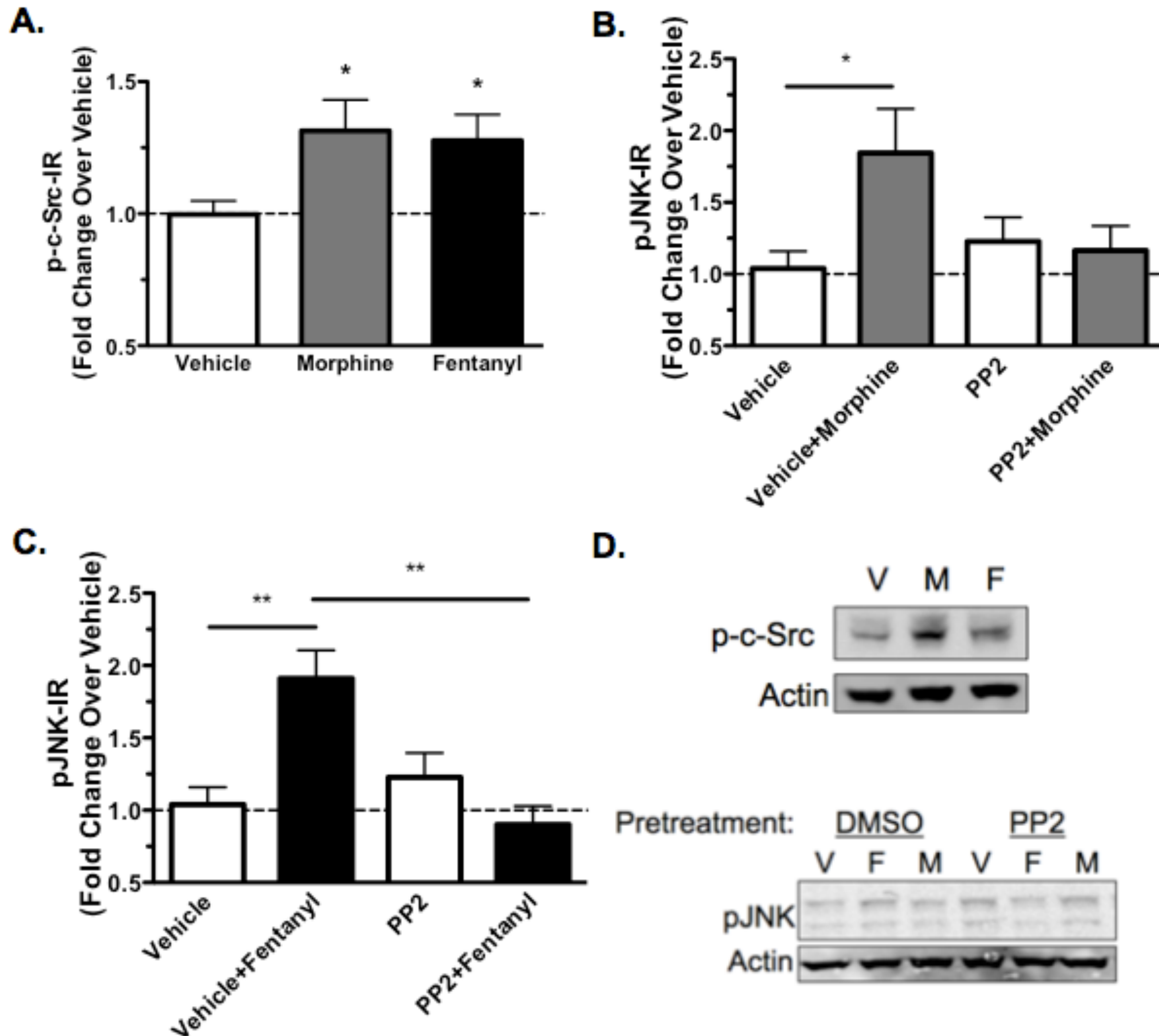

Figure 2.5 - Src is Required for JNK Activation by Morphine and Fentanyl.

MOR-GFP expressing HEK293 cells were treated with vehicle (H_2O), 10µM morphine, or 10µM fentanyl for 30 min before lysis and analysis of phospho-Src-IR. Similar treatments were performed following a 30 min pretreatment with 5µM PP2 or an equivalent volume of DMSO vehicle followed by cell lysis and analysis of phospho-JNK-IR for each sample. *A*, phospho-Src-IR was increased over baseline following a 30 min morphine treatment or fentanyl treatment. *B*, Morphine increased phospho-JNK-IR in the presence of DMSO, but this effect was blocked by pre-incubation with PP2. PP2 did not increase phospho-JNK-IR on its own. *C*, Similar results were observed following fentanyl treatment. *E*, Representative westerns are shown for phospho-Src analysis and phospho-JNK analysis. n=4-13; Data analyzed by one-way ANOVA with Newman-Keuls post-test.

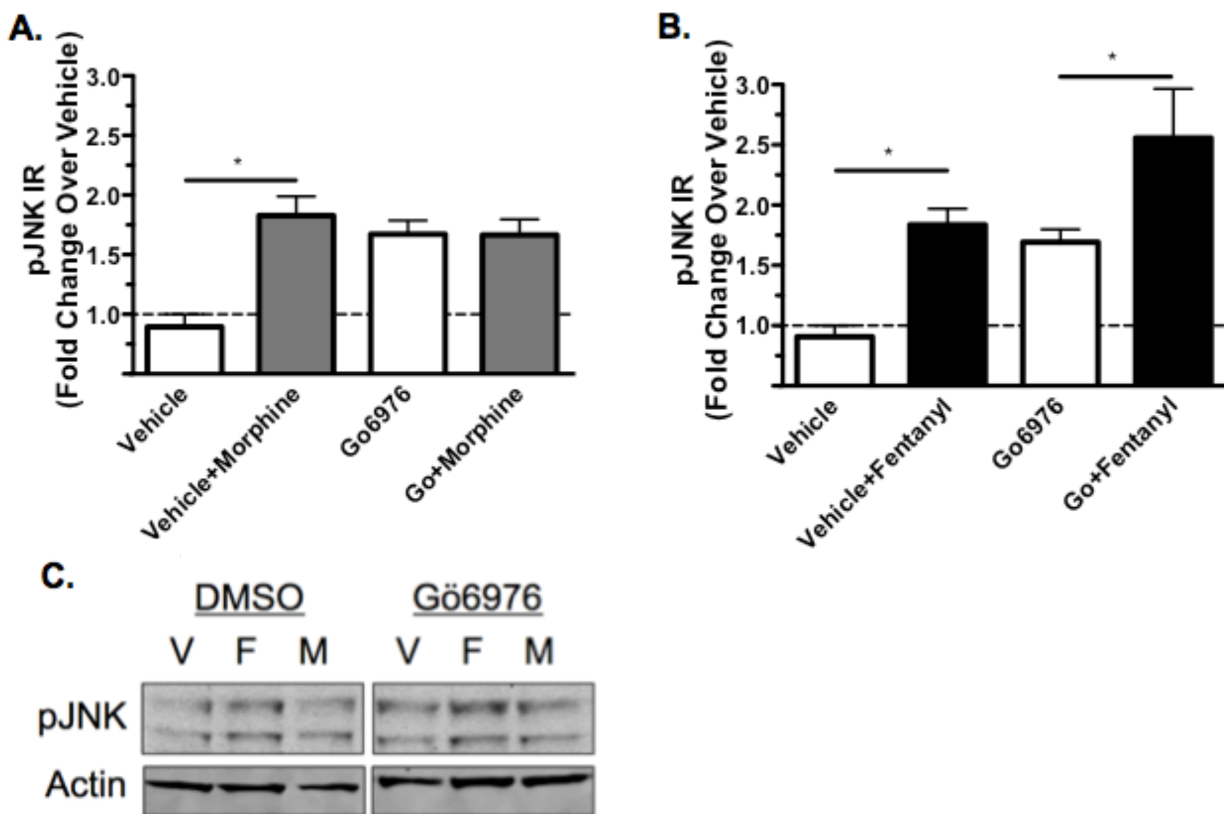

Figure 2.6 - PKC is Required Morphine Activation of JNK.

MOR-GFP expressing HEK293 cells were treated with 5μM Gö6976 or an equivalent volume of DMSO (vehicle) for 30 min. Cells were subsequently treated with vehicle (H_2O), 10μM morphine, or 10μM fentanyl for 30 min before lysis and analysis of phospho-JNK-IR for each sample. *A*, phospho-JNK-IR was increased following morphine treatment in the presence of DMSO, but this effect was blocked by the inhibition of PKC with Gö6976 pretreatment, although Gö6976 elevated phospho-JNK-IR over baseline as well. *B*, Fentanyl treatment, however, led to an increase in phospho-JNK-IR even in the presence of Gö6976, suggesting that PKC is not required for fentanyl-mediated JNK activation. *C,* Representative westerns are shown for each phospho-JNK analysis. n=7-11; Data analyzed by one-way ANOVA with Newman-Keuls post-test.

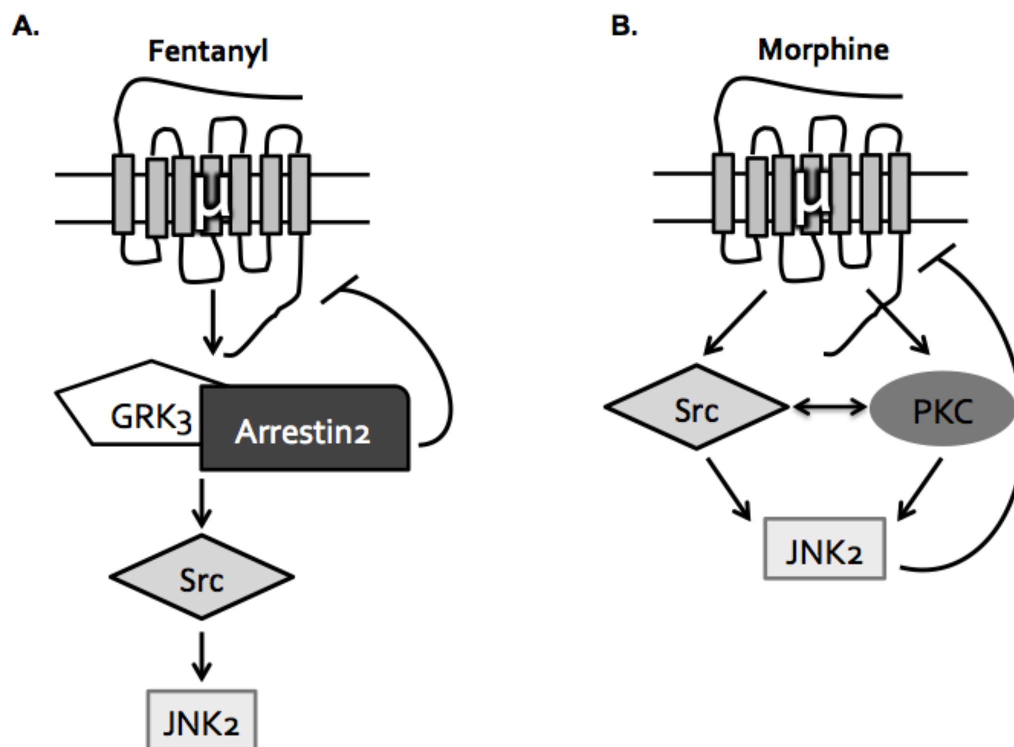

Figure 2.7 - Arrestin-Dependent and Arrestin-Independent Mechanisms of JNK Activation.

A, Fentanyl leads to MOR inactivation through a GRK3/arrestin-2 dependent mechanism. Src was also required for fentanyl-mediated JNK2 activation. *B,* Morphine led to MOR inactivation through a JNK2 dependent mechanism and activates JNK2 specifically. Both PKC and Src were required for JNK activation by morphine, suggesting an arrestin-independent mechanism of JNK activation.

Chapter 3. C-JUN N-TERMIMAL KINASE-MEDIATED INACTIVATION OF μ- AND κ-OPIOID RECEPTORS IS MEMBRANE DELIMITED

This work is in preparation as: "Schattauer SS, Kuhar JR, Land BB, Ong SE, Chavkin C (2015). Opioid Receptor Inactivation is Mediated by $G\alpha_i$ Depalmitoylation.

3.1 INTRODUCTION

Spinally-mediated tolerance following administration of the MOR agonist morphine requires activation of c-Jun N-terminal Kinase 2 and JNK-dependent KOR inactivation has been found to be responsible for norBNI's long-duration of action (Bruchas, Yang, *et al.*, 2007; Melief *et al.*, 2010; 2011). While the durations of action of a broad range of KOR antagonists, including norBNI, positively correlate with the ability of the antagonist to activate JNK1, there is no correlation between duration of antagonist action and drug clearance (Melief *et al.*, 2011; Munro *et al.*, 2012). Previous data also suggests JNK activation ultimately results in receptor uncoupling (Melief *et al.*, 2010). We originally hypothesized that JNK activation results in a direct phosphorylation of a component of the receptor-signaling complex leading to recruitment of an arrestin like molecule. To distinguish between direct (phosphorylation of the receptor or receptor signaling complex) and indirect effects (changes in gene expression or modulation of downstream signaling cascades) responsible for receptor uncoupling, this study uses an *in vitro* JNK kinase assay to assess if JNK can directly affect opioid stimulated $[^{35}S]GTP\gamma S$ binding.

To determine potential candidates responsible for receptor desensitization, we used Stable Isotope Labeling of Amino Acids in Culture (SILAC) to identify proteins bound to myc-KOR following treatment with norBNI. We identified enhanced receptor association with $G\alpha_i$

and this was also replicated in myc-MOR-expressing HEK293 cells. We did not, however, identify any arrestin-like candidates, which has led to our revised hypothesis that $G\alpha_i$ must undergo a structural modification leading to reduced function. This finding not only identifies a mechanism for JNK-mediated inactivation of opioid receptors but also suggests that this mechanism might result in reduced function of other $G\alpha_i$ protein-coupled receptors expressed in the same neurons as MOR or KOR.

I contributed the data demonstrating that JNK-mediated inactivation of MOR and KOR is membrane delimited in both spinal cord (Fig 3.1).

3.2 Materials and Methods

Reagents. DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; Sigma-Aldrich), U69,593 (NIDA), [^{35}S]GTPγS (Perkin-Elmer), GDP (Sigma-Aldrich), and GTPγS (Sigma-Aldrich) were dissolved in GTPγS binding buffer (50mM HEPES, 100mM NaCl, 5mM $MgCl_2$, 1mM EDTA, 0.1% BSA, 1mM DTT, pH 7.4). JNK1α1/SAPK1c (EMD Millipore) and ATF-2 (aa 19-96; EMD Millipore) were diluted in kinase dilution buffer (50mM Tris/HCl, 0.1mM EGTA, 0.1% 2-mercaptoethanol, 0.1% BSA, pH 7.5).

Animals. WT male mice (20-30g) were generated in house or purchased from Charles River. All mice were on a C57BL/6 background. Animals 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 of the National Institutes of Health on the care and use of animals.

Membrane Preparation. For *in vitro* kinase assay spinal cord membrane preparation, the lumbar region of the spinal cord was removed and homogenized in 2 mL membrane buffer

(300mM NaCl, 1mM EDTA, 1mM Na_3VO_4. 1mM NaF, 1x protease inhibitor, 1x phosphatase inhibitor (Calbiochem)) using a Polytron homogenizer at setting 1 (Kinematica, Littau-Lucern, Switzerland). Homogenates were centrifuged at 15,000x*g* at 4°C for 20 min. Supernatant was discarded and the pellet was washed in 2 mL membrane buffer, rehomogenized, and recentrifuged twice before freezing the pellet at -80°C until use.

Kinase Reaction. 10-20 μg membrane proteins or 2.7 μg ATF-2 (aa 19-96) were incubated with 160 ng active JNK1α1 or an equivalent volume of kinase dilution buffer in 20 μl kinase reaction buffer (50mM Tris/HCl, 150mM NaCl, 50mM MgCl, 0.5mM ATP, 0.1mM EGTA, 0.5mM DTT, 1mM 1mM Na_3VO_4, [+ or -] 0.5mM ATP, pH 7.5) or GTPγS binding buffer ([+ or -] 0.5mM ATP) at 25-30°C for 30 min. The reaction was terminated by placing tubes rapidly on ice and samples were immediately subjected to [^{35}S]GTPγS binding.

[^{35}S]GTPγS binding. 20 μg membrane proteins from *in vitro* JNK kinase assay treated preparations were incubated with 1μM DAMGO, 1μM U69,593, 1μM GTPγS, or an equivalent volume of GTPγS binding buffer in 50mM GTPγS binding buffer at 30°C for 1 hr in the presence of 0.1nM [^{35}S]GTPγS and 10mM GDP. Bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by rapid filtration using a Brandel cell harvester onto GF/B filters pre-soaked in 50mM Tris/0.1%BSA. Filters were washed 3x with 50mM Tris/0.1%BSA. Bound [^{35}S]GTPγS was measured using a liquid scintillation counter (Packard TriCarb 1900CA). Data were normalized to percent of binding in vehicle-stimulated controls.

Western Blotting. ATF-2 *in vitro* kinase assay samples, described above, were analyzed for pan-phospho-serine/threonine immunoreactivity using western blot analysis. Samples were mixed with 4x Laemmli sample buffer (Bio-Rad) and were heated at 100°C for 5 min and resolved on a 10% Bis-Tris polyacrylamide gel (Invitrogen Life Technologies, Grand City, NY)

at 120V for 2 hr. Blots were transferred to nitrocellulose membranes (Whatman, Middlesex, UK) for 1.5–2 hr at 30V. Membranes were blocked with 5% BSA-TBST for 1 hr at 23°C and blotted for pan-phospho-serine/threonine (1:1000, BD Biosciences) immunoreactivity (-IR) in 5% BSA-TBST overnight at 4°C. Membranes were washed 3x5 min in TBST and incubated in IRdye secondary (Li-Cor Biosciences, Lincoln, NE; 1:10,000) in 1:1 5% milk-TBST and Odyssey buffer (Li-Cor Biosciences, Lincoln, NE) for 1 hr at 23°C. Membranes were washed for 3x5 min in TBST and scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences; Lincoln, NE). Relative fluorescent band intensity was measured using the Odyssey software. All westerns were run blinded to treatment.

Analysis. Student's t-test was used to assess statistical significance of the data (α=0.05).

3.3 RESULTS

In GTPγS binding buffer, JNK robustly phosphorylated its known substrate, ATF-2, only in the presence of ATP (Fig. 3.1A). This confirmed that in our membrane preparation and the subsequent *in vitro* JNK kinase assay, JNK would only be capable of phosphorylating potential substrates when co-incubated with ATP. Spinal cord membranes pre-incubated with inactive JNK (-ATP) showed robust MOR and KOR function, as measured by DAMGO or U69,593 stimulated [^{35}S]GTPγS compared to vehicle , respectively (Fig. 3.1B). However, pre-incubation with active JNK (+ATP) resulted in significantly reduced DAMGO (p=0.0342) and U69,593 (p=0.0206) stimulated [^{35}S]GTPγS compared to the inactive JNK incubated membranes. This suggests that the JNK target responsible for uncoupling MOR and KOR in spinal cord is membrane delimited.

3.4 Discussion

The principal findings of this study are that JNK is able to directly uncouple MOR and KOR and the putative JNK substrate is present in the cell membrane. In separate studies, we have also established that following treatment with norBNI or morphine, $G\alpha_i$ is tightly associated with the KOR and MOR receptor complex, respectively, despite being unable to activate downstream signaling mechanisms. We also identified peroxiredoxin 6 as a $G\alpha_i$ interactor. Therefore, our current hypothesis is that JNK phosphorylates a membrane bound substrate, leading to a modification of $G\alpha_i$ that blocks the G protein's intrinsic ability to exchange GDP for GTP. The work performed in this chapter helps confirm that JNK-mediated inactivation of MOR and KOR is not due to transcriptional changes or targeting of cytosolic proteins, but instead is the result of direct phosphorylation of a membrane bound substrate. This establishes that JNK-mediated MOR and KOR inactivation is membrane delimited.

While the data presented in this study advances our understanding of JNK-mediated opioid receptor inactivation, the precise JNK substrate has yet to be identified. Our hypothesis is that $G\alpha_i$ undergoes a structural modification, specifically decreased palmitoylation. This is based on the ability of peroxiredoxin 6 to cause an increase in reactive oxygen species at the cell membrane (Chatterjee *et al.*, 2011) leading to cysteine modification and ultimately reduced palmitoylation. Canonically, activated GPCRs catalyze the exchange of GDP for GTP and the G protein's intrinsic GTPase activity converts it back to GDP, thus inactivating the G protein and terminating signaling. It is also known, however, that covalent modifications of G proteins can lead to functional modulation. Specifically, lipid modifications have been shown to be required for efficient G protein function. This is partially due to membrane anchoring functions, but lipid

modifications can also alter other protein-protein interactions and depalmitoylation has been hypothesized to dampen G protein signals (Wedegaertner *et al.*, 1993; 1995).

In summary, this work establishes that the mechanism of JNK-mediated opioid receptor inactivation is direct. Future work will focus on identifying the precise membrane-associated JNK substrate. It is important to note that this result might also be applicable to additional GPCR systems and this mechanism should be considered when investigating GPCR desensitization. Additionally, it will be important to identify if $G\alpha_i$ depalmitoylation results in inactivation of other GPCRs coexpressed in MOR or KOR containing cells.

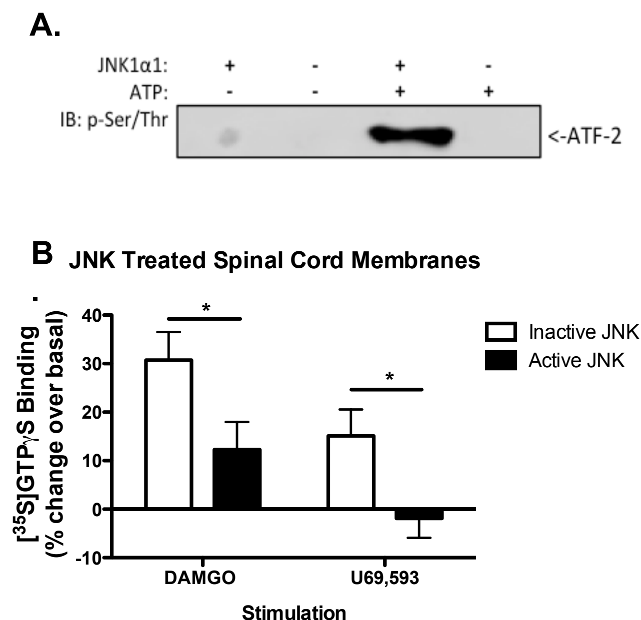

Figure 3.1 – JNK Directly Uncouples MOR and KOR

To investigate if JNK could directly uncouple MOR and KOR, an *in vitro* JNK kinase assay was used. *A,* To determine if JNK was capable of phosphorylating its substrates in this assay we used the known substrate ATF-2 to demonstrate that JNK can only phosphorylate this substrate when both JNK and ATP are present in the kinase assay. *B,* Isolated spinal cord membranes were the subjected to a 30 min kinase assay, followed by DAMGO or U69,593 stimulated [^{35}S]GTPγS binding to measure MOR and KOR function, respectively. Preincubation with active JNK resulted in reduced [^{35}S]GTPγS binding at MOR (p=0.0342) and KOR (p=0.0206) compared to membranes preincubated with inactive JNK, demonstrating that JNK causes direct receptor uncoupling in spinal cord. n=11; Data analyzed by student's t-test with welsh's correction when necessary.

Chapter 4. CONTRIBUTIONS TO ADDITIONAL STUDIES INVESTIGATING MECHANISMS OF OPIOID RECEPTOR SIGNALING

4.1 Pentazocine Analgesia is μ-Opioid Receptor Mediated

This work was originally published in: "Schattauer SS, Miyatake M, Shankar H, Zietz C, Levin JR, Liu-Chen LY, Gurevich VV, Rieder MJ, Chavkin C (2012). Ligand directed signaling differences between rodent and human κ-opioid receptors. *JBC.* 287(50): 41595-607.

Pentazocine is a mixed acting opioid analgesic with agonist activity at KOR and DOR and antagonist or partial agonist activity at MOR (Emmerson *et al.*, 1996; J Zhu *et al.*, 1997). When given to humans, pentazocine elicits classic KOR side effects, including dysphoria and hallucinations (Beaver *et al.*, 1966; Hamilton *et al.*, 1967; Jasinski *et al.*, 1970; Zacny *et al.*, 1998). However, some patients report pentazocine-elicited euphoria that is blocked by a MOR selective dose of naloxone (Preston and Bigelow, 1993). Previous studies demonstrated that in mice, thermal analgesia can be MOR-mediated (Ide *et al.*, 2011; Shu *et al.*, 2011), although results depend on the mouse strain and pain modality used (Suzuki *et al.*, 1991; Chien and Pasternak, 1995; Ide *et al.*, 2011; Shu *et al.*, 2011). It has been hypothesized that pentazocine analgesia is mediated by KOR in humans, although this has not been rigorously tested.

Further complicating KOR compound testing in animals is the sequence difference between human and rodent KOR (hKOR and rKOR) where the amino acid responsible for GRK-mediated KOR phosphorylation and subsequent p38 activation, which results in dysphoria (Bruchas, Land, *et al.*, 2007; Land *et al.*, 2009; Bruchas *et al.*, 2011), is not conserved despite 94% sequence similarity (J Li *et al.*, 2002). This study investigates the ability of pentazocine to activate p38 in hKOR and rKOR expressing HEK293 cells to determine if this differential

phosphorylation site has an effect on GRK/arrestin-dependent signaling. We found that although hKOR and rKOR are capable of activating p38, pentazocine is 30-fold more potent for p38 activation in hKOR compared with rKOR although pentazocine is equally potent for arrestin-independent activation of ERK1/2 in hKOR and rKOR. Additionally, pentazocine produced conditioned place aversion and thermal analgesia in mice, but both effects were MOR-mediated. These results suggest that caution should be taken when drawing conclusions about KOR agonists in humans based only on rKOR models and supports the idea that G protein biased KOR agonists hold promise for future development of analgesics with reduced abuse potential and aversive properties.

I contributed data investigating the opioid receptor mediating pentazocine analgesia described below (Fig. 4.1).

Methods

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 *et al.*, 1999; Clarke *et al.*, 2002).

Conditioned Place Preference/Aversion. C57Bl/6 mice were trained in a balanced, unbiased three-chamber conditioning apparatus as described previously (Land *et al.*, 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 intraperitoneal of 15% DMSO, 5% Cremophor and 80% sterile saline), and the afternoon session was paired 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, mice were allowed to roam freely between all three compartments and the amount of time spent in each compartment was recorded. Test sessions took place midday. Conditioned place preference or aversion 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, 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, Marton-Popovici, *et al.*, 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.

Results

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 5 mg/kg naloxone. Mice showed a 200 s place preference for the pentazocine-paired chamber that was not affected by pretreatment with the KOR-selective antagonist norBNI. Pretreatment with naloxone reversed the preference for the pentazocine-paired chamber and unveiled an aversion that was not blocked by pretreatment with norBNI (Fig. 4.1A). These results suggest that pentazocine did not produce KOR-mediated aversion in mice, in contrast to reports of KOR-mediated aversive side effects in humans.

To assess the receptor selectivity of pentazocine in mice, 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. 4.1B). 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 antinociceptive response was not significantly different between wild type and $KOR^{-/-}$ mice, whereas $MOR^{-/-}$ had significantly reduced analgesia as compared with wild type and $KOR^{-/-}$ mice. Furthermore, pretreatment with norBNI produced no reduction in pentazocine-induced analgesia in $MOR^{-/-}$ mice (data not shown).

To determine the locomotor effects of pentazocine, total distance traveled was measured during drug training sessions for conditioned place preference. Pentazocine produced neither hypolocomotor effects (typical of KOR agonists) nor hyperlocomotor effects (typical of MOR agonists; Fig. 4.1C).

4.2 Nalfurafine Analgesia is κ-Opioid Receptor Mediated and Produces Acute Analgesic Tolerance

This work has been submitted to *Cellular Signaling* as: "Schattauer SS, Kuhar JR, Song A, Chavkin C (2015). Nalfurafine is a G Protein Biased Agonist Having Significantly Greater Bias at the Human than Rodent Form of the Kappa Opioid Receptor.

Functionally selective KOR agonists that preferentially activate G protein signaling over arrestin-dependent signaling might be good candidates for safer analgesics that avoid the side effects typically associated with MOR selective compounds and KOR p38 signaling (Chavkin, 2011). While numerous functionally selective KOR agonists have been identified, none of them have desirable drug-like qualities (Rives *et al.*, 2012; Schmid *et al.*, 2013; K White *et al.*, 2015). However, the partially selective KOR agonist nalfurafine (TRK820) is in clinical trials for the treatment of uremic pruritus (Nagase and Fujii, 2011). Nalfurafine also has pharmacological properties suggestive of functional selectivity, such as analgesia with a low incidence of dysphoria (Nagase *et al.*, 1998; Hasebe *et al.*, 2004). Using KOR-expressing HEK293 cells, we found that nalfurafine was G protein biased with 20x and 250x lower potency for p38 activation compared to G protein-mediated ERK1/2 activation by rKOR and hKOR, respectively.

Additionally, we found that nalfurafine at 50 µg/kg and 150 µg/kg produced peak thermal analgesia that was blocked by norBNI, indicating that nalfurafine analgesia in this assay is KOR-mediated. KOR-dependence had not previously been demonstrated for thermal pain assays. Further, this study found that high doses of nalfurafine are capable of inducing acute cross-tolerance that resolves within 24 hrs. These studies suggest that future development and testing of nalfurafine analogs could provide key insights into the structural basis of KOR signaling bias and lead to development of more selective KOR ligands with reduced side effects.

I contributed data investigating nalfurafine thermal analgesia (Fig. 4.2) and tolerance (Fig. 4.3).

Methods

*Animals***.** Male C57Bl/6 mice (20-30g) were group-housed, kept on a 12-h light/dark cycle, and provided with food and water *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.

Antinociceptive Testing. Antinociceptive responses were measured using the warm-water tail-withdrawal assay (McLaughlin, Marton-Popovici, *et al.*, 2003). The response latency for an animal to withdraw its tail after being immersed in 52.5°C water was measured before treatment with (-/+)U50,488 or nalfurafine. After drug administration, responses were measured at 30 min post drug administration. After dose response testing was complete, animals were injected with saline or norBNI and retested 3-5 days later before and 30 min after (-/+)U50,488 or nalfurafine. For tolerance studies, animals were injected with saline or 1mg/kg nalfurafine. 3 hr later, baseline analgesia was recorded followed by administration of U50,488. A second response post-drug was recorded 30 min post U50,488. The same mice were treated in a similar fashion 24 hr following the initial treatment with saline or nalfurafine. A 15 sec maximal immersion duration was used as a cut off to prevent tissue damage. Analgesia testing was done by an investigator blinded to drug treatment.

Results

Does Nalfurafine Produce KOR-Mediated Thermal Analgesia in C57Bl/6 Mice? While the analgesic effects of nalfurafine have been demonstrated to be KOR-dependent in the acetic acid writhing (Endoh *et al.*, 1999) and rat paw pressure test (Endoh *et al.*, 2000), KOR-dependence has not been established for nalfurafine thermal analgesia. The warm-water tail-withdrawal assay was used to assess the analgesic properties of nalfurafine and to determine if

nalfurafine analgesia is KOR-mediated in this test. Treatment with U50,488 resulted in a dose dependent increase in tail withdrawal latency ($p<0.001$), indicative of analgesia (Fig. 4.2A), and an effect of drug compared to baseline ($p<0.01$; interaction $p<0.05$). Similarly with nalfurafine, drug treatment significantly increased latency to withdrawal (Fig. 4.2B; $p<0.001$; interaction $p<0.01$). Specifically at 15 and 30 mg/kg U50,488, animals showed a significant analgesic response to drug (2.9±1.1 seconds and 2.8±0.9 sec, respectively; Bonferroni $p<0.001$) and at 50 and 150 μg/kg nalfurafine (1.5±0.7 sec and 1.7±0.4 sec, respectively; Bonferroni $p<0.01$ and $p<0.001$, respectively).

To determine if this analgesia was KOR-dependent, mice were injected with norBNI (10 mg/kg) 24 hr prior to treatment with U50,488 or nalfurafine. As expected, norBNI blocked the U50,488-mediated increase in withdrawal latency ($p<0.01$, Fig. 4.2C), with a tail withdrawal latency of 0.7±0.2 sec and 0.76±0.2 seconds after 15 and 30 mg/kg U50,488. norBNI also blocked the nalfurafine-mediated increase in withdrawal latency ($p<0.05$, Fig. 4.2D), with a tail withdrawal latency of 0.6±0.1 sec and 0.7±0.2 sec after 50 and 150 μg/kg nalfurafine. These results demonstrate a KOR-dependent analgesic response to 50 and 150 μg/kg nalfurafine in this assay.

Does Nalfurafine Produce Acute Tolerance? Prior studies have found that nalfurafine produces minimal analgesic tolerance in the acetic acid writhing and herpetic pain assays (Suzuki *et al.*, 2004; Takasaki *et al.*, 2004). To determine if high doses of nalfurafine are capable of producing analgesic tolerance in the warm-water tail-withdrawal assay, U50,488-induced increases in tail withdrawal latency were used to measure cross-tolerance at KOR following a pre-injection of nalfurafine. Mice were pretreated with saline or nalfurafine (1 mg/kg, i.p.). After 3 hr, tail withdrawal latency was measured prior to and 30 min following a U50,488 injection (15

mg/kg, i.p.; Fig. 4.3A). Twenty-four hours after the initial pretreatment, tail flick withdrawal latency was measured again prior to and after U50,488 treatment (Fig. 4.3B). Three hours after pretreatment, prior nalfurafine significantly decreased U50,488-induced analgesia, which was not observed with saline pretreated mice (effect of U50,488 $p<0.05$; interaction between nalfurafine pretreatment and U50,488 pretreatment $p<0.05$; two-way ANOVA with repeated measures, n=13 for saline, n=12 for nalfurafine). This was not a consequence of elevated baseline following nalfurafine pretreatment, as no difference in response latency was observed between saline and nalfurafine pretreated mice prior to U50,488 injection ($p>0.05$; student's t-test), indicating that high doses of nalfurafine are capable of producing an acute cross-tolerance in this assay. Twenty four hours after pretreatment, U50,488 stimulated an increase in response latency regardless of pretreatment (effect of U50,488 $p<0.05$; interaction $p>0.05$; two-way ANOVA with repeated measures), demonstrating a lack of residual analgesic tolerance at 24 hr. Together, these results indicate that high doses of nalfurafine (1mg/kg) produce acute analgesic tolerance, but that tolerance is resolved rapidly, with normal KOR function one day later.

4.3 κ-Opioid Receptor Induced Aversion Requires p38 MAPK Activation in VTA Dopamine Neurons

This work has been accepted pending text revision at *Journal of Neuroscience* as: "Ehrich JM, Messinger DI, Knakal CR, Kuhar JR, Schattauer SS, Bruchas MR, Zweifel LS, Kieffer BL, Phillips PEM, Chavkin C (2015). Kappa Opioid Receptor induced aversion requires p38 MAPK activation in VTA dopamine neurons.

Finding a non-euphorigenic opioid analgesic has been a central goal of opioid medicinal chemistry for over 100 years, but the prediction that G biased KOR agonists that do not activate p38 signaling would not be dysphoric requires a better understanding of how activation of KOR either pharmacologically or by stress-induced dynorphin release produces dysphoria. A series of recent studies indicate that stress exposure produces its dysphoric, anxiogenic, and proaddictive

effects through activation by extrahypothalamic corticotrophin releasing factor (CRF), which releases the endogenous dynorphin opioid neuropeptides in brain (Land *et al.*, 2008; Mantsch *et al.*, 2015). Dynorphin selectively activates KOR, and rodents treated with KOR antagonists or genetically lacking either prodynorphin or KOR have reduced immobility in forced swim assays (typical of antidepressant drug actions), reduced social avoidance behaviors following repeated social defeat, reduced escalation of psychostimulant self-administration, and reduced reinstatement of extinguished ethanol or cocaine self-administration (Bruchas *et al.*, 2010; Van't Veer and Carlezon, 2013; Lalanne *et al.*, 2014). Recent clinical studies confirm that drugs having KOR antagonist activity significantly reduce depressive symptoms in humans with treatment-resistant depression and reduce nicotine consumption in dependent individuals (Saxon *et al.*, 2005; Ehrich *et al.*, 2014)

At the circuit level, KOR activation in the serotonergic neurons of the dorsal raphe nucleus (DRN), the dopaminergic neurons of the ventral tegmental area (VTA), and neurons of the nucleus accumbens (NAc) have been implicated in the aversive properties of stress response (Bruchas *et al.*, 2010; Van't Veer and Carlezon, 2013). These results suggest that dynorphin regulates mood by controlling serotonergic and dopaminergic inputs to the NAc, but these mechanisms are not yet clear.

There are four principal findings of this study. First, KOR activation of p38 within the VTA is required for the aversive properties of KOR stimulation. Second, the aversive properties of KOR activation are rescued in $KOR^{-/-}$ mice by viral restoration of KOR signaling to VTA dopamine neurons specifically. This behavioral restoration is dependent on the phosphorylation of KOR at Ser^{369}, which is required for p38 activation (Land *et al.*, 2008). Third, the aversive properties of KOR activation are dependent on the expression of p38α in dopaminergic neurons,

but the ability of KOR agonists to inhibit dopamine release is not dependent on p38 activation within dopaminergic neurons. Fourth, KOR activation has complex, time-dependent effects on VTA neuron firing rates, which likely involves p38-induced GIRK phosphorylation and regulation of excitability. Together, these findings suggest that activation of p38 by KOR in dopaminergic neurons is required to elicit the aversive properties of KOR agonists, and this process occurs independently of KOR's ability to inhibit dopamine release.

I produced the Cre inducible KOR and KOR S369A (KSA) viral vectors to selectively restore KOR, with or without the key GRK3 phosphorylation site, to dopaminergic neurons in the VTA, which was used to produce the results presented in Figure 4.4.

Methods

Animals. Male C57Bl/6 mice (Charles River, Wilmington, MA) >50 days old were used and given *ad libitum* access to food and water. Global gene deletion of KOR ($Oprk1^{-/-}$) on C57Bl/6 background was accomplished as previously described (Hough *et al.*, 2000). In some instances conventional KOR gene knockout mice were bred to also carry the DAT-Cre transgene. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Stereotaxic Injection. Mice were anesthetized with isoflurane and mounted on a Model 1900 stereotaxic alignment system (David Kopf Instruments). For VTA injections, a 32 gauge needle with a 45 degree beveled tip (Hamilton Company) was lowered bilaterally (A/P = -3.30mm, M/L = ± 0.30mm from bregma) and 0.5 µl was injected at D/V -4.70-4.75mm and at -4.40-4.45mm from bregma. Injection locations were confirmed via postmortem immunohistochemistry.

Vector design and production. AAV-DIO KOR-GFP and AAV-DIO KSA-GFP were developed using the AM/CBA-DIO-poly vector (Cardin *et al.*, 2009). Rat KOR-GFP or KSA-GFP was subcloned into the AAV-DIO vector using AgeI and NotI in the reverse coding orientation following a chicken beta-actin promoter so that expression would only be driven in the presence of Cre recombinase.

After confirming sequence fidelity, HEK293T cells were transfected with 10 µg AAV and 20 µg pDG1 vector per 10 cm plate. Three days post-transfection, cells were harvested and subjected to three freeze thaw cycles. The supernatant was transferred to a Beckman tube containing a 40% sucrose cushion and spun at 27,000 rpm overnight at 4°C. Pellets were resuspended in CsCl at a density of 1.37 g/ml and spun at 5,000 rpm overnight at 4°C. The following day, 1 ml CsCl fractions were run on an agarose gel and genome containing fractions were selected and spun again at 5000 rpm overnight at 4°C. 1 mL fractions were collected again and genome containing fractions were dialyzed overnight. The filtered solution was transferred to a Beckman tube and spun at 27,000 rpm overnight at 4°C. The pellet (containing purified AAV) was resuspended in 100 µl 1xHBSS.

Conditioned Place Preference (CPP). Mice were trained in a balanced three-chamber apparatus consisting of chambers of equal size with horizontal or vertical stripes, and a third smaller chamber in the center. The paradigm consisted of tests on day one and four and training on days two and three. On test days the mice were placed in the center chamber then allowed to roam freely through all three chambers for 30 min. Time spent in each chamber was recorded with a video camera (Canon DC310) and analyzed using Ethovision (Noldus, Wageningen, Netherlands). On training days, mice received saline vehicle (10 mL/kg, i.p.) while restricted to one chamber in the morning and U50,488 (2.5-5.0 mg/kg at 10 mL/kg, i.p.) in the other chamber

in the afternoon. The drug-paired chamber was assigned based on the day one pre-test to ensure that the average time spent in each chamber was the same for all groups. The final preference score was determined based on the difference in the amount of time spent in the drug-paired chamber on the day four post-test and the day one pre-test. All mice were handled 1-2 times a day for four days the week prior to the day one pre-test.

*Immunohistochemistr*y. Mice were anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and intracardially perfused with ice-cold phosphate-buffered saline (PBS) and 4% paraformaldehyde. Brains were stored overnight in 4% paraformaldehyde, then cryoprotected in 30% sucrose (wt/vol) in phosphate buffer (PB) solution. At least 48 hours later, brains were sliced at 30 μm using a microtome (Leica). Slices were stored at 4°C in PB with 0.1% sodium azide. VTA slices were then taken and washed for 3 x 10 minutes in PBS before blocking in PBS containing 5% normal goat serum and 0.3% Triton X-100. Slices were then incubated for 36-48 hours in blocking solution containing chicken anti-GFP (1:3000, AB13970, Abcam) and mouse anti-tyrosine hydroxylase (1:500, T1299, Sigma). Slices were then washed for 6 x 15 minutes in PBS and incubated in blocking solution containing Alexa 488 and Alexa 555 (1:500, Life Technologies) tagged to IgG of the appropriate species. The slices were then washed for 3 x 10 minutes in PBS and 2 x 10 minutes in PB. Slices were then mounted onto Superfrost Plus slides (Fisher) with Vectashield mounting media and sealed with nail polish for microscopy. All slices were imaged at the W.M. Keck Center for Advanced Studies in Neural Signaling (Seattle, WA) on either a Leica SL confocal or a Nikon Upright with QImaging Camera.

Results

Selective Restoration of KOR Activation to VTA Dopamine Neurons of KOR$^{-/-}$ Mice Rescues Aversion in a Phosphorylation-Dependent Manner. Although we demonstrated that

KOR activation and phosphorylation within VTA neurons was sufficient to support U50,488 CPA (data not shown, presented in original manuscript), it did not clarify which cell types are required. To determine if KOR expression within dopaminergic neurons of the VTA alone was sufficient to support KOR-CPA, we developed an adeno-associated double-floxed inverse open reading frame (AAV-DIO) viral construct (Cardin *et al.*, 2009) directing expression of GFP-tagged KOR or KSA selectively in Cre-expressing neurons (Fig. 4.4A; DIO-KOR-GFP or DIO-KSA-GFP). The number of cells showing colocalization between GFP-IR and immunoreactivity for tyrosine hydroxylase (TH-IR), a selective marker for dopamine neurons, was significantly increased in $DAT^{Cre/+}$mice relative to $DAT^{+/+}$ mice (Fig. 4.4B,C; two-way ANOVA, main effect of genotype, $F_{(1,43)}=110.7$, $p < 0.0001$), demonstrating selectivity of expression. Although WT control mice showed a normal CPA to U50,488, $DAT^{Cre/+}$ $KOR^{-/-}$ mice showed U50,488 CPA after intra-VTA injection with DIO-KOR-GFP but not DIO-KSA-GFP (Fig. 4.4D; unpaired t-test, $t_{(29)}=2.767$, $p < 0.01$). This demonstrates that KOR activation in VTA dopamine neurons alone is sufficient to support CPA, but this requires an intact GRK3 phosphorylation site.

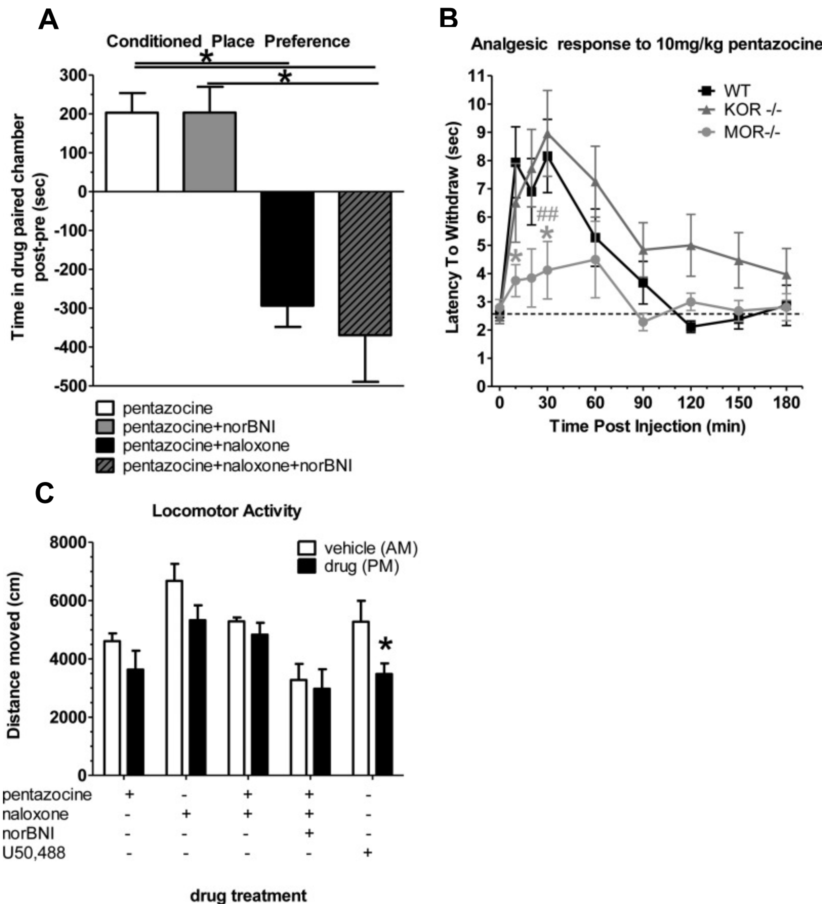

Figure 4.1 - Behavioral Effects of Pentazocine in Mice.

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 (10 ml/kg intraperitoneal of 15% DMSO, 5% Cremophor, and 80% sterile saline), and the afternoon session was paired with drugs (pentazocine, 10 mg/kg intraperitoneal, pentazocine + norBNI 10 mg/kg intraperitoneal, pentazocine 10 mg/kg + naloxone 5 mg/kg intraperitoneal, or pentazocine 10 mg/kg + norBNI 10 mg/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 during the post-test. Significant effect of drug treatment was based on one-way ANOVA ($p < 0.0001$, $n = 4–10$), with the Bonferroni-Dunn post-hoc test with correction for multiple comparisons. *B*, wild type, $KOR^{-/-}$, and $MOR^{-/-}$ C57Bl/6 mice were injected with 10 mg/kg pentazocine intraperitoneally, and tail flick withdrawal latency was recorded over 180 min 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 ($p < 0.0001$) ($n = 13–19$). Significance according to the Bonferroni-Dunn post-hoc test of the differences as compared with wild type or $KOR^{-/-}$ is indicated by * 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 *versus* vehicle ($p < 0.01$) based on two-way repeated measures ANOVA is shown. Significance according to Bonferroni-Dunn post-hoc test of the differences for drug *versus* vehicle ($p < 0.05$) is indicated by the *.

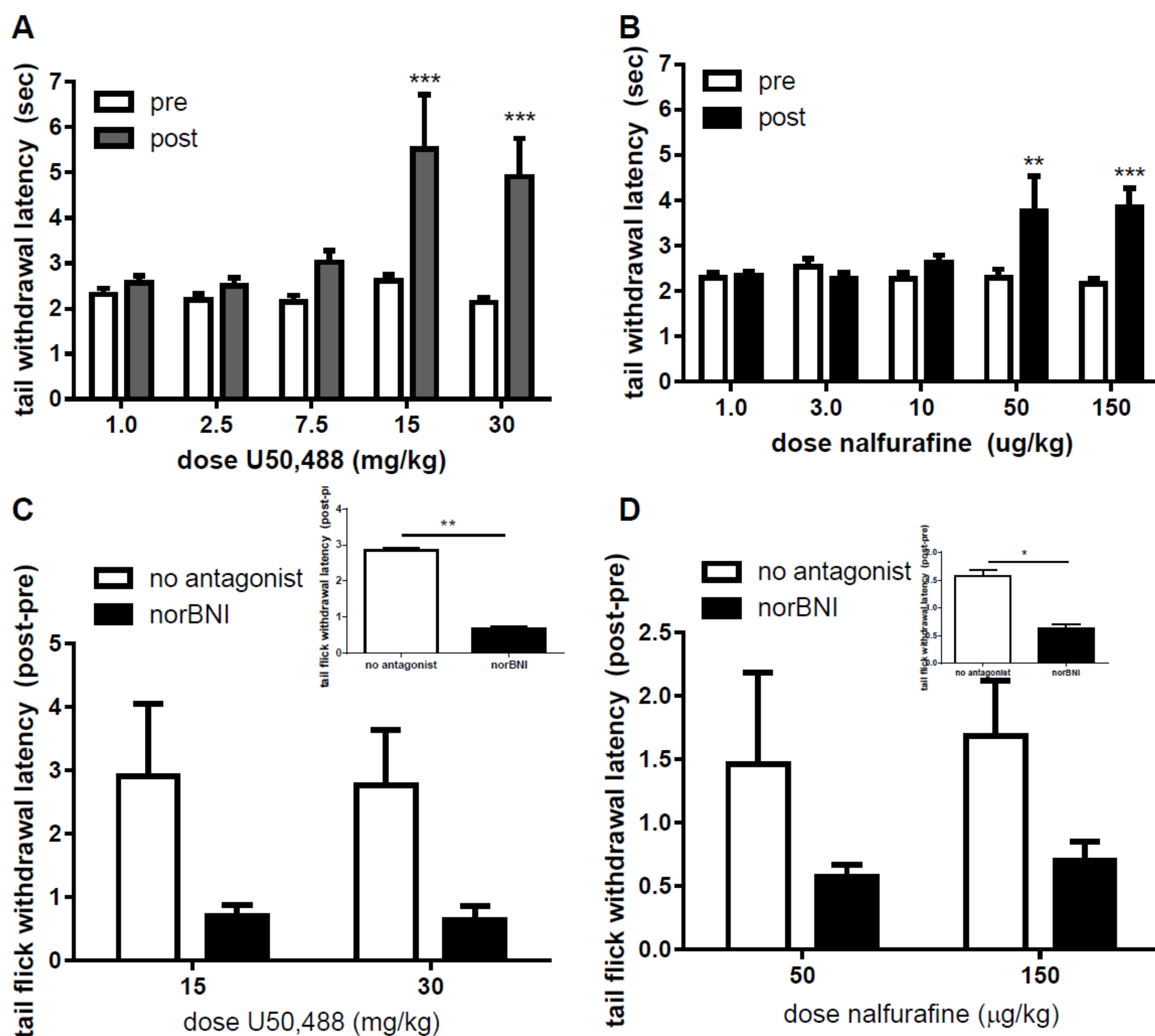

Figure 4.2 - Nalfurafine has KOR-Mediated Analgesic Effects in the Warm-Water Tail-Withdrawal Assay.

Wild type C57Bl/6 mice were injected with the indicated doses of *A,* U50,488 or *B*, nalfurafine i.p, and tail flick withdrawal latency was recorded before and 30 min post-injection. For U50,488, a significant interaction ($p<0.001$), effect of drug treatment ($p<0.0001$), and effect of dose ($p<0.001$) was observed based on two-way ANOVA with repeated measures ($n=16$). For nalfurafine, a significant interaction ($p<0.001$) and significant effect of drug treatment ($p<0.001$) was observed based on two-way ANOVA with repeated measures ($n=16$). Significance according to Bonferroni post-hoc test, as compared to pretest is indicated by ** ($p<0.01$) or *** ($p<0.001$). KOR-dependence of increased tail flick withdrawal latency by *C*, U50,488 and *D,* nalfurafine was determined by measuring tail flick withdrawal latency following pretreatment with norBNI. A significant effect of antagonist (depicted by inset) was observed for U50,488 ($p<0.001$, $n=16$) and nalfurafine ($p<0.05$, $n=16$) based on two-way ANOVA with repeated measures followed by student's t-test (inset).

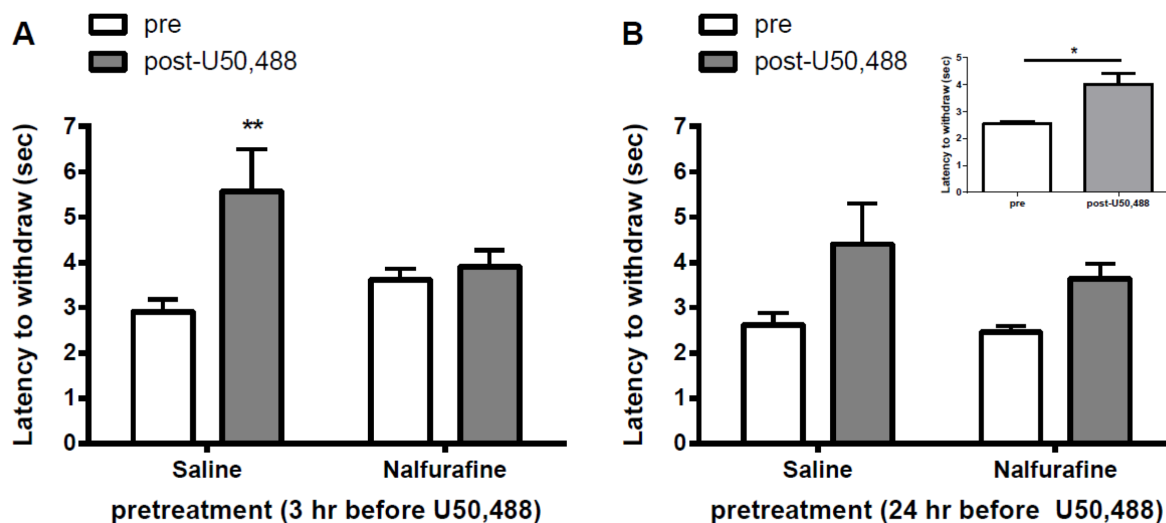

Figure 4.3 - Nalfurafine Results in Acute but not Prolonged Tolerance.

Wild type C57Bl/6 mice were pretreated with saline or nalfurafine (1 mg/kg). Tail flick withdrawal latency was measured before and 30 min after injection of U50,488 (15 mg/kg, i.p.) at *A,* 3 hr and *B,* 24 hr following pretreatment with saline or nalfurafine. At 3 hr following pretreatment, a significant interaction ($p<0.05$) and significant effect of time ($p<0.05$) were observed based on two-way ANOVA with repeated measures (n=13 for saline, n=12 for nalfurafine). At 24 hr following pretreatment, a significant effect of time ($p<0.05$, inset) was observed, but no interaction or effect of pretreatment. Significance according to Bonferroni post-hoc test, as compared to pretest, is indicated by ** ($p<0.01$).

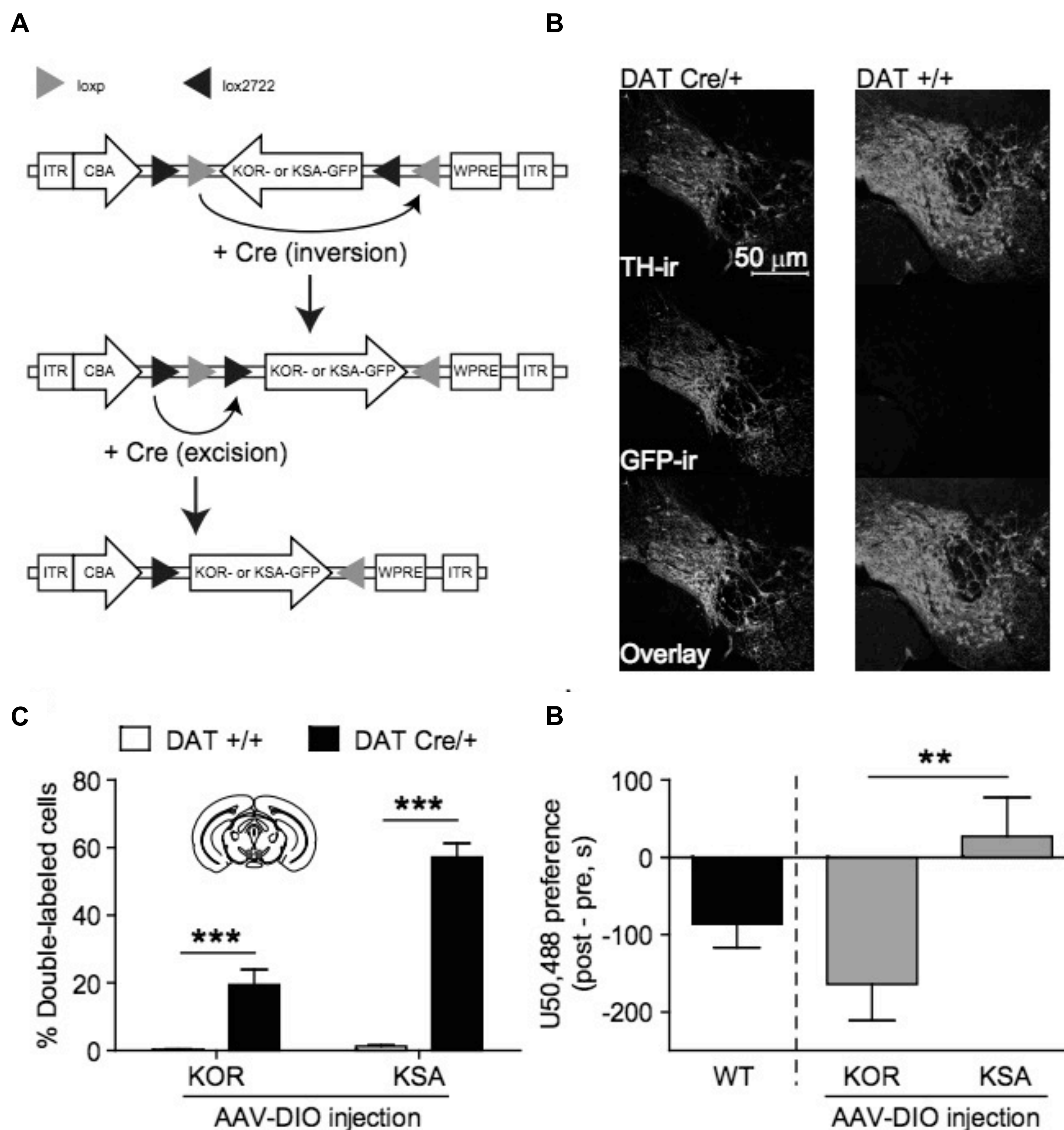

Figure 4.4 - KOR Activation Selectively in VTA Dopamine Neurons is Sufficient for KOR CPA.

A, Schematic shows the design of AAV-DIO viral construct, along with inversion of KOR GFP/KSA-GFP gene and excision of loxp/lox2722 sites in the presence of Cre. *B,* Representative IHC images showing DIO-KSA-GFP selectively expressed in VTA TH-ir neurons in $DAT^{Cre/+}$ but not $DAT^{+/+}$ animals after VTA injection (TH-r, red; GFP-ir, green; colabeling, yellow). *C,* Cell counts after VTA injection of DIO-KOR-GFP or DIO-KSA-GFP show a significant increase in cells double-labeled with TH-ir and GFP-ir in $DAT^{Cre/+}$ animals relative to $DAT^{+/+}$ littermates (n=7-16; ***, $p < 0.001$; Bonferroni post-hoc after two-way ANOVA). *D,* U50,488-CPA was selectively restored after injection of DIO-KOR-GFP into the VTA of $DAT^{Cre/+}$, $KOR^{-/-}$ mice, but not after injection of DIO-KSA-GFP (n=14-17; **, $p < 0.01$; t test).

Chapter 5. DISCUSSION

The data presented in this thesis expands our understanding of opioid receptor signaling, specifically in relation to classic side effects associated with therapeutic opioid use. Agonists targeting MOR are effective analgesics, but their clinical use is hindered by side effects, including tolerance and addiction. KOR agonists also produce analgesia, but clinical use of these compounds has remained minimal due to aversive properties in humans. The data presented in this thesis provides insight into the molecular mechanisms contributing to these side effects with the ultimate goal of developing improved opioid therapeutics.

C-JUN N-TERMINAL KINASE-MEDIATED OPIOID RECEPTOR INACTIVATION

Opioid receptors are phosphorylated on their C-tail by GRKs following agonist stimulation, resulting in arrestin recruitment and termination of receptor signaling. However, work in our lab and others has demonstrated that morphine-stimulated MOR does not engage this pathway. In addition to arrestin-mediated receptor inactivation, arrestins can also activate a separate set of signaling cascades. It is likely that individual agonists promote unique receptor conformations that result in distinct signaling events. Elucidating the precise molecular mechanism contributing to a specific behavioral outcome provides the framework for a better understanding of ligand-directed signaling events at GPCRs and provides better screening tools for the development of novel therapeutics.

Prior work demonstrated that in addition to the canonical GRK/arrestin-mediated mechanism of receptor desensitization, both MOR and KOR were capable of engaging a JNK-mediated pathway resulting in termination of receptor signaling (Bruchas, Yang, *et al.*, 2007; Melief *et al.*, 2010; 2011; Mittal *et al.*, 2012). The work presented in this thesis extends and

substantiates those findings by demonstrating that a similar mechanism regulates receptor desensitization in centrally-mediated pain circuits (Fig. 2.1). In the case of MOR, however, numerous other pathways have been identified as mediators of signaling termination, including PKC and Src (Kovoor *et al.*, 1998; J Zhang *et al.*, 1998; E Johnson, 2006; Bailey, Llorente, *et al.*, 2009; Bailey, Oldfield, *et al.*, 2009; Dang *et al.*, 2009). This thesis identifies a ligand-directed mechanism of JNK activation that can consolidate these findings into a single mechanism (summarized in Fig. 2.7), where morphine activates JNK2 through a PKC- and Src family kinase-dependent mechanism, resulting in receptor inactivation, whereas fentanyl activates JNK2 through a GRK3-, arrestin-2-, and Src-dependent mechanism and results in receptor inactivation through the canonical GRK3/arrestin-2-mediated pathway. The different arrestin-independent and -dependent mechanisms of JNK activation by morphine and fentanyl also provides an explanation for how both compounds are capable of activating JNK2 (Fig. 2.2) yet this JNK activation leads to distinct behavioral consequences, since arrestin in known to regulate cellular localization of signaling molecules (Tohgo *et al.*, 2003; Kobayashi *et al.*, 2005; Hanson *et al.*, 2007).

GPCRs can be categorized by their unique interactions with arrestin. One group of GPCRs, including MOR, preferentially binds arrestin-3 and forms more transient interactions with arrestin while the second group binds arrestin-2 and arrestin-3 with equal affinity and forms more stable complexes (Oakley *et al.*, 2000). It is thought that transient interactions tend to result in activation of MAPKs that translocate to the nucleus and regulate gene transcription through transcription factor regulation, whereas tighter arrestin and GPCR associations lead to retention of MAPKs in the cytosol (Tohgo *et al.*, 2003). This is not unique to arrestins, as numerous scaffolding molecules have been shown to regulate the subcellular localization of MAPKs,

including JNK (Garrington and Johnson, 1999). It is likely that the mechanistic differences in JNK activation between morphine and fentanyl lead to retention of JNK in distinct cellular compartments and ultimately distinct behavioral consequences of JNK activation. It is also possible that the lack of an effect of JNK inhibition on fentanyl tolerance arises from a steric block of the JNK substrate by arrestin in addition to or alternative to cytosolic sequestration.

It was hypothesized that fentanyl activated JNK3, as this isoform had previously been shown to be scaffolded and activated by arrestin-3 (McDonald *et al.*, 2000; Zhan *et al.*, 2013; 2014). Chapter 2, however, demonstrates that fentanyl promotes JNK2 activation through an arrestin-2- rather than arrestin-3-mediated mechanism, suggesting that arrestin-2 might also be capable of scaffolding JNK2. This would suggest that arrestin-2 might be capable of scaffolding all three JNK isoforms, similar to arrestin-3. It is likely that the arrestin scaffold specificity could lead to distinct recruitment of upstream activators and therefore different consequences of JNK activation (Garrington and Johnson, 1999), as the MAPK response is regulated by a unique combination of different MKKK–MKK–MAPK modules.

While Chapter 2 describes ligand-directed mechanisms of JNK activation at MOR, it was still unclear how JNK activation by morphine and norBNI ultimately resulted in receptor inactivation. We hypothesized that JNK activation phosphorylated a component of the receptor-signaling complex. This could occur through direct phosphorylation of the receptor or receptor signaling complex similar to GRK. Alternatively, JNK could mediate opioid receptor function through indirect changes, including changes in gene expression or modulation of downstream signaling cascades, resulting in receptor uncoupling. Results in Chapter 3 demonstrate that the JNK phospho-target mediating MOR and KOR desensitization is membrane delimited through use of a JNK kinase assay followed by measurement of opioid stimulated [^{35}S]GTPγS binding.

This establishes that JNK mediates opioid receptor inactivation through a direct mechanism. This substantiates the hypothesis that the JNK target directly alters receptor coupling and again highlights the critical role of JNK in this mechanism. Additional work in the lab has shown that both morphine and norBNI treatment result in a long-term increase in $G\alpha_i$ association with the receptor complex, although the receptor is in an inactivate state. The work presented in Chapter 3 combined with this finding has led to the current hypothesis that the JNK target mediating MOR and KOR inactivation is membrane bound and blocks the ability of the G protein to initiate exchange of GDP for GTP, resulting in long-term $G\alpha_i$ association with the receptor complex and signaling blockade.

Additional work in the lab has identified peroxiredoxin 6 (PRDX6) as a norBNI regulated $G\alpha_i$ interacting protein. Peroxiredoxins are antioxidants that can catalyze the removal of H_2O_2 from the cell (Rhee *et al.*, 2005) and PRDX6 is unique in that it also has phospholipase A2 (PLA2) activity. It has been shown that PRDX6 is capable of activating NADPH oxidases (NOX) (Fisher, 2011), which generate reactive oxygen species (Leavey *et al.*, 2002), and this can be mediated by MAPK phosphorylation and membrane localization of PRDX6 (Wu *et al.*, 2009; Chatterjee *et al.*, 2011). Cysteine is highly reactive in its thiolate form and therefore cysteines are a target of oxidative posttranslational modification (Reddie and Carroll, 2008; Roos and Messens, 2011; H Chung *et al.*, 2013). Additionally, cysteine oxidation prevents palmitoylation (Burgoyne *et al.*, 2012) since palmitoylation requires cysteines in their reactive deprotonated thiolate form (Dietrich and Ungermann, 2004). Therefore, we have further expanded our hypothesis and believe that PRDX6 is modulated by JNK activation, which leads to an accumulation of reactive oxygen species at the cell membrane. This results in oxidation of cysteine thiols on $G\alpha_i$, leading to decreased palmitoylation and an inhibition of G protein

function despite increased receptor association (Wedegaertner *et al.*, 1995). This hypothesis requires additional validation but represents a plausible mechanism of JNK regulated opioid receptor inactivation. Palmitoylation of $G\alpha_i$ in this context has not yet been explored, but it is known that this posttranslational lipid modification regulates GPCR signaling, both enhancing and dampening it (Resh, 2006). This novel pathway also might represent a unique therapeutic target capable of reducing tolerance; for example use of an antioxidant in combination with opioid therapy.

These findings further elucidate a novel mechanism of opioid receptor regulation. While the work described focuses on opioid receptor signaling, it is likely that JNK also results in a similar desensitization mechanism across the GPCR family. Additionally, regulation of G protein expression and function has been implicated in tolerance to other drugs of abuse (Kitanaka *et al.*, 2008). Therefore, understanding this mechanism sheds new light of GPCR pharmacology and is important not only for the development of novel opioids with reduced risk of tolerance, but for compounds targeting other GPCRs where receptor desensitization limits clinical utility.

IN SEARCH OF A NON-AVERSIVE κ-OPIOID RECEPTOR ANALGESIC

Chapter 4 describes a series of studies investigating KOR-dependent p38-mediated aversion to help inform the development of novel KOR analgesics, which could have a reduced addiction potential compared with more commonly prescribed MOR analgesics. Numerous studies have demonstrated that p38 signaling results in KOR-mediated aversion and this signaling pathway is downstream of GRK/arrestin (Bruchas *et al.*, 2006; Bruchas, Land, *et al.*, 2007; Land *et al.*, 2008; 2009; Bruchas *et al.*, 2011). Therefore, identifying a G biased ligand would eliminate aversive side effects.

Both pentazocine and nalfurafine have been used clinically and represent unique compounds that can act as a base for future drug development. However, the studies described in Chapter 4.1 and 4.2 demonstrate that an amino acid difference in hKOR and rKOR can result in different agonist potencies for p38 activation dependent on species. This is an important cautionary note for future development of KOR analgesics that avoid dysphoria, since animal models might not accurately predict the side effects found in humans. Additionally, Figure 4.1 demonstrates that the analgesic properties of pentazocine are MOR-mediated and development of this compound is likely to be hampered by the same side effects currently limiting the use of MOR analgesics. Therefore, pentazocine is not a good candidate scaffold for future KOR analgesic development.

Nalfurafine represents a more promising partially selective compound. Chapter 4.2 demonstrates that nalfurafine is a highly G biased agonist both at hKOR and rKOR in contrast to pentazocine. Pentazocine is more efficacious and potent for p38 activation in hKOR-expressing HEK293 cells, but was G protein biased in rKOR expressing HEK293 cells. Nalfurafine analgesia is KOR-mediated, although much lower doses are required for an analgesic response in comparison to U50,488. Additionally, nalfurafine produces acute tolerance but this effect is resolved after 24 hours, suggesting that nalfurafine does not engage a long-term JNK-mediated mechanism of receptor desensitization. Since p38 plays an important role in the aversive effects of KOR agonists, the G protein bias of nalfurafine may help explain the reported lack of aversive side-effects to nalfurafine in clinical trials.

The KOR ligand 6'GNTI has been reported to be G protein biased, but is a peripherally restricted partial agonist (Rives *et al.*, 2012). In contrast, nalfurafine is a full KOR agonist indicating that partial agonism is not required for signaling bias. While the selectivity of

nalfurafine for KOR is not optimal for therapeutic use, it is a centrally-active and G protein biased agonist. Therefore, nalfurafine can provide a base for future analgesic drug development and testing of nalfurafine analogs can provide insight into the structural basis of signaling bias at KOR.

Although identifying compounds that do not activate p38 remains the goal of KOR analgesic development, this thesis also helps explain the underlying circuitry that contributes to KOR-mediated aversion and dysphoria (Pfeiffer *et al.*, 1986; Shippenberg and Herz, 1986). The prevailing hypothesis was that this behavior resulted from an inhibition of dopamine signaling, specifically in the nucleus accumbens, in animal models (Di Chiara and Imperato, 1988; Spanagel *et al.*, 1990; Tejeda *et al.*, 2012; Carroll and Carlezon, 2013). However, this hypothesis was based on correlative data and dopamine receptor antagonists are not consistently aversive. Previous work also identified a serotonergic nucleus, the dorsal raphe, as a key brain region involved in p38-dependent KOR-mediated aversion (Land *et al.*, 2009; Bruchas *et al.*, 2011). Additionally, it was demonstrated that p38 activation results in increased membrane expression of the serotonin reuptake transporter (SERT), leading to a hyposerotonergic state in the ventral striatum (Schindler et al., 2012). KOR and p38 activation is necessary for aversion in both classes of neurons (Land *et al.*, 2009; Bruchas *et al.*, 2011) and in the prefrontal cortex (Tejeda *et al.*, 2013). Through development of a Cre-inducible KOR and KSA virus, Chapter 4.3 demonstrates that p38 activation in the VTA is also sufficient to induce KOR-mediated aversion. This implies that aversion results from KOR activation of p38 at multiple sites in this neuronal circuit and aversion can be disrupted by perturbing any portion of this circuit. KOR activation in different brain regions might also mediate different types of aversion, resulting from anhedonia, depression, dysphoria, or anxiety. Future experiments could focus on distinguishing between

these different states to determine if each is modulated by KOR and p38 activation in specific circuits. This work expands our understanding of the circuits and cell types involved in this behavior and demonstrates the importance of both serotonergic and dopaminergic cells. Altogether, the data presented in Chapter 4 furthers our understanding of KOR-mediated aversion and the work presented can inform future studies focused on developing novel therapeutics.

Conclusions and Future Directions

Opioids remain a mainstay of pain treatment and pain is a component of virtually all clinical pathologies. Therefore, it is critical to better understand opioid receptor function so novel therapeutics can be developed. Additionally, since opioids are members of the rhodopsin family of GPCRs, understanding opioid receptor signaling can shed light on other GPCR systems as well. My work has identified JNK as a key modulator of morphine-induced tolerance in novel pain circuits, substantiating the concept that this novel mechanism of opioid GPCR inactivation is evident in numerous cells types and circuits. My work describes novel mechanisms of ligand-directed JNK activation to demonstrate that JNK mediated MOR inactivation results from distinct upstream signaling mechanisms involving PKC and Src (Fig. 2.7). Finally, this thesis demonstrates that JNK directly targets the MOR and KOR receptor signaling complex. In addition to JNK mediated receptor inactivation resulting in tolerance, this work has also added to our understanding of G biased ligands targeting KOR, which might prove to be useful therapeutics in the clinic that avoid dysphoria in humans. The potential for KOR-mediated analgesia that is independent of the associated p38-mediated dysphoria and JNK-mediated inactivation is a target of ongoing research. Future work developing novel opioid therapeutics will benefit from the signaling mechanisms described in this thesis. GPCRs are the third largest

gene family in humans and the complexities of their signaling mechanisms continue to emerge, providing novel targets for therapeutics and a richer understanding of their ability to regulate such a diversity of physiological functions.

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Education

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Advisor: Charles Chavkin, Ph.D.

- Molecular Medicine Certificate
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Bachelor of Science: Neuroscience and Behavioral Biology, Minor: Japanese
Kansai Gaidai University, Osaka, Japan 8/2007-12/2007
Study Abroad Program, Japanese Studies

Research Experience

Graduate Student Researcher, *UW Dep. of Pharmacology*, Seattle, WA 9/2010-8/2015
Principal Investigator: Charles Chavkin, Ph.D.

- Investigated cellular mechanisms of morphine tolerance using cell culture models, biochemistry, and proteomics with the goal of designing new pain medications with reduced side effects

Research Technician, *Emory University, Dep. of Pharmacology*, Atlanta, GA 6/2009-8/2010
Principal Investigator: Raymond Dingledine, Ph.D.

- Independently designed and executed experiments to investigate the role of specific proteins in learning and memory before and after seizures

Student Assistant, *Yerkes National Primate Research Center*, Atlanta, GA 8/2008-9/2008
Principal Investigator: Michael Davis, Ph.D.

- Ran behavioral tests to investigate how cocaine affects startle in rats

Teaching Experience

Teaching Assistant, *University of Washington School of Medicine*, Seattle, WA 9/2011-3/2012
Department of Pharmacology, General Pharmacology I/II

- Organized weekly quiz sections for pharmacy students to review course material and prepare for exams

Teaching Assistant, *Emory University*, Atlanta, GA 1/2007-5/2007
Department of Biology, Introduction to Biology Lab

- Helped undergraduates understand lab procedures and answered questions regarding lab material and concepts.

Publications (née Levin)

Kuhar JR, Bedini A, Melief EJ, Chiu YC, Striegel HN, Chavkin C (2015). Mu opioid receptor stimulation activates c-Jun N-Terminal kinase 2 by distinct arrestin-dependent and independent mechanisms. *Cellular Signaling*. 27(9): 1799-1806.

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Kuhar JR, Schattauer SS, Land BB, Chavkin C. (2015) G protein-coupled receptor kinase (GRK) regulation of opioid receptors. *In preparation*.

Chavkin C, Schattauer SS, **Levin JR**. (2014) Arrestin-mediated activation of p38 MAPK: molecular mechanisms and behavioral consequences. *Handb Exp Pharmacol*. 219: 281-92.

Schattauer SS, Miyatake M, Shankar H, Zietz C, **Levin JR**, Liu-Chen LY, Gurevich VV, Rieder MJ, Chavkin C. (2012). Ligand directed signaling differences between rodent and human κ-opioid receptors. *Journal of Biological Chemistry*. 287: 41595-607.

Levin JR*, Serrano G*, Dingledine R (2012). Reduction in delayed mortality and subtle improvement in retrograde memory performance in pilocarpine-treated mice with conditional neuronal deletion of cyclooxygenase-2 gene. *Epilepsia.* 53(8):1411-20. *co-first author

Abstracts and Presentations

Kuhar JR, Schattauer SS, Melief EJ, Chavkin C (2015). c-Jun N-terminal Kinase Mediated Inactivation of Opioid Receptors. Kappa Therapeutics Conference (p*oster presentation).*

Bedini A, **Kuhar JR,** Melief EJ, Chavkin C (2014). Ligand-directed signaling at mu opioid receptors: differential mechanisms of MAPK activation by morphine and fentanyl. International Narcotics Research Conference. (p*oster presentation).*

Kuhar JR, Bedini A, Melief EJ, Chiu YC, Striegel HN, Chavkin C (2014). Arrestin-Dependent and -Independent Mechanisms of Mu Opioid Receptor Inactivation. Society for Neuroscience. (*poster presentation*).

Levin JR, Schattauer SS, Melief EJ, Groblewski PA, Chavkin C (2013). JNK Dependent Mechanism of Opioid Receptor Inactivation. Kappa Therapeutics Conference (p*oster presentation).*

Levin JR, Schattauer SS, Melief EJ, Groblewski PA, Chavkin C (2013). Distinct JNK and GRK3 Dependent Mechanisms of Mu and Kappa Opioid Receptor Inactivation. ASPET GPCR Colloquium (*poster presentation*).

Levin JR, Melief EJ, Groblewski PA, Chavkin C (2012). JNK and GRK3 Dependent Mechanisms of Ligand-Directed Opioid Receptor Inactivation. Symposium on Molecular Pharmacology (*poster presentation)*.

Levin JR (2010). Understanding the Effects of Epilepsy on Learning and Memory Using Transgenic Mice. Georgetown Day High School (*oral presentation)*.

Additional Experience

Market Analyst, *UW Coulter Translational Research Partnership*, Seattle, WA 1/2015-8/2015

- Assessed the market for submitted proposals to inform funding decisions
- Managed funded teams and oversaw commercialization strategies

Technology Licensing Fellow, *UW CoMotion,* Seattle, WA 7/2013-12/2014

- Analyzed commercial potential of technologies disclosed to CoMotion and presented findings in a weekly written report, which influenced next steps taken by the inventor and UW

Additional Projects and Committees

President; VP Relations*, UW Science and Engineering Business Association* 9/2012-8/2015

- Coordinated events and volunteer opportunities to teach business skills to science and engineering students (e.g. SEBA Career Fair [$100,000 annual revenue] and the Foster Business Plan Competition)
- Promoted and developed relationships between SEBA and the Seattle business community

Committee Member, *Steering Committee, Life Science Innovation Northwest* 9/2014-7/2015

Volunteer, *Life Science Strategy Project, WA State Department of Commerce* 9/2014-Present

Committee Member, *Commercialization Committee, Washington Biotechnology & Biomedical Association* 9/2014-7/2015

Chair; External Relations; Marketing Director, *UW Bioscience Careers* 6/2011-6/2014

- Coordinated monthly seminars featuring professionals with Ph.D.s in nonacademic careers to expose graduate students and post-doctoral fellows to career options
- Managed a $10,000 budget and 12 person committee responsible for operating the seminar series

Executive Committee Member, UW *Molecular Medicine Certificate Program* 5/2012-3/2014

- Planned the development and implementation of the Molecular Medicine Ph.D. program
- Acted as student liaison for the Molecular Medicine Certificate Program

Honors

2015 HHMI/UW Molecular Medicine Scholar
7/1/2011- 6/30/2014 NIDA Predoctoral Training Award T32-DA07278