Novel KOR Antidepressant and Analgesic Strategies

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Abstract

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The goal of this thesis has been to gain a better understanding of the mechanisms involved in depression and pain. These disorders are highly prevalent in our society and present a great burden on the individuals affected by them, the healthcare system, and the economy. Although current therapeutics have demonstrated effectiveness against these symptoms, many patients experience treatment resistance to first line antidepressants and increased risk for abuse of prescribed opiates. The kappa opioid receptor (KOR) presents an alternative therapeutic target, as it is a key mediator of the stress response and has been extensively studied in both animal models and clinical trials investigating pain and depression. Further, activation of this receptor by the endogenous stress peptide dynorphin produces signaling through both the G proteins and β-arrestin to produce both analgesia and dysphoria, respectively. Although KOR-directed therapeutics are currently being developed, more research is necessary before they are ready to compete with currently prescribed treatments. This thesis begins by describing two key issues in the use of KOR therapeutics by identifying

both A) sexually dimorphic signaling in response to KOR activation mediated by the presence of estrogen in female mice; and B) a novel administration schedule of the receptor-inactivating KOR antagonist norBNI, producing long-lasting inactivation of KOR at dramatically lower doses than previously used. The results of these studies will aid in the design of both KOR-directed drugs and clinical trials in the treatment of pain and depression. The effects of stress mediated activation of KOR on the serotonin system are also investigated**.** First, it is demonstrated that repeated forced swim stress significantly increases serotonin transporter reuptake rates as mediated by translocation to the plasma membrane in the ventral striatum. Lastly, stress-induced changes in gene expression in the dorsal raphe nucleus are examined utilizing RiboTag protocols. RNAseq was used to analyze changes in this population and the effect of pretreating animals with a KOR antagonist was evaluated. The results of these studies identify novel risk factors and targets in the development of kappa therapeutics for the treatment of pain and depression.

Dedication

To my family: Marty, Marilyn, Dan, and Sunny

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Chapter 1

Introduction

1.1 Introduction to anxiety, depression, addiction and the role of the kappa opioid receptor

More than twenty percent of adults in the United States will suffer from a mood disorder in their lifetime, with 85% of all cases producing moderate to severe impairment in the afflicted patients (Kessler 2005). Comorbidity of mood disorders is extremely common, with depression and anxiety disorder comorbidity measured at 50-70% (Ishak 2014; Zhou 2017). The occurrence of these disorders produce extensive behavioral symptoms including cognitive, emotional, motivational, and physiological manifestations.

Interestingly, many of these disorders also produce deficits in reward signaling and response to positive emotional stimuli. One of the most common forms of these deficits is anhedonia, which is the loss of the ability to experience pleasure from rewarding stimuli such as food, sex, and social interactions and is a prominent symptom in major depressive disorder. This change in reward signaling is likely responsible for high prevalence of substance abuse comorbidity, which was estimated at over 20% (Conway 2006). The same study estimates that a staggering 40% of patients suffering from substance abuse were comorbid for a mood disorder, suggesting a high degree of overlap in the systems responsible for these disorders. Comorbid substance abuse disorders are associated with significantly worse outcomes in patients with both depression and bipolar disorder (Agosti 2006) and it is estimated that about 50% of all patients

with depressive disorders exhibit treatment resistant symptoms (Culpepper 2010). With the economic burden of depressive disorders costing $200 billion in the U.S. alone, improved treatments are imperative.

Extensive research has demonstrated that the dynorphin/kappa opioid receptor (KOR) system plays an important role in regulating the emotional state. Dynorphins are a class of peptides produced from the precursor prodynorphin. The precursor protein is cleaved by proprotein convertase 2, producing seven dynorphin peptides including dynorphin A, dynorphin B, and α- and β-neoendorphin (Merg 2006). Dynorphins have a significantly higher affinity for KOR than for μ or δ opioid receptors, with dynorphin A having the highest affinity for KOR. Unlike classical neurotransmitters (i.e. GABA and glutamate), neuropeptides like dynorphin and the other endogenous opioids are released from large dense core vesicles in response to sustained activation at both synaptic and extrasynaptic sites. Once released, neuropeptides can diffuse relatively long distances, as compared to neurotransmitters, and produce broad regional activation of their receptors (Drake 1994). Thus, neuropeptide signaling enables coordinated activation or inhibition across broad networks of neurons. These characteristics should be distinguished from the faster, classical neurotransmitter signaling produced by an acute release of these molecules from the presynaptic terminal and activation of receptors at the postsynaptic target, limiting communication to a single pair of neurons. These differences allow neuropeptides to act as neuromodulatory signals which produce a broad response to repeated activation without affecting information processing at existing circuits during conditions of high neuronal activity, including stress.

KORs are G protein-coupled receptors which signal with the inhibitory $G_{\alpha i}$ protein. Once bound by its endogenous ligand, KOR signaling produces inhibition of adenylyl cyclase, increased current through inwardly rectifying potassium channels, and decreased calcium conductance (Grudt and Williams 1995). These G protein-mediated signals produce cellular inhibition and prevent neurotransmitter release. Additionally, KOR agonism activates mitogen activated protein kinase (MAPK) pathways which modulate a variety of cellular functions including proliferation, differentiation, and gene expression. The combination of effects on ion channels and MAPK signaling allow KOR to produce both rapid changes in neuronal signaling responsible for the acute effects of stress and delayed alterations in gene transcription responsible for the effects of chronic stress (Knoll and Carlezon 2010).

It is important to acknowledge the strong evidence suggesting that GPCRs are capable of taking on multiple conformations, depending on their ligand binding state and association with other proteins in the signaling complex (Urban 2007). The conformational state of GPCRs, including KOR, confer differences in functional selectivity for specific stimulus response. This phenomenon has potential utility in the therapeutic targeting of KOR to produce a more restricted range of pharmacological responses, as this and other GPCRs are capable of transducing an array of downstream signals. As described above, KOR activation allows for release of the Gβγ subunit which goes on to activate inwardly rectifying potassium channels and inhibit voltage-gated calcium channels to prevent depolarization of the cell. Meanwhile,

the G_α subunit inhibits cellular activity by decreasing adenylyl cyclase activity. These mechanisms of neuronal inhibition are responsible for the robust analgesic effects of KOR agonists. Additionally, $G_{\beta/\gamma}$ activation promotes the phosphorylation of the extracellular signal-regulated kinase ERK1/2 and c-Jun-N-terminal kinase (JNK). Previously, JNK activation has been implicated in environmental stress, inflammation pathways, cytokine activation, and animal models of pain (Minden and Karen 1998). Phosphorylated JNK goes on to activate the transcription factor c-Jun, creating a mechanism for stress-mediated changes in gene transcription.

In addition to G protein-mediated signaling, activation of KOR can also lead to β-arrestin-dependent signaling. Here, agonist binding results in G protein-coupled receptor kinase 3 (GRK3) phosphorylation of the KOR carboxyl-terminal and subsequent recruitment of β-arrestin to the receptor (Cheng 1998). β-arrestin has been shown to activate a "late phase" ERK1/2 phosphorylation capable phosphorylating the transcription factor CREB in the nucleus accumbens of stressed mice (McLennan 2008; Bruchas 2008). Interestingly, previous research has also implicated phospho-ERK1/2 in the activation of CREB and the transcription factor has been demonstrated to tightly regulate dynorphin expression (Carlezon 2005). KOR recruitment of β-arrestin has also been shown to activate p38 mitogen activated protein kinase (MAPK), a kinase known to be activated by environmental stress and inflammation. Previously p38 MAPK was demonstrated to be critical for producing cytokines and modulating chronic pain (Ashwell 2006). More recently, our lab has shown that KOR activation leads to GRK3 phosphorylation of

p38 MAPK and that this kinase is necessary for KOR-mediated aversion behaviors in mice (Bruchas 2006). While the KOR agonist U50,488 produces robust condition place aversion in mice, pretreatment with the selective p38 inhibitor SB203580 significantly blocks the KOR-mediated aversion (Bruchas 2007). Further, KOR-activated p38 MAPK in the dorsal raphe nucleus has been demonstrated to be necessary for both aversive behaviors and translocation of the serotonin transporter, suggesting the kinase plays a critical role in modulating the serotonin system in response to stress (Land 2009; Schindler 2012).

The distinct G protein-mediated and β-arrestin-mediated pathways provide an example for how ligand directed signaling, also referred to as biased agonism, may be utilized to design unique therapeutically effective molecules. As discussed previously, binding of different ligands produces different conformational changes in the kappa receptor which promote activation of one or both downstream signals. Although the endogenous ligand dynorphin promotes both the G protein-mediated and β-arrestin-mediated pathways, researchers have been designing drugs that selectively activate the analgesic G protein pathway. These drugs are being designed to produce robust opiate analgesia without altering the patient's mood. Unlike traditional opiates that produces both powerful pain relief either euphoric or dysphoric effects, molecules designed to specifically activate KOR-mediated G protein-signaling represent an important step in combating the current opioid epidemic in the U.S. In contrast, drugs that are currently described as long-lasting KOR antagonists may also be considered biased agonists as they activate c-Jun N-terminal kinase in a mechanism that leads to inactivation of the receptor. This

class of drugs, as well as shorter acting antagonists, show promise for treating depression and addiction and will be elaborated on in chapter 3.

1.2 Animal models of stress and the methods used in this thesis to analyze them

Behavioral stress causes rapid release of both stress hormones (i.e. CRF, corticosterone, and glucocorticoids) and the stress peptide dynorphin (Marinelli and Piazza 2002). While agonism at all of the opioid receptors produces analgesia, only activation of the KOR produces an aversive state in humans and depressive-like behaviors in animal models (Wadenberg 2003; Mague 2003). These events in KOR signaling may serve as evolutionarily adaptive responses allowing animals to increase physical abilities (through analgesia) and increasing motivation to escape environmental dangers (through aversion). However, there is evidence suggesting that chronic stress may lead to maladaptive changes in behavior such as depressive-like and anxiety-like symptoms (Nestler and Carlezon 2006).

To further study the effects of repeated stress on KOR activation and downstream signaling, several studies in this thesis utilized a modified Porsolt forced swim stress paradigm that was previously demonstrated to induce release of dynorphin and activation of KOR (Porsolt 1977; Mague & Carlezon 2003). As previously discussed, agonism at kappa receptors produces analgesia through inhibition of synaptic transmission. This generally occurs in the well-defined pain circuits of the dorsal root ganglia, periaqueductal gray, sensory thalamus, and limbic regions (Gutstein 1998). Early studies of the stress response identified the phenomenon of

stress-induced analgesia as an adaptive response to increase survival ability (Maier 1980). Later, a role of KOR in stress-induced analgesia was suggested as administration of subanalgesic doses of the stress peptide dynorphin A prolonged the effect, while pretreatment with the antagonist norbinaltorphimine blocked the effect (Katoh 1990; Watkins 1992). To test the hypothesis that repeated stress recruits the dynorphin/KOR system to produce analgesic and depressive-live behavior, McLaughlin et al. (2003) subjected mice to a 2-day, modified Porsolt forced swim stress. In this study, mice were repeatedly swam four times on the second day, leading to increasing measures of immobility, a behavior associated with depressive-like symptoms. Interestingly, this effect was blocked in animals pretreated with the antagonist norBNI and in transgenic dynorphin knockout animals. Decreased immobility time in the forced swim assay is commonly used as a measure of efficacy when investigating the properties of selective serotonin reuptake inhibitors, suggesting norBNI may have strong antidepressant properties. Further, the repeated forced swim paradigm produced robust analgesia in the warm water tail withdrawal assay. This effect was also blocked by norBNI and transgenic knockout of dynorphin. These experiments demonstrate that the forced swim stress protocol used produces significant increases in immobility and stress-induced analgesia. The same repeated forced swim paradigm was used throughout this thesis to provide a model for robust, stress-induced KOR activation in order to further study the actions of dynorphin at its receptor and discover molecular pathways that may provide insight into development of novel therapeutics for pain, depression, and addiction.

The warm water tail flick nociception assay used to measure KOR-mediated analgesia above provides a quick and reproducible measure of pain sensitivity in animals. In this thermal latency task, the tip of an animal's tail is placed into warm water and the time it takes for the animal to flick or withdraw its tail is recorded (Janssen 1963). A couple of concerns with this assay have been discussed in the literature, including altered response due to learned avoidance behavior and restraint stress from researcher handling (Berge 1988; Deuis 2017). These are valid concerns for this assay and analysis of the behavior of control mice was used to determine the effect of these confounds on the accuracy and reproducibility of these data. Discussed further in chapter 3, repeated injections of saline and repeated measurement of tail flick analgesia using this assay over the course of thirty days produced remarkably consistent data. This is likely because any stress the animals experience from restraint is very brief, usually lasting under ten seconds. Further, inter-researcher variance among our lab members is minimal with only slight variance in the magnitude of responses. Overall, this assay has consistently proven to be an efficient and reliable measure of KOR signaling and KOR-mediated analgesia.

Critical to the study outlined in chapter five, the RiboTag transgenic mice and methods for cell-type specific RNA isolation from complex tissue provide a novel method for examining changes in the ribosome-associated transcriptome (Sanz 2009). This technology utilizes transgenic mice expressing a ribosomal protein (RPL-22) with the final exon flanked by LoxP recombination sites and an additional exon containing a triple hemagglutinin (HA) tag. Using cell-type specific Cre recombinase driver lines (Pet1-Cre in these studies), we are able to remove the final wild-type

exon of RPL-22 and express this epitope-tagged exon in distinct populations of cells. During tissue processing, incubation with monoclonal antibody against HA allows for immunoprecipitation of the ribosome-associated transcriptomes from the specific cell type. This technique is especially useful when investigating changes in gene expression in heterogeneous brain regions like the dorsal raphe nucleus as different and allows researchers to identify gene expression profiles unique to a particular cell type, whereas this change may be lost when measuring differences in expression across all cell populations.

Previous methods have achieved cell-type specific isolation of RNA from heterogenous samples, but each have significant drawbacks compared to the RiboTag approach. Fluorescence-activated cell sorting (FACS) techniques, laser capture micro-dissection, and aspiration of patched cells after electrophysiological recording allow for sequestration of target cells, but each of these methods require expensive equipment and/or complex techniques (Lobo 2006; Yao 2005). Additionally, many of these approaches introduce experimental noise due to tissue fixation, dissociation, or extraction of cells from tissue (Heiman 2014). In comparison, RiboTag is performed on tissue *in situ*, providing a higher degree of sensitivity and allowing for extraction of mRNA from the whole cell, not just the soma. Another advantage of RiboTag is that it identifies the translating mRNA profile in a sample, which more closely matches the protein content and functional changes induced by an experimental group as compared to the total RNA expression profile. Overall, RiboTag provides a powerful method for examining gene expression changes in complex tissues when combined with qPCR and RNA sequencing. Chapter five will further elaborate the methods and capability of this technique, providing a

genome wide analysis of transcriptional changes induced by stress and the significance of KOR signaling in regard to these changes.

1.3 Investigation of KOR system towards improved therapeutics

The implications for the dynorphin/KOR system in pain pathways and various disorders including generalized anxiety, depression, and substance abuse suggest that it could be an important therapeutic target in treating these disorders. As the research described above clearly demonstrates, this system is responsible for integrating the aversive component of stress and pretreatment of KOR antagonists significantly block these signals in animal models (Land 2008). Furthermore, if these stress signals are the cause of psychiatric illness, then successfully inhibiting them may prove useful in also preventing the onset of these disorders. In comparison to current treatments for mood disorders, KOR antagonists have demonstrated a combination of both acute antidepressant-like and anxiolytic-like effects, whereas reuptake inhibitor antidepressants have demonstrated acute anxiogenic effects. Lastly, several clinical trials have demonstrated both the utility and the adverse side effects of targeting KOR in the treatment of depression and substance abuse. With substantial evidence implicating KOR in psychiatric conditions, the aim of the studies in this thesis is to further investigate the downstream effects of KOR signaling in order to identify novel therapeutic targets and design improved therapeutic models for treating depression and substance abuse.

Chapter 2

Estrogen inactivates downstream signaling of KOR

(adapted from Abraham AD, Schattauer SS, Reichard KL, **Cohen JH**, Fontaine HM, Song AJ, Johnson S, Land BB, and Chavkin C. "Estrogen regulation of GRK2 inactivates kappa opioid receptor signaling mediating analgesia, but not aversion." Submitted to J. Neuro.)

All qPCR data for this manuscript was generated by me. I also performed ventral striatum dissections and tissue prep for all samples used to explore kinase phosphorylation and coimmunoprecipitation with the help of Dr. Selena Schattauer.

Introduction

The need for safer treatments for chronic pain is clear from the current crisis in opioid abuse and the growing number of overdose deaths in the United States (Volkow and Collins, 2017). G biased kappa opioid receptor (KOR) agonists are potentially safer analgesics because they lack the addictive properties of mu opioid receptor agonists and lack the dysphoric effects of non-biased KOR agonists (Shippenberg and Herz, 1986; Pfeiffer et al., 1986; Bruchas and Chavkin, 2010; Chavkin and Koob, 2016; Brust et al., 2016). The analgesic and antipruritic effects of KOR agonists occur via G protein-mediated signaling (Land et al. 2009, Schattauer et al., 2017), whereas the dysphoric properties of KOR agonists are dependent on G protein-coupled receptor kinase 3 (GRK3)/p38 MAPK and β-arrestin activation (Land et al., 2009; Ehrich et al., 2015). This dissociation between the analgesic and dysphoric properties of KOR actions has stimulated the development of G protein-biased KOR agonists (Brust et al., 2016) and some are in clinical trials for pain and itch disorders (Eisenach et al., 2003; Delvaux et al., 2004).

Chronic pain disorders are more prevalent in women (Berkley, 1997), but KOR agonists have low or inconsistent analgesic efficacy in female rodents (Mogil et al., 2003; Stoffel et al., 2005; Craft, 2008). Female C57BL/6J and DBA/2J mice are significantly less sensitive to the analgesic effects of KOR agonists compared to males (Mogil et al., 2003). Dysphoric effects of prototypical KOR agonists (i.e. U50,488) are present in males and female rodents (Chartoff and Mavrikaki, 2015), but females have dose- and species-dependent differences in reactivity to the anhedonic and aversive effects of KOR agonists. Female rats show decreased KOR agonist-mediated suppression of intracranial self-stimulation (ICSS; Russell et al., 2014), whereas female California mice show conditioned place aversion to a lower dose of U50,488 compared to males (Robles et al., 2014). These affective changes produced by KOR agonists are hypothesized to be estrous cycle-independent (Russell et al., 2014), but KOR-mediated analgesia has been shown to be modulated by estradiol (Mogil et al., 2003). The present study was designed to identify the intracellular signaling mechanisms responsible for sexually dimorphic analgesic responses to opioid activation with the goal of providing insights to guide the development of better analgesics.

Here, we confirm that KOR activation produced conditioned place aversion and potentiated cocaine conditioned place preference in both male and female C57BL/6N mice, but analgesic effects of KOR activation were attenuated in female mice. The analgesic effects of KOR activation were found to be estradiol-sensitive in female mice. We then demonstrated that activation of the estradiol sensitive G protein-coupled receptor kinase 2 (GRK2) in females increased sequestration of G_{α} signaling and reduced effects of KOR activation on intracellular

signaling, inhibition of dopamine release, and opioid agonist-mediated analgesia. Together, these studies identify an estradiol-mediated intracellular signaling mechanism leading to sexually dimorphic responses that may generalize to other G protein-coupled receptors (GPCRs) acting through Gαi/o protein signaling.

Methods

Isolation of proteins

Mice were decapitated at the specified times following drug administration. Ventral striata, periacqueductal gray (midbrain), and spinal cord were dissected and homogenized with a Dounce homogenizer on ice in MAPK lysis buffer composed of 50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 10% Glycerol, protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail (Millipore). For analysis of GRK2, lysis buffer was supplemented with 1% triton-X100. Samples were centrifuged 25 min at 30,000g at 4°C. Protein concentrations in supernatant were determined by bicinchoninic colorimetric assay (Thermo-Fisher), mixed with 4x LDS (Thermo-Fisher) supplemented with 8% β-mercaptoethanol and stored at -20°C until analysis by western blot.

Quantitative RT-PCR analysis of KOR and GRK2 mRNA levels

Mice were decapitated, and ventral striata were dissected on ice. RNA was isolated using the Qiashredder and RNeasy Plus Mini kits (Qiagen). RNA was amplified on a Stratagene Mx3000P (Agilent) using the Thermo Power SYBR Green RNA-to-CT™ 1-Step Kit (Thermo-Fisher) according to manufacturer instructions, with the standard cycle settings: 50°C 30 min, 95°C 5 min, 40x

(95°C 15 sec, 62°C 1 min), followed by a dissociation curve ramping from 95°C to 55°C and back to 95°C. For KOR, the following primers were used: GTGGGCTTAGTGGGCAATTCT, AGTGGTAGTAACCAAAGCATCTG. For GRK2, the following primers were used: GCGCCAGCAAGAAGATCCT, GCAGAAGTCCCGGAAAAGCA. Peptidylprolyl isomerase A (PPIA) was selected as a reference gene after experiments demonstrated no difference in expression between
male and female mice, using the following primers: CACCGTGTTCTTCGACATCA and CAGTGCTCAGAGCTCGAAAGT. All samples were run in duplicate (20 ng RNA) with a standard curve on each plate. Data were analyzed with the MxPro v3.20 software (Agilent). Transcript levels were calculated from the standard curve, with KOR and GRK2 transcript levels then normalized to PPIA transcript levels.

Western blot analysis

Samples were loaded (30 µg protein for phospho-ERK1/2 or 40 µg protein for phospho-p38 MAPK) onto 10% Bis-Tris precast gels (Thermo-Fisher) and running at 100 V for 2 hr. Blots were transferred to nitrocellulose (Fisher-Scientific) for 1.5 hr at 30 V. The nitrocellulose was blocked with 5% BSA-TBS 1 hr at room temperature and stained overnight at 4 °C for phospho-ERK1/2 (Cell Signaling #cs9101, lot 28) and ERK2 (Santa Cruz #sc1647, 1:2000), phospho-p38 (Cell Signaling #cs9211, lot 23, 1:1000) and p38α (Cell Signaling #cs9228, lot 2, 1:1000), or phospho-GRK2(S670) (Sigma-Aldrich #MABS155, lot 2792488, 1:250) and GRK2 (Sigma-Aldrich #SAB4500592, lot 218278, 1:500) in 5% BSA-TBS. Blots were incubated in IRdye secondary (Li-Cor Biosciences #926-68070 lot C50721-05 and #926-32211 lot C506602-05, 1:10,000) in 1:1

Odyssey buffer (Li-Cor) and 5% milk-TBS 1 hr at room temperature then scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences). Band intensity was measured using the Odyssey software and expressed as phospho-immunoreactive protein intensity over the homologous protein band intensity. For total protein quantification, protein was normalized to Revert protein stain (Thermo-Fisher). Data were normalized to percentage of control sample (saline, 100%). The Gβ antibody detected a single band at 37 kD. ERK1/2 and phospho-ERK1/2 antibodies each detected two bands at 42 and 44 kD. The antibody for p38α identified a single band at the predicted weight of ~42 kD. In addition to the expected band, the phospho-p38 antibody also detected nonspecific bands at ~45 kD; only the band overlapping with the p38α band was quantified (Bruchas et al., 2007b). The GRK2 antibody detected bands at the predicted weight of ~80 kD and at ~55 kD, while the phospho-GRK2 antibody detected multiple nonspecific bands but a single band at ~80 kD; the ~80 kD band was identified as the specific band by GRK2 overexpression in HEK293 cells combined with Phorbol-12-myristate-13-acetate (PMA) treatments (Pitcher et al., 1999).

Ovariectomy

Ovaries were removed from female mice under isoflurane anesthesia (Smith et al., 2005). Mice were allowed 3 weeks for recovery from surgery prior to behavioral or biochemical experiments. For estradiol replacement experiments, mice received a priming injection of 50 µg/kg estradiol 3 days prior to testing, then a second injection of 50 µg/kg estradiol 60 min prior to behavioral testing or tissue collection (Green et al., 1970).

Coimmunoprecipitation

Mice were decapitated at the specified times following drug administration. Ventral striata and spinal cord were dissected and homogenized with a Dounce homogenizer on ice in coimmunoprecipitation buffer (20 mM Tris, 135 mM NaCl, 0.5 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1% triton-x100, 10% glycerol, protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail (Millipore), pH 7.5). 700 µg of protein was incubated overnight at 4°C with 5 µl GRK2 antibody (Cell Signaling #cs3982, lot 1) prior to incubation for 2 hr with 25 ul washed magnetic protein A/G beads(Thermo-Fisher) at 4°C. Samples were washed twice with buffer prior to elution at room temperature with 40 ul 4x LDS. Samples were loaded onto 4-12% Bis-Tris precast gels (Thermo-Fisher) and analyzed as described above, blotting for GRK2 (as above) and Gβ (Santa-Cruz #sc-378, lot C0515, 1:500) Gβ band intensity was normalized to GRK2 band intensity.

Results

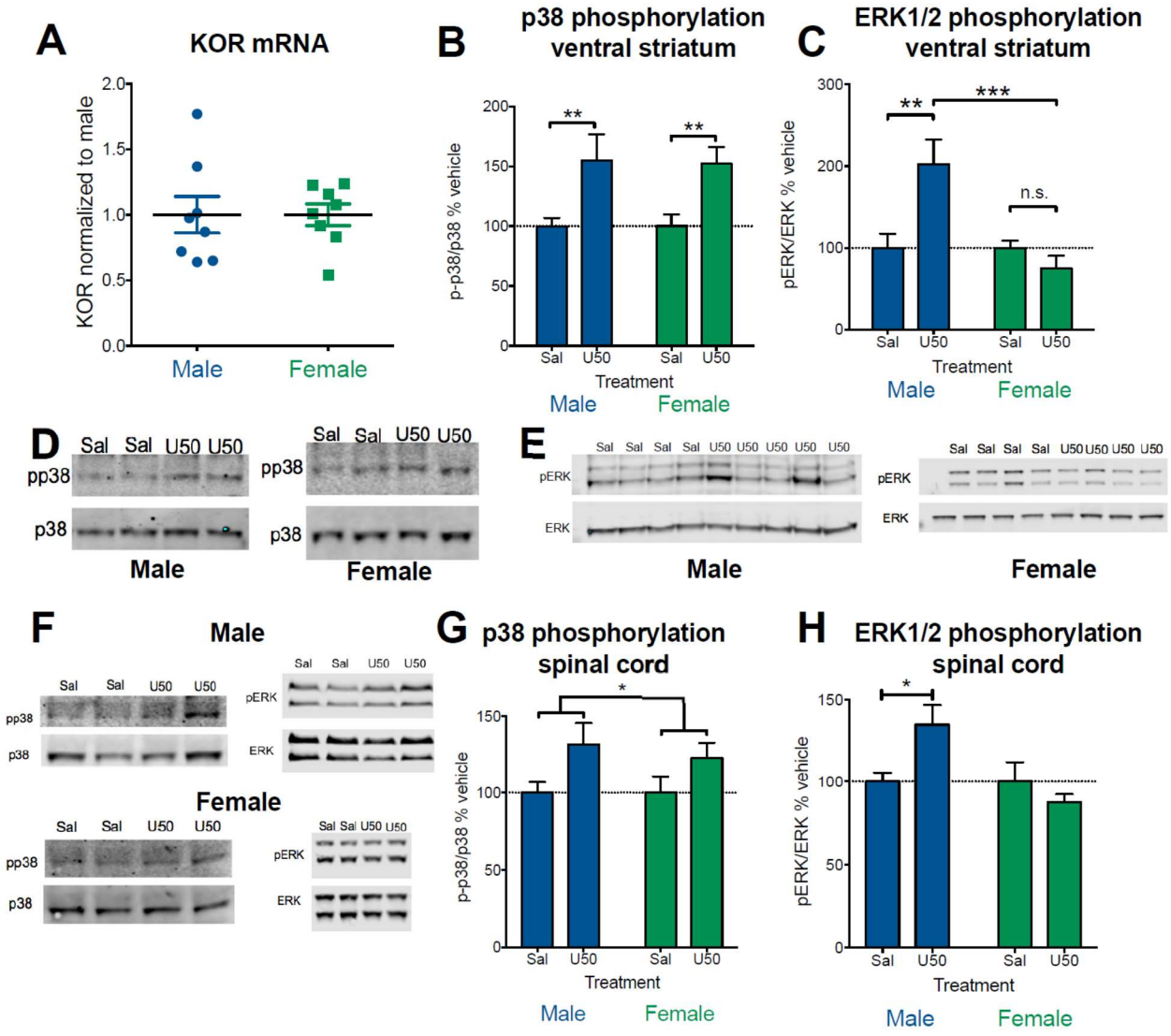
KOR activation produces p38 MAPK phosphorylation in males and females, but not ERK 1/2 phosphorylation in females.

To determine whether differences in KOR signaling could be due to altered basal expression of KOR, we measured levels of KOR mRNA in males and females using quantitative RT-PCR in striatal homogenates. We found no significant differences in male and female KOR mRNA in the striatum (Fig. 2-1A). Based on our behavioral data, we hypothesized that KOR activation should produce equivalent arrestin-mediated signals in males and females, but G protein-mediated

signals should not be present in females. Western blot analysis demonstrated that there was a significant effect of KOR activation on phosphorylation of striatal p38 MAPK ($F(1,34) = 12.35$, $p = 0.001$), with no effect of sex and no interaction (Fig. 2-1B). In contrast, there was no main effect of KOR agonist on striatal extracellular regulated kinase 1/2 (ERK1/2) phosphorylation (Fig. 2-1C) but there was an interaction between sex and KOR agonist ($F(3,37) = 4.98$, $p = 0.005$). There was no significant difference in total ERK1/2 (males = 102±9%; females = 98±7% of average expression) or total p38 (males = 112±17%; females = 88±17% of average expression) protein levels. Post hoc analyses showed that ERK1/2 phosphorylation in males treated with KOR agonist were significantly different from saline-treated males ($p = 0.007$) and from females treated with KOR agonist ($p = 0.0003$); whereas U50,488 treatment did not significantly increase striatal pERK in females (Fig. 2-1C). Representative Western blots for 3A-3C are shown in 3D-3E. Representative blots for 3G-3H are shown in 3F. Western blot analysis of spinal cord homogenates 30 min following systemic U50,488 (10 mg/kg) administration showed increased p38 phosphorylation in spinal cord in males and females ($F(1,30) = 5.97$, $p = 0.021$), with no significant effect of sex or interaction between sex and treatment (Fig. 3G). U50,488 significantly increased pERK in males, but not females at 30 min, with a significant interaction between sex and treatment ($F(1,29) = 14.38$, $p = 0.019$). Post hoc analysis showed that saline-treated males were significantly different from U50,488-treated males ($p = 0.038$). There was no significant effect on pERK at 15 min in males or females, suggesting an altered time course for KOR signaling in the spinal cord compared to brain tissue. In midbrain samples targeted to dissect the periaqueductal gray, a region known to mediate opioid analgesia, p38 phosphorylation (n = 7-9) at 30 min was non-significantly increased in males (132±22% of

average expression) compared to females (104±20% of average expression). Similar to p38 phosphorylation, pERK (n = 4-5) at 15 min was also non-significantly increased in males (144±21% of average expression) compared to females (74±20% of average expression) in the midbrain.

Figure 2-1



KOR activation produces p38 MAPK phosphorylation in males and females, but not ERK 1/2 phosphorylation in females. (A) Striatal mRNA from male and female mice was isolated and

analyzed for KOR expression by qRT-PCR. No difference in KOR expression was observed (n=8 per group) (B-C). Mice were injected with U50,488 (10 mg/kg, i.p.) or saline 15 or 30 min prior to isolating striatal proteins. Phosphorylation of p38 (B) and ERK1/2 phosphorylation (C) in ventral striatum of male and female mice by western blot analysis. (B) U50,488 induced p38 phosphorylation in male and female mice. There was a significant effect of U50,488 ($p<0.001$), with no difference between males (n=11) and females (n=8). (C) U50,488 induced ERK1/2 phosphorylation ($p<0.001$) in male (n=5-6) but not female (n=8) mice, and males were significantly different from females ($p < 0.001$). (D) Representative blots for (B). (E) Representative blots for (C). (F) Representative blots for (G) and (H). (G) U50,488 induced p38 phosphorylation in male and female mice (n=7-9 per group) in spinal cord homogenates (Bracket indicates main effect of treatment; $p<0.05$). (H) U50,488 induced ERK phosphorylation in male (n = 7-9 per group) but not female (n = 9 per group) mice ($p < 0.05$). Error bars indicate SEM.*$p<0.05$, **$p<0.01$; ***$p<0.001$, n.s., not significant.

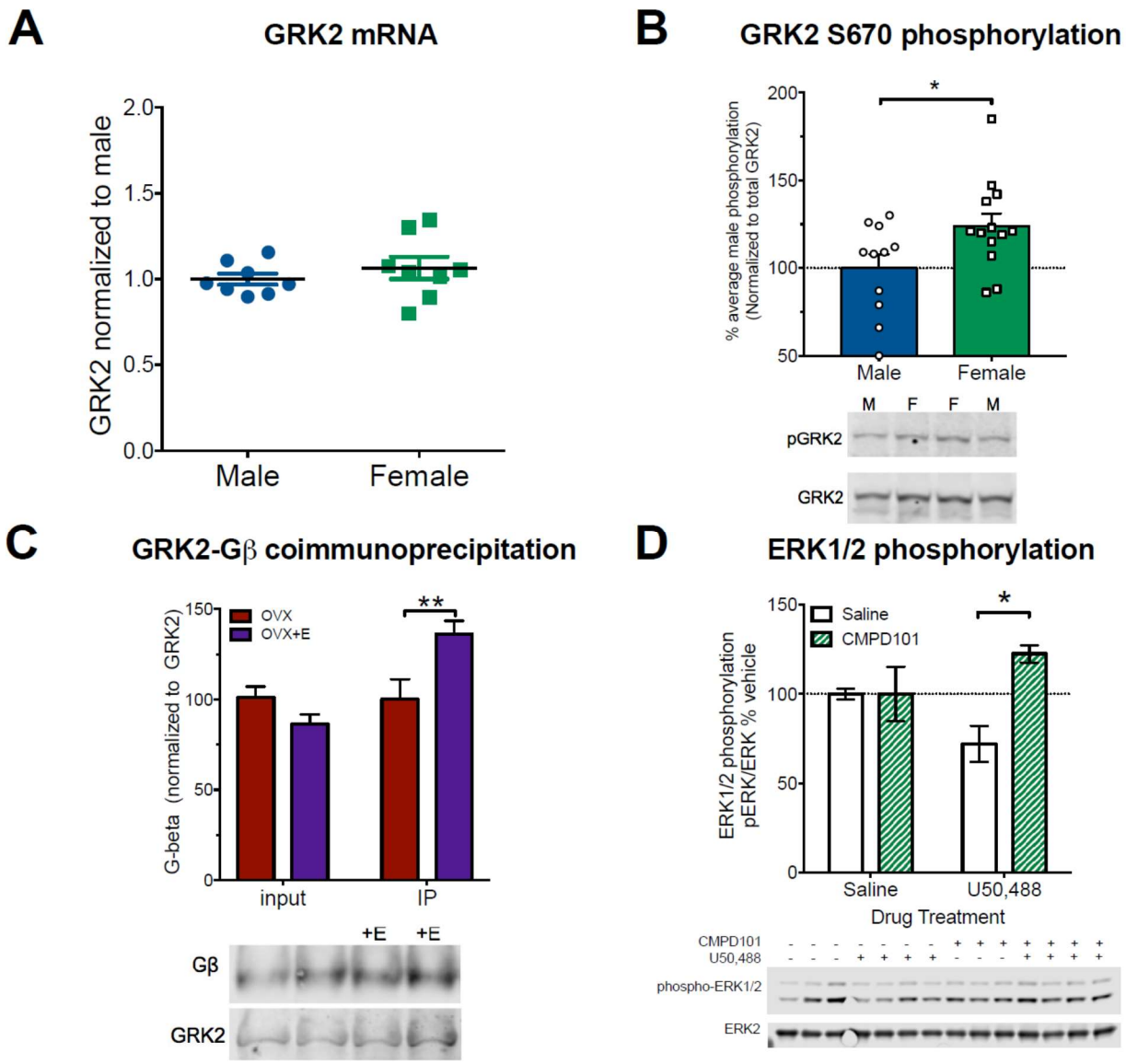
GRK2 sequesters Gβ/γ to decrease KOR-mediated ERK phosphorylation in females.

G protein-coupled receptor kinase 2 (GRK2) can be modulated by estradiol via phosphorylation of the S670 GRK2 site (Dominguez et al., 2009) and GRK2 can sequester components of signaling pathways upstream from ERK1/2 phosphorylation, such as Gβ/γ and MEK (Daaka et al., 1997; Pitcher et al., 1999; Raveh et al., 2010). GRK2 activity can blunt KOR-mediated effects in cardiac tissue (Chen et al., 2017), suggesting that there may be direct interactions between GRK2 and KOR in the brain. Using quantitative RT-PCR in striatal homogenates, we determined that total GRK2 levels were not significantly different between males and females (Fig. 2-2A). However, phosphorylation of the estrogen target S670 site of GRK2 was significantly greater in females ($t(22) = 2.64$, $p = 0.034$) compared to males (Fig. 2-2B), and there was an estradiol-sensitive increase in GRK2 association with Gβ in ovariectomized females (Fig. 2-2C). There was a significant effect of GRK2 immunoprecipitation compared to input ($F(1,13) = 10.01$, $p = 0.004$) and a significant interaction between sample group and estradiol treatment ($F(1,13) = 10.74$, $p = 0.003$). Post hoc analysis showed that estradiol treatment significantly increased ($p = 0.005$)

co-immunoprecipitation between GRK2 and Gβ/γ in the immunoprecipitate, but not input sample. A similar effect was observed in spinal cord (n = 3-5), with a non-significant increase in GRK2 association with Gβ/γ in the immunoprecipitate following estradiol treatment (142±19% of average expression) compared to the input sample treated with estradiol (104±19% of average expression), suggesting that GRK2 could modulate KOR signaling in the spinal cord.

Gβ/γ regulates ERK phosphorylation via phosphoinositide 3-kinase γ (PI3Kγ) and Ras activity (Lopez-Ilasca et al., 1997; Belcheva et al., 1998), and we hypothesized that inhibition of GRK2 would reveal G protein-mediated effects of KOR activation in females. We first tested whether the selective GRK2/3 inhibitor CMPD101 (Thal et al., 2011) (1.25 mg/kg; Tocris Bioscience) could unmask KOR-mediated ERK phosphorylation in females (Fig. 2-2D). There was a significant effect of sex ($F(1,11) = 8.64$, $p = 0.014$), and an interaction between sex and drug treatment ($F(1,11) = 8.64$, $p = 0.0135$). KOR activation in females treated 30 min after CMPD101 significantly increased ERK phosphorylation ($p = 0.002$) compared to females pre-treated with saline. These results suggested that KOR/Gβγ-mediated intracellular signaling effects in the striatum were evident in females following GRK2/3 inhibition.

Figure 2-2



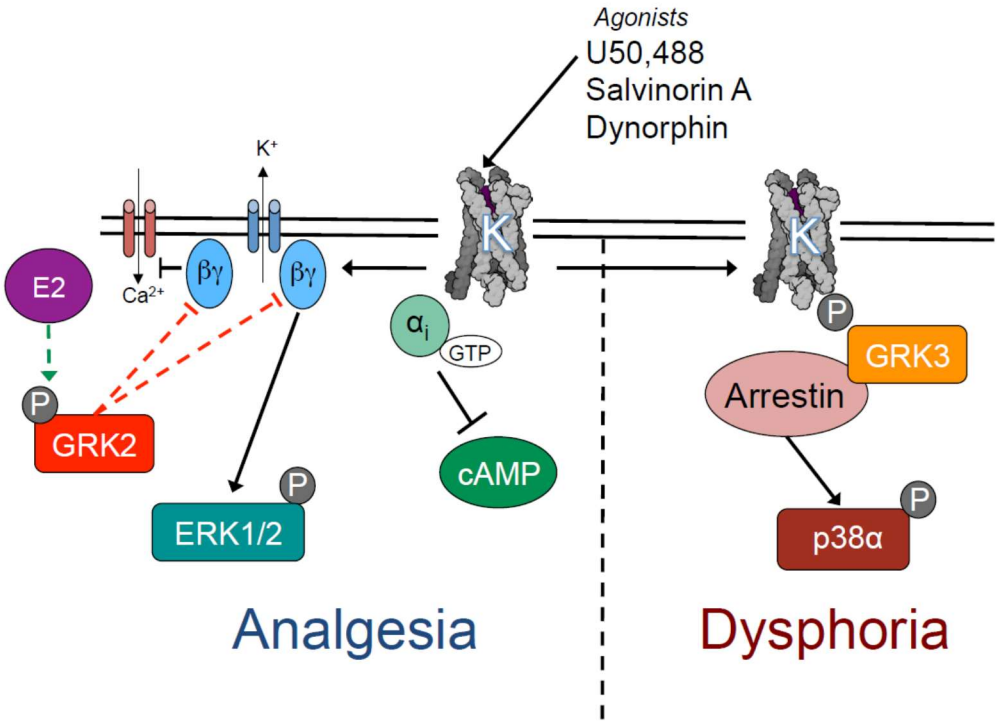
GRK2 sequesters Gβ/γ to decrease KOR-mediated ERK phosphorylation in females. (*A*) Striatal mRNA from male and female mice was isolated and analyzed for GRK2 expression. No difference in GRK2 expression was observed (n=8 per group). (*B*) Striatal protein from male and female mice was isolated and analyzed for GRK2 phosphorylation at Ser670 by western blot. Phosphorylation of Ser670 was significantly elevated in female mice, compared to male mice ($p < 0.05$, n=11-13). (*C*) GRK2 was immunoprecipitated from striatal homogenates of ovariectomized female mice treated with vehicle or estradiol (50 µg/kg; 60-min prior to tissue collection). Samples were then immunoblotted for GRK2 and Gβ. No differences were observed in Gβ in total protein, but estradiol increased Gβ in GRK2 immunoprecipitates ($p<0.005$, n = 8-10). (*D*) Female mice were treated with CMPD101 (1.25 mg/kg) 30 min prior to U50,488 (10

mg/kg, 15 min). Striatal proteins were isolated and analyzed for ERK1/2 phosphorylation (n = 3-4). CMPD101 pretreatment significantly ($p<0.01$) elevated ERK1/2 phosphorylation following U50,488.

Proposed model

These studies demonstrate that estradiol stimulates GRK2 phosphorylation to increase Gβ/γ sequestration and block G protein-mediated biochemical, circuit, and behavioral effects of opioid receptor activation (Schematic, Fig. 2-3).

Figure 2-3



Proposed model: Elevated estradiol (E2) in female mice results in GRK2 phosphorylation and enhanced sequestration of the Gβγ subunits. This results in reduced Gβγ signaling, including a reduction in analgesic responses. In contrast, GRK3/arrestin-mediated signaling is left largely intact, maintaining the aversive response.

Discussion

These studies demonstrate that females show blunted intracellular responses to the G protein-mediated, but not arrestin-mediated actions of KOR agonists. In particular, elevated estradiol was found to underlie the reduction in KOR function in females. Furthermore, we specifically identified estradiol upregulation of GRK2 activity to mediate the observed sex differences in opioid responses. We found that p38 MAPK phosphorylation was increased in both B6 males and females following KOR activation. In contrast, G protein-mediated ERK1/2 phosphorylation was blunted following KOR activation in B6 females.

Few selective KOR agonists have been tested for sex differences in analgesic efficacy in humans (Coffin et al., 1996, Delvaux et al., 1999) but nonselective MOR/KOR agonists have been reported to produce greater analgesia in women than men (Gear et al., 1996). The discrepancy between rodent and human studies may be due to innate signaling differences in KOR function between rodents and humans (Schattauer et al., 2012; Broad et al., 2016) or synergistic interactions between MOR/KOR. In female rats, increased MOR/KOR heterodimerization in the spinal cord during periods of high estrogen or progesterone is associated with increased analgesic responses to intrathecal morphine administration (Chakrabarti et al., 2010; Liu et al., 2011; Liu et al., 2017). In addition to species differences, there may also be differences in spinal and supraspinal KOR responses (Lawson et al., 2010) that produce inconsistencies in KOR-mediated analgesia. Reviews of the literature describing analgesic effects of selective kappa opioid receptor agonists in rodents concluded that there were mixed effects of KOR agonists in

females (Craft, 2003; Fillingim and Gear, 2004; Rasakham and Liu-Chen, 2011), but the mechanisms underlying this variability were not previously known.

Mogil et al. (2003) showed that ovariectomy restored KOR-mediated analgesia and this effect was blocked by estradiol replacement. In agreement with Mogil et al. (2003), our results show that estradiol blunts KOR-mediated analgesia and suggests that reported inconsistencies in KOR-mediated analgesia in females may be due to differences in estrogen levels between study subjects. Progesterone may also alter KOR function in female rats, as systemic progesterone administration in gonadectomized or progesterone receptor antagonism alters analgesic responses to U50,488 (Stoffel et al., 2005; Liu et al., 2011). Changes in either or both of these hormones may underlie the analgesic effects we observed in pregnant female mice. The multiple sites of action underlying sex differences in KOR analgesia (Chakrabarti et al., 2010; Mogil et al., 2003), indicates that there are complex interactions between agonist dose, administration route, rodent species, and behavioral assays for pain that could be explored in future studies to determine optimal treatment strategies in humans.

Estradiol can produce complex effects on mood and behavior through estrogen receptor activation or interactions with G protein-coupled receptor signaling (Martinez et al., 2016; Gillies and MacArthur, 2010). Based on the observation that KOR activation did not produce analgesia or ERK phosphorylation, we hypothesized that an estradiol-regulated signaling system that controls G protein-biased signals was likely to be a mechanistic target for sexually dimorphic responses. G protein-coupled receptor kinase 2 (GRK2) is phosphorylated following

estradiol treatment, likely through estrogen receptor α (Dominguez et al., 2009), sequesters Gβ/γ subunits (Lodowski et al., 2003), and decreases ERK activation (Pitcher et al., 1999). GRK2 activity also desensitizes KOR activity in cardiac tissue (Chen et al., 2017), suggesting that estradiol modulation of GRK2 activity could underlie sex differences in KOR activity. Our results showed that in females, GRK2 phosphorylation is increased, and there was no change in GRK2 mRNA levels. We also observed increased association of GRK2 with Gβ/γ subunits following estradiol treatment in ovariectomized females, indicating that estradiol increased GRK2 sequestration of Gβ/γ to blunt G protein-mediated signals.

We found that pharmacological inhibition of GRK2 with compound 101 (CMPD101) attenuated sex differences following KOR activation. CMPD101 is a selective inhibitor for members of the GRK2 subfamily (GRK2 and GRK3), with little activity at GRK1 or GRK5 (Thal et al., 2011). To ensure that the effects that we observed were selective to GRK2, we tested the effect of CMPD101 in female GRK3 KO mice and found that the CMPD101-mediated increase in KOR analgesia was not GRK3 dependent. Genetic deletion of GRK2 in mice causes death during embryonic development (Jaber et al., 1996), preventing an assessment of CMPD101 in GRK2 knockout mice. In addition to sequestration of Gβ/γ, GRK2 can also interact with mitogen-activated protein kinase kinase (MEK) to inhibit phosphorylation of ERK, suggesting an additional level of regulation over GPCR signaling that may contribute to our observed GRK2-mediated effects (Jurado-Pueyo 2008). GRK2 has also been shown to desensitize melanocortin-1 receptor (Sánchez-Más et al., 2005), which may contribute to sex-dependent analgesic effects

of KOR agonists (Mogil et al., 2003). Together, these studies showed that GRK2 inhibition is sufficient to decrease KOR-mediated sex differences.

Our studies demonstrate that KOR activation produces distinct effects in males and females in an estradiol- and GRK2-regulated manner. G protein-biased KOR analgesic compounds that are in clinical development may have sex-dependent effects, and careful consideration of estradiol interactions with opioid receptor actions is required for the implementation of biased KOR agonists in clinical populations. Further understanding of the mechanisms underlying sexually dimorphic effects of opioid receptor activation could guide the development of highly efficacious non-addictive opioid drugs for analgesia in males and females.

Conclusions

This study demonstrates that although there is no difference in KOR mRNA levels in the ventral striatum of male and female mice, KOR agonism with U50,488 fails to increase the phosphorylation state of ERK1/2 in female mice. However, p38 MAPK is phosphorylated in both sexes in response to KOR activation. Further, GRK2 mRNA levels do not differ in the ventral striatum between sexes. GRK2 S670 residue phosphorylation is increased in females, an effect of estradiol previously described by Dominguez et al. (2009). GRK2 coimmunoprecipitation with the Gb protein was significantly increased in ovariectomized females injected with estradiol, suggesting that phosphorylated GRK2 interacts with Gβ/γ. Finally, pretreatment with the GRK2/3 inhibitor CMPD101 restores KOR-mediated ERK1/2

phosphorylation. These data suggest that GRK2 sequesters Gβ/γ to decrease KOR-mediated ERK phosphorylation in females.

Chapter 3

Developing a novel schedule for receptor-inactivating KOR antagonists to optimize therapeutic utility

Introduction

Kappa opioid receptors are widely expressed throughout the body and in brain regions implicated in the modulation of reward, emotional state, and cognitive function including the ventral tegmental area, nucleus accumbens, prefrontal cortex, striatum, dorsal raphe nucleus, and others (Simonin 1995). Further, stress-induced release of dynorphin and activation of the KOR has been extensively studied, demonstrating that this system promotes aversive, depressive-like behaviors in both animal and preclinical studies. This suggests that KOR antagonists may provide effective treatment for psychiatric conditions including stress, depression, and substance abuse (Carroll and Carlezon, 2013).

As discussed in chapter 2, KOR activation by agonists or the endogenous peptide dynorphin can activate two separate signaling pathways. Firstly, KOR agonism can phosphorylate GRK3, which recruits β-arrestin, leading to downstream activation of p38α MAPK (Bruchas 2006). Additionally, the G protein-mediated pathway is $G_{i/o}$ coupled, leading to the inhibition of adenylyl cyclase, increased conductance of potassium channels, and activation of ERK1/2 and c-Jun N-terminal kinase (JNK) (Belcheva 1998; Kam 2004). While the β-arrestin pathway has been

shown to modulate the aversive effects of KOR activation, the G protein activation is responsible for the receptor's analgesic effects (Bruchas 2010). Interestingly, KOR antagonists can also activate JNK through ligand-directed signaling (Bruchas 2007; Melief 2011). In this mechanism, a drug that acts as an antagonist to prevent activation of the receptor by typical agonists, is itself activating a specific downstream kinase in JNK.

Recently, our lab has identified a role for JNK recruitment of peroxiredoxin 6 to the plasma membrane upon KOR binding of the antagonist norBNI (Schattauer 2017). Here, the enzyme locally activates NADPH oxidase, stimulating the production of reactive oxygen species which oxidize the Gai subunit. The oxidation of the G protein causes its depalmitoylation and subsequent jamming of the GPCR complex in an inactive state. This method of KOR antagonism leaves the receptor system inactive for four weeks in vivo (Horan 1992; Bruchas 2007). In contrast, typical competitive, short-acting antagonists, like buprenorphine and CERC-501 (previously known as LY-2456302), occupy receptor binding sites to prevent agonist binding at KOR. These competitive antagonists have advanced the furthest in clinical trials because of their relatively short duration of activity. In fact, ALKS5461 (a 1:1 combination of buprenorphine and the mu antagonist samidorphan) is currently under FDA review for approval to treat reuptake-drug resistant depression. However, previous competitive antagonists have failed clinical trials because the dose administered was too conservative, leading to low clinical efficacy. Alternatively, other studies have administered drugs at a dose that produced toxic side effects, resulting in immediate termination of the trial (Buda 2015).

The mechanism of receptor-inactivating antagonists has been a topic of concern in approving clinical trials. While the effects of competitive antagonists are short lived, there is concern that receptor inactivating antagonists may produce prolonged toxicity and poor outcomes. To address this concern, our lab has developed a novel administration schedule for the long lasting KOR antagonist norBNI. In this study, we investigate the effect of daily administration of norBNI at doses 10-100 fold lower than used in acute antagonist studies. Our hypothesis is that lowering the dose of antagonist will avoid off target effects and that repeated norBNI administration will progressively inactivate enough receptors to produce high clinical efficacy. The results demonstrate that this novel schedule of receptor-inactivating antagonist successfully prevents KOR activation when administered as low as 0.1mg/kg and produces long lasting effects.

Methods

Subjects

Male and female C57BL/6N (B6) mice (n = 26) ranging from 8-16 weeks of age were used in these experiments. Breeding stocks were maintained in the UW Vivarium. All experimental procedures were approved by the University of Washington Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) "Principles of Laboratory Animal Care" (NIH Publication No. 86-23, revised 1985). All testing was during the light phase of the 14-h light/dark cycle.

Ovariectomy

Ovaries were removed from female mice under isoflurane anesthesia (Smith et al., 2005). Mice were allowed 3 weeks for recovery from surgery prior to behavioral experiments.

Drugs

norbinaltorphimine (norBNI) and U50,488 (NIDA Drug Delivery program) were dissolved in sterile saline (0.9%) at injected at 10mL/kg.

Tail-Flick Analgesia

Mice were administered norBNI or saline daily at the specified dose (0.1-10mg/kg, i.p.) and for the specified duration (1-30 days). 24hrs after the last norBNI injection, the response latency for the mouse to withdraw its tail from immersion into 52.5 +/- 0.05 °C water was measured using a stopwatch before and 30 min after U50,488 administration (10 mg/kg, i.p.). Data is expressed as maximum possible effect, normalized to time matched saline/U50,488 controls on the day of testing.

Statistics:

Group differences were determined using t-test or ANOVA and post hoc comparisons analyzed with Dunnett's multiple comparison test with α set to 0.05.

Results

Daily administration of low dose norBNI blocks U50,488-mediated analgesia

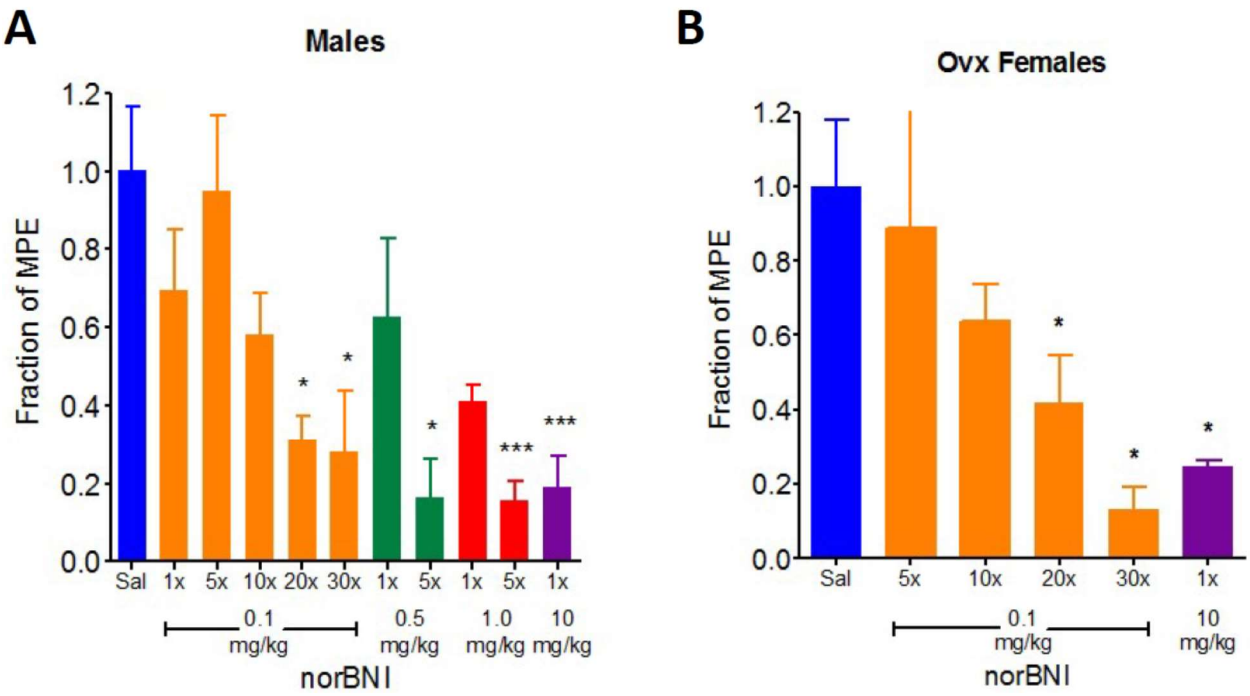
To determine if lower doses of the receptor inactivating KOR antagonist norBNI are effective, we designed a novel, daily administration schedule. norBNI or saline was injected i.p. daily at a volume of 10 mL/kg for a period of 1-30 days. On test days, KOR function was tested by observing U50,488-mediated analgesia in the warm water tail withdrawal test. Previous experiments have shown that a single dose of 10mg/kg norBNI 24 hours before testing provides a significant blockade of KOR-mediated analgesia as measured by this assay (Bruchas 2007). Here, we were able to replicate this effect of norBNI at 10mg/kg (Fig. 3-1A; $p < 0.001$). However, since Bruchas et al. (2007) also demonstrated that the effects of norBNI lasted up to 28 days, we wanted to know if repeated administration of the receptor inactivating antagonist would produce a cumulative effect over the course of an extended schedule.

We began by injecting animals with 1mg/kg or 0.5mg/kg norBNI (10-20 times lower dose than previous studies) 24 hours before measuring tail flick analgesia. Although there was not a significant block of KOR-mediated analgesia after a single injection, 5 daily injections were sufficient to produce a significant effect at both of these doses (Fig. 3A; $p < 0.001$ at 1mg/kg, $p < 0.05$ at 0.5mg/kg). These results suggested that the effect of daily antagonist administration could indeed be cumulative. Administering 0.1mg/kg norBNI (100 times lower than the dose previously used in this assay) we found no effect on U50,488 tail withdrawal latency after 1, 5,

or 10 injections. However, after 20 and 30 daily injections of antagonist at this low dose, we observed a significant blockade of KOR-mediated analgesia compared to saline injected control mice (Fig. 3A; $p < 0.05$). Overall, there was a significant effect of norBNI on tail flick analgesia with several administration schedules providing significant reduction (Fig3-1A; ANOVA $F = 3.599$, $p < 0.05$).

As outlined in chapter 2, estrogen-mediated sequestration of GRK2 inhibits downstream G protein signaling in response to KOR agonism. To overcome the effect of estrogen on ERK signaling and the analgesic effects of KOR activation, we used ovariectomized mice in this study. Similar to male mice, ovariectomized females also demonstrated significant blockade of U50,488 analgesia when pretreated with norBNI at the previously studied dose of 10mg/kg (Fig. 3-1B; t-test, $p < 0.05$). Female mice injected with 0.1mg/kg norBNI for 20 or 30 days also failed to show KOR-mediated analgesia, compared to saline injected control mice (Fig. 3-1B; t-test, $p < 0.05$). These results demonstrate that both male and female mice respond to extremely low doses of norBNI. Furthermore, because lower doses fail to produce KOR-mediated effects after a single injection, this suggests that a similar method of administration could mitigate off target effects of KOR therapeutics in clinical trials.

Figure 3-1



Daily administration of low dose norBNI blocks U50,488-mediated analgesia. (A-B) norBNI or saline were administered i.p. daily until 24 hours before tail withdrawal assay. Tail withdrawal latencies were measured before and after a 10mg/kg U50,488 injection. Data are reported as maximum possible effect of time and injection matched saline controls. (A) Males showed a significant effect of drug. A single injection of 10mg/kg and multiple injections of 1mg/kg, 0.5mg/kg, and 0.1mg/kg significantly reduced U50,488-mediated analgesia compared to saline control. (n = 4-9; ANOVA F = 3.599; *, P<0.05, ***, P<0.001 Dunnett's posthoc comparing all groups to saline control). (B) Female mice were ovariectomized to limit estrogen modulation of G protein signaling (Schattauer 2017). Ovariectomized females treated once with 10mg/kg or 20-30 times with 0.1mg/kg showed significantly less analgesia that saline controls. (n=5; t-test; *, P<0.05).

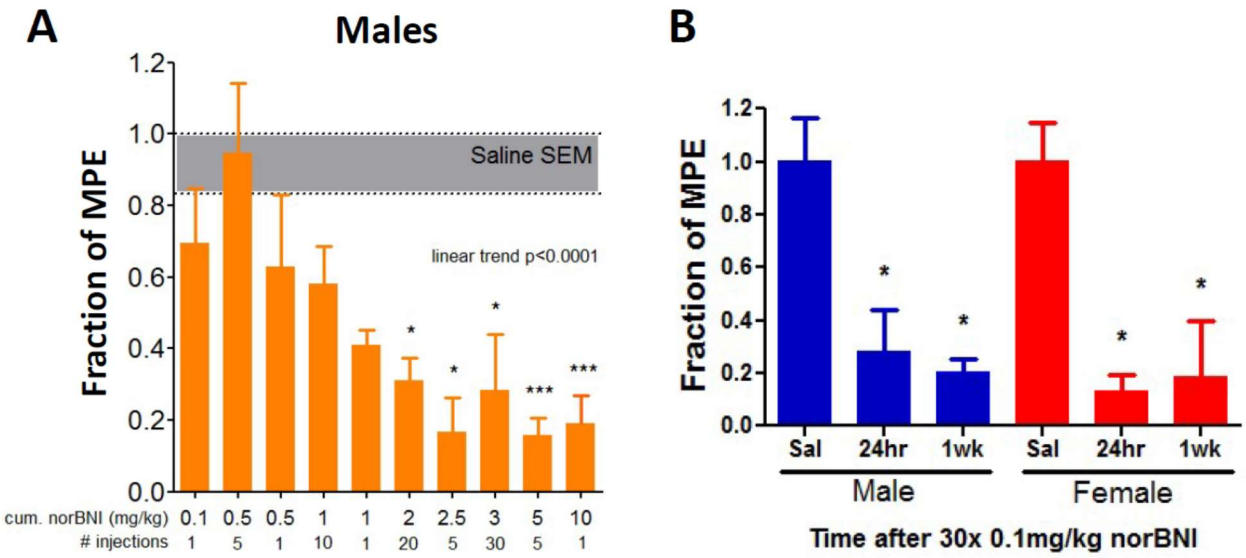
Daily administration of low dose norBNI produces dose-dependent, long lasting blockade or KOR-mediated analgesia

Since the effects of receptor inactivating antagonists persist for 28 days (Bruchas 2007), multiple doses in this period combine to produce a cumulative dose with similar effects to that of a larger single dose. When considering cumulative norBNI dose in this study, all

administration schedules amounting to doses over 2mg/kg produced significant block of U50,488 analgesia in the tail withdrawal assay (Fig. 3-2A; ANOVA $F = 3.599$, $p < 0.05$; Dunnett's post hoc $p < 0.05$ or $p < 0.001$). Additionally, post-hoc analysis determined there is a significant linear trend for decreased analgesic response with increasing cumulative dose norBNI (Fig. 3-2A; $p < 0.0001$).

The long lasting effects of norBNI were also seen drug administration was stopped. After the thirtieth day of 0.1mg/kg norBNI or saline injections, animals were allowed to rest in their home cages for 7 days without drug. On the eighth day, mice were tested again for KOR-mediated analgesia. Interestingly, mice receiving thirty days of norBNI injection remained insensitive to the analgesic properties of KOR activation by U50,488 (Fig. 3-2B; ANOVA $F = 3.599$, Dunnett's post hoc $p < 0.05$). These data suggest that norBNI inactivation of KOR is long lasting when administered at doses as low as 0.1mg/kg over the course of 30 days.

Figure 3-2



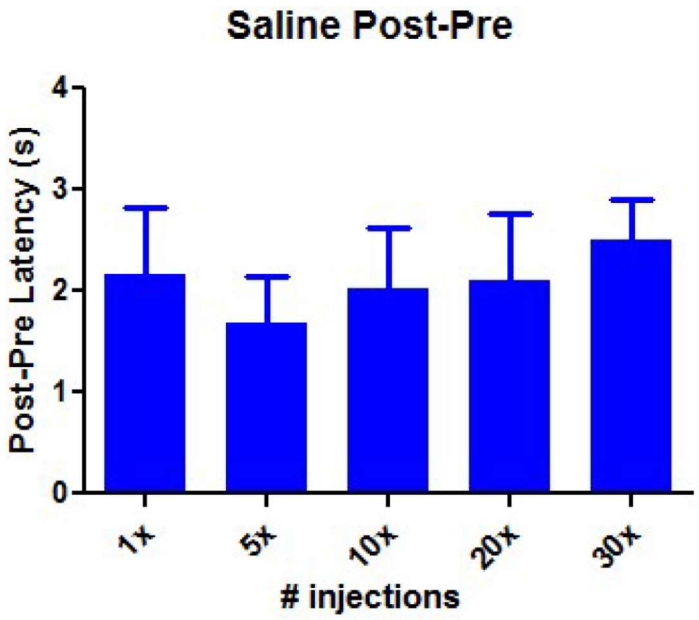
Daily administration of low dose norBNI produces dose-dependent, long lasting blockade or KOR-mediated analgesia. (A) Plotting data from Fig 3-2A as a function of cumulative dose. Males demonstrated a significant linear effect of dose on analgesic response in the tail withdrawal assay, with posthoc analysis showing that cumulative doses of norBNI 2mg/kg or higher produced blockade of U50,488 analgesia 24 hours after last injection. (n = 4-9; ANOVA F = 3.599; *, P<0.05, ***, P<0.001 Dunnett's posthoc comparing all groups to saline control, posthoc for linear trend comparing effect of increasing dose on analgesic response) (B) 30 daily injections of 0.1mg/kg norBNI produced long lasting receptor inactivation in both male and female mice as demonstrated by lack of U50,488 analgesia in these mice one week after the last norBNI injection. Data are reported as maximum possible effect of time and injection matched saline controls. (male data: n = 4-9; ANOVA F = 3.599; *, P<0.05, ***, P<0.001 Dunnett's posthoc comparing all groups to saline control; female data: n=5; t-test; *, P<0.05).

Daily saline injections do not affect the magnitude of U50,488 analgesia in the tail withdrawal assay

In order to confirm that repeated injections of saline do not alter the magnitude of analgesic response to KOR agonist U50,488 I have included this data here. For mice treated with saline, the latency to withdraw the tail from 52.5°C water after U50,488 minus the latency before KOR agonist was maintained at around two seconds for the duration of the experiment (Fig. 3-3; p =

0.909) . This is important to note because all data is normalized to saline controls in Figures 3-1 and 3-2 to aid comparisons. Had the magnitude of analgesia changed with increased saline injection, these comparisons would not be valid.

Figure 3-3



Daily saline injections do not affect the magnitude of U50,488 analgesia in the tail withdrawal assay. Raw analgesia scores for saline controls shows that normalizing to these animals does not bias data demonstrating long lasting norBNI blockade of KOR activation. (n = 4-9; ANOVA F = 0.245)

Discussion

These studies demonstrate that daily administration of norBNI at 10-100 fold lower than the typical dose inactivates kappa receptors in both male and ovariectomized female mice. In particular, administering the lowest dose of 0.1mg/kg norBNI i.p. daily for 30 days produced

significant reduction in U50,488-mediated analgesia as measured by the tail flick assay 24 hours after the last treatment. Additionally, these effects were demonstrated to be long lasting as mice receiving this schedule of norBNI administration maintained significant block of KOR agonist analgesia for at least one week after the last norBNI injection. Interestingly, these effects seem to be caused by a cumulative effect of drug dose, regardless of the amount of time required to achieve this dose (up to 30 days). Animals receiving a cumulative dose of norBNI amounting to at least 2mg/kg over the course of 1-30 days showed significant blockade of KOR activation, suggesting that repeated norBNI administration produces a long-lasting, additive effect on receptor inactivation. Although ovariectomized females were used in this study in order to overcome the inhibitory effect of estrogen on G protein mediated KOR analgesia, it is encouraging that these mice mimicked the long-lasting effect of norBNI that had previously been shown in males. Further studies must be done to probe the effectiveness of this schedule in preventing the aversive component of stress through KOR in both sexes.

The role of the dynorphin/KOR system on anxiety-, depressive-, and addiction-like behaviors in animals has been extensively studied. Early investigation of the potential therapeutic effects of KOR antagonists demonstrated that norBNI dose dependently decreases immobility in the forced swim test, a measure commonly associated with the effectiveness of antidepressants (Mague 2003). In addition to having antidepressant-like effects, the prototypical antagonists norBNI and JDTic also demonstrated anxiolytic-like effects as measured through open arm exploration in the elevated plus maze and decreased fear conditioning in the fear potentiated startle paradigm (Knoll 2007). The combination of antidepressant and anxiolytic characteristics

of these drugs suggest that KOR antagonists may be especially useful in treated those with comorbid depressive and anxiety disorders. Further, as stress and depression have been linked to relapse to drug abuse, the role of the dynorphin/KOR system on drug taking behaviors has also been investigated. The KOR antagonist JDTic was shown to prevent foot shock-induced stress reinstatement of drug seeking in rats previously trained to lever-press for cocaine, suggesting that KOR inhibition may attenuate the effects of anxiety or depression on relapse to drug use (Beardsley 2005).

The potential for KOR antagonists in the treatment of anxiety and depression related disorders is welcome in a field dominated by reuptake inhibitor drugs. Although this class of therapeutics has demonstrated good efficacy and large improvements over previous generations of antidepressants, there are distinct drawbacks that can be improved upon. Patients beginning SSRI treatment commonly report anxiogenic effects of the drug in the first week, leading to issues with compliance. Conversely, KOR antagonists demonstrate acute anxiolytic- and antidepressant-like effect in preclinical models and lack the initial anxiogenic side effect common to reuptake inhibitor treatment. Additionally, the stress-mitigating and long-lasting effects of these drugs suggest a potential for prophylactic treatment. Although predicting stressors is not always possible, the protective effects of KOR antagonists may be possible in soldiers going into combat or emergency response personnel. Additionally, a prophylactic could be useful in persons predetermined to be sensitive to the effects of stress (based on diagnosis or genetics), limiting the ability of these experiences to produce long-term psychiatric consequences.

These characteristics make KOR antagonists an intriguing improvement over existing treatments. Current studies investigating the use of KOR antagonists in clinical trials have produced mixed results. While GNTI and JDTic have demonstrated strong therapeutic effects in preclinical models of depression, anxiety, and drug abuse, their clinical value has been diminished by poor bioavailability and toxic effects in clinical studies (Mague 2003; Knoll 2007; Buda 2015). The mechanism by which JDTic produced ventricular tachycardia in clinical trials is still unknown. Additionally, ventricular tachycardia (VT) has been observed, at lower rates, in response to administration of the nonselective opioid antagonist naloxone (Hunter 2005) and the selective antagonist LY2456302 (Lowe 2014). Although KOR is expressed in heart tissue, the physiological effect of these receptors has not been identified, making it unclear whether the VT is produced by an off-target effect of JDTic and other opioid antagonists or a general property of the drug class. Further studies investigating the mechanism of KOR antagonism in cardiac tissue is necessary to elucidate the cause of VT seen in patients.

Interestingly, the nonselective KOR antagonist buprenorphine has been well tolerated and was demonstrated to be effective in clinical trials for the treatment of substance abuse, treatment resistant depression, social stress, and suicide (Montoya 2004; Karp 2014; Bershad 2015; Yovell 2015). Because buprenorphine also modulates the activity of mu opioid receptors, studies have investigated the drugs clinical effectiveness when administered with the mu antagonist samidorphan (Fava 2016). The 1:1 ratio of these drugs has been developed by Alkermes

(ALKS5461) and is currently under FDA approval for the treatment of reuptake inhibitor resistant depression.

Although pending approval of the short acting drug ALKS5461 represents a significant development in the field of KOR targeted therapeutics, the advantages of receptor inactivating KOR antagonists are another attractive prospect for improving current treatment options. This study demonstrates that daily administration of sub-therapeutic doses of a receptor inactivating KOR antagonist produces significant and long-lasting blockade of KOR activation by the agonist U50,488. This schedule of administration could be useful for future clinical studies, suggesting a dosage that is low enough to avoid any potential off target effects while also providing a cumulative therapeutic effect on kappa receptor inactivation. While this treatment schedule may affect the acute therapeutic efficacy of KOR antagonists, 1mg/kg administered once daily for just five days was sufficient to block behavioral effects of KOR activation, a period much shorter than the duration necessary for the therapeutic effect of SSRIs. Further studies demonstrating the effect of this schedule on depression and anxiety behaviors will be necessary to demonstrate that this method of KOR inactivation prevents downstream p38 activation/aversion in both males and intact females. It is understandable that clinical trials have focused on short-acting, competitive KOR antagonists to maintain the ability to reverse unanticipated side effects, but the novel administration method in this study suggests a potentially safer way to administer receptor inactivating antagonists.

Conclusions

This study demonstrates that daily administration of the long-lasting kappa antagonist norBNI at low doses effectively blocks KOR-mediated analgesia. Specifically, the lowest dose of 0.1mg/kg administered for 30 days significantly inhibited U50,488 induced analgesia measured by the tail flick assay in both male and ovariectomized female mice. Further, this effect lasted for a minimum of 7 days in both sexes. Analyzing the effect of cumulative norBNI dose, regardless of the amount of injections, demonstrated a significant linear trend. These results suggest a novel schedule of administration for receptor-inactivating KOR antagonists that has the potential to produce robust receptor inhibition and low risk of off target effects.

Chapter 4

Stress activation of dyn/KOR system increases p38-mediated SERT reuptake in the ventral striatum

Introduction

The stress response is an evolved, adaptive behavior which allows animals to cope with challenging situations. However, the benefits of this system begin to break down when animals are faced with repeated stress, leading to maladaptive responses including increased risk for cognitive impairment, mood disorders and drug addiction (Krishnan 2008). Studies from our lab have suggested that activation of the dynorphin-kappa opioid receptor (dyn/KOR) system plays a critical role in producing these dysfunctional responses to stress (Land 2008). When this pathway is disrupted either pharmacologically with the KOR antagonist norBNI or by genetic deletion of the G protein receptor kinase 3, mice fail to demonstrate depression-like and addiction-like behaviors normally displayed by chronically stressed animals (Bruchas 2007). p38 mitogen activated protein kinase (MAPK) signaling has proven to be necessary not only for these behavioral outcomes, but also for stress-induced alterations in the serotonergic system. Changes in serotonin transporter (SERT) localization and function have been investigated in the past, with studies linking decreased SERT activity with anxiety-, depression-, and addiction-like behaviors (Lesch 1996; Lira 2003; Heinz 1998). Further, multiple publications from Blakely and Ramamoorthy labs have demonstrated that SERT activity is altered by phosphorylation and the kinase responsible may be p38 (Ramamoorthy 1998; Zhu 2004; Samuvel 2005).

Recently, our lab found that disruption of this signaling in serotonergic neurons of the dorsal raphe nucleus is sufficient to block maladaptive responses to stress (Bruchas 2011). Further investigation into the role of the serotonergic system has shown that behavioral or pharmacological activation of the dyn/KOR system promotes translocation of the serotonin transporter to the plasma membrane and increases the rate of reuptake in whole brain synaptosomes isolated from repeatedly stressed mice in a p38 MAPK dependent manner (Schindler 2012). It is presumed that this change in SERT expression produces a hyposerotonergic state in the ventral striatum that is responsible for the negative effects of stress in these mice (Schindler 2012). Alterations in 5-HT levels, serotonin transporter (SERT) expression, and studies have suggested a possible effect of SLC6A4 variants on stress vulnerability (Saveanu and Nemeroff, 2012). Individuals presenting these genetic characteristics have low stress-resilience and therefore react more strongly to moderate stress, which can trigger clinical depression. By further studying the mechanisms of chronic stress, we hope to discover potential targets to promote stress resilience and prevent comparable destructive responses observed in the human population.

While the research published by Schindler in 2012 provided strong evidence for increased SERT-mediated reuptake in the ventral striatum, the rotating disk electrode voltammetry (RDEV) experiments were only conducted in whole brain synaptosomes. Schindler demonstrated through western blotting of surface biotinylated synaptosomes isolated from the ventral

striatum that SERT surface expression was increased in this region, but not other regions, in response to stress. To investigate the functional effect of this translocation in the ventral striatum, I purified synaptosomes from this region and measured SERT specific reuptake using RDEV. In this study, I found that activation of the KOR through both administration of 10mg/kg U50,488 and repeated forced swim stress (R-FSS) significantly increases uptake through SERT in synaptosomes isolated from the ventral striatum as measured by rotating disk electrode voltammetry. I suggest that this stress-induced change in ventral striatal SERT expression and activity produces a localized hyposerotonergic state that may underlie anxiety-, depression-, and addiction-like behaviors.

Methods

Subjects

C57BL/6N (B6) mice (n = 74) ranging from 8-16 weeks of age were used in these experiments. Breeding stocks were maintained in the UW Vivarium. All experimental procedures were approved by the University of Washington Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985). All testing was during the light phase of the 14-h light/dark cycle.

Drugs

U50,488 (NIDA Drug Delivery program) was dissolved in sterile saline (0.9%) at injected at 10mL/kg.

Forced swim stress

Mice were exposed to a modified Porsolt forced swim stress as described previously (McLaughlin 2003). Briefly, mice were exposed to one 15 min swim, and 24 h later were exposed to four 6 min swims, each separated by a 6 min break in the home cage, 10 min before decapitation and synaptosome generation.

Preparation of synaptosomes

Synaptosomes were prepared as described in Schindler 2012.

Purification, biotinylation, and Western blotting of synaptosomes

Surface protein synaptosomal samples were generating as described in Schindler 2012. This paper also describes the conditions and antibodies used for immunoblotting of the serotonin transporter.

Rotating disk electrode voltammetry (RDEV)

RDEV was performed as previously described in Hagan 2010 and Schindler 2012.

Data analysis

Data are expressed as means +/- SEM. Experimental groups were compared using student's unpaired, two-sample t-tests.

Results

Repeated swim stress increases SERT surface expression in the ventral striatum

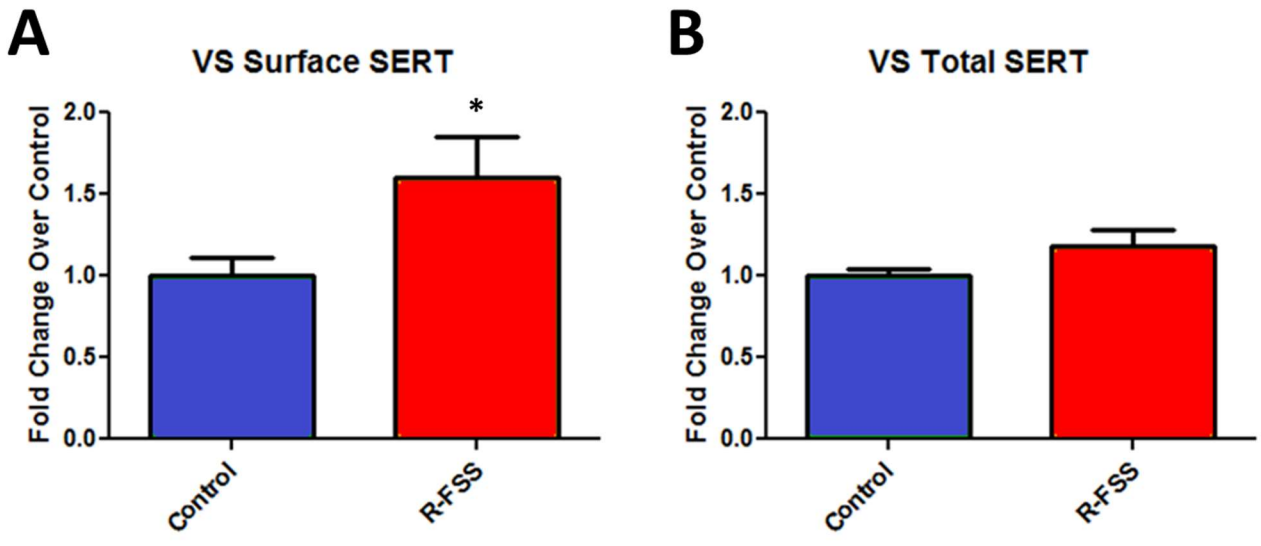
Previous studies in the lab demonstrated that KOR activation through repeated swim stress produces a change in uptake rates through the serotonin plasma membrane transporter. Rotating disk electrode voltammetry (RDEV) was used to obtain kinetics data by measuring SERT uptake rates in R-FSS and control mice. After injection of several concentrations of 5-HT, analysis demonstrated that increased 5-HT uptake rates were due to increased Vmax and not changes in SERT Km (Schindler 2012). This suggests that changes to serotonin uptake are due to either increased surface expression or increased synthesis of the transporter (due to Vmax) and not due to increases in catalytic activity of the protein (due to Km). These kinetics experiments were performed in synaptosomes isolated from whole brain tissue, but previous studies suggest the serotonin plasma membrane transporter is widely expressed in brain regions with serotonergic projections (Torres 2003).

To investigate both the mechanism behind changes in kinetics and the region responsible for the effects seen in whole brain isolates, I purified synaptosomes from ventral striata of controls and mice subjected to repeated forced swim and biotinylated them to selectively label cell surface proteins. Utilizing this method, I was able to use a pull down assay with streptavidin beads to obtain both surface protein samples and total protein samples and processed each for Western blotting with SERT antibody. I found that R-FSS increased surface SERT expression compared to unstressed controls (Fig. 4-1A; $p < 0.05$). Additionally, R-FSS did not change total

SERT expression in these samples (Fig. 4-1B), suggesting R-FSS induced increases in Vmax are due to increased surface trafficking of the transporter and not increased synthesis.

These findings replicate studies published in Schindler et al. (2012), demonstrating the reproducibility of these effects and my ability to successfully isolate intact synaptosomes using this protocol. Interestingly, the Schindler study found no stress-induced changes in SERT localization prefrontal cortex, hippocampus, dorsal striatum, amygdala, or dorsal raphe nucleus. Furthermore, this phenomenon was demonstrated to be norBNI sensitive and requires both GRK3 and p38α MAPK, suggesting it is mediated by downstream β-arrestin pathways.

Figure 4-1



Repeated swim stress increases SERT surface expression in the ventral striatum. Striatal tissue was extracted and used for synaptosome purification. Intact synaptosome samples were then

biotinylated to allow for labeling and subsequent isolation of surface proteins (A) from total protein (B). (A) Animals subjected to repeated forced swim stress expresses significantly higher levels of serotonin transporter at the plasma membrane than control animals ($p< 0.05$, $n = 6-7$). (B) However, the level of total SERT protein did not differ in ventral striatal synaptosomes extracted from stressed and unstressed animals, suggesting a change in SERT trafficking but not SERT synthesis following stress.

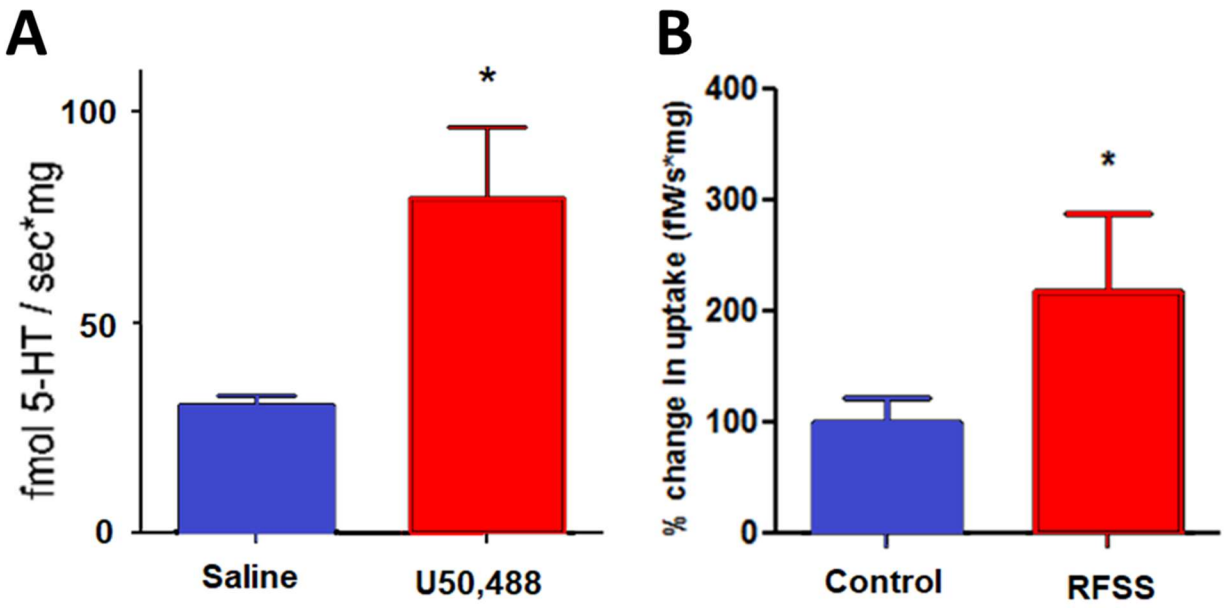
Activation of dyn/KOR system increases reuptake through SERT in the ventral striatum

The previously published Schindler study used rotating disk electrode voltammetry to investigate the effects of stress on SERT function following injection of serotonin into a suspension of synaptosomes isolated from whole brain tissue. Serotonin content was measured as current produced by redox reactions in the suspension and SERT mediated uptake was calculated through subtraction of paroxetine insensitive currents as previously described (Hagan 2010). This study demonstrated that repeated swim stress produces increased serotonin uptake through SERT in synaptosomes isolated from whole brain tissue (Schindler 2012). Additionally, the increase in SERT uptake rate was blocked by inhibition of KOR signaling through pretreatment with norBNI or transgenic deletion of downstream kinases GRK3 and p38a MAPK.

To determine if the regional specificity of changes in SERT uptake rate matched the previously identified changes in SERT trafficking, I isolated ventral striatal synaptosomes from control mice and mice with pharmacologically or behaviorally activated KOR signaling. Ventral striatal synaptosomes harvested from mice injected with the KOR agonist U50,488 produced SERT uptake rates several times higher than saline injected controls (Fig. 4-2A; $p < 0.05$). Similarly, VS synaptosomal suspensions from mice exposed to repeated swim stress recorded significantly

higher paroxetine sensitive uptake rates than unstressed controls (Fig. 4-2B; $p < 0.05$). These data demonstrate that the previous finding of increased SERT surface expression in the ventral striatum produces increased uptake of serotonin in this brain region. Increased uptake rates in the ventral striatum suggest the production of a hyposerotonergic state, which could elucidate a mechanism for how KOR activation produces a dysphoric state as previously suggested (Land 2008; Koob 2008; Bruchas 2011). Further, these results will be critical in guiding future studies examining the effect of ventral striatal serotonin signaling in stress potentiation of drug seeking and drug reinstatement.

Figure 4-2



Activation of dyn/KOR system increases reuptake through SERT in the ventral striatum. Rotating disk electrode voltammetry (RDEV) was used to determine the rate serotonin uptake in synaptosomes isolated from ventral striatum of control mice compared to either (A) mice treated with the KOR agonist U50,488 or (B) exposed to repeated forced swim stress (R-FSS). (A) The rate of paroxetine sensitive uptake by synaptosomes isolated from the ventral striatum

of mice treated with 10mg/kg U50,488 was significantly higher than synaptosomes isolated from control mice ($p < 0.05$, $n = 7$). (B) Ventral striatal synaptosomes from mice exposed to R-FSS also showed significantly greater paroxetine sensitive serotonin uptake than synaptosomes isolated from unstressed control mice ($p < 0.05$, $n = 5$).

Discussion

This study further identifies a novel stress signaling mechanism through the dynorphin/KOR system. While there is strong evidence demonstrating the role of KOR and SERT in the aversive effects of stress, this study elucidates a link between the two systems which may be modulating the anxiogenic, prodepressive and proaddictive properties of KOR activation. Specifically, this study has identified the afferent nerve terminals from the dorsal raphe to the ventral striatum as a key region of KOR activation and subsequent regulation of serotonin signaling.

Our lab and others have demonstrated repeatedly that the dynorphin/KOR system is activated in response to stress. KOR mediates the anxiogenic and depressive-like effects of stressors such as forced swim stress, foot shock stress, and social defeat stress supporting a clear role for the receptor in the generalized stress response (Carlezon 2006; McLaughlin 2006; Land 2008). The connection between stress and serotonin regulation have also been thoroughly examined. In humans, tryptophan depletion studies demonstrated no effect in subjects with no personal or family history of depression. However, similar methods produced mild depressive symptoms in healthy subjects with a family history of depression and more pronounced symptoms in patients previously diagnosed with depression (Benkelfat 1994; Delgado 1994). These results

suggest that a decrease in serotonin levels is especially problematic for vulnerable individuals, leading to negative mood and anhedonia. Altered expression of the serotonin transporter has also been associated with depression in human studies. Differences in SERT density have been discovered in subjects diagnosed with depression or who have committed suicide, and SSRIs targeting this transporter have become one of the most commonly prescribed classes of psychiatric drug in the world (Drevets 1992). However, no connection has previously been made between kappa receptor activation and the regulation of serotonin or its transporter. We believe that KOR activation modulates serotonin signaling in the ventral striatum through increased trafficking of SERT to the plasma membrane which is critical in the expression of aversive, norBNI sensitive behaviors including forced swim immobility and potentiation of drug seeking in cocaine CPP.

I began this study by replicating the surface protein biotinylation assay previously described in Schindler 2012. Briefly, I purified synaptosomes through a series of centrifugation steps, biotinylated surface proteins, homogenized samples, and precipitated surface proteins bound to streptavidin beads. Looking at region specific changes in SERT protein expression, I found that repeated forced swim stress significantly increased expression at the plasma membrane but did not change total expression in the sample in synaptosomes isolated from animals subjected to repeated forced swim stress. These results replicate what has previously been demonstrated in this region in response to forced swim. In order to investigate the functional consequences of this change in transporter localization, I performed rotating disk electrode voltammetry (RDEV) on similar synaptosomal preparations.

RDEV is a method for studying the activity of neuronal membrane transporters for monoamines including serotonin and dopamine. During measurements, an electrode with voltage set to detect the analyte of interest is placed into a solution and rotated at a constant velocity. This rotation produces laminar flow, drawing the solution up to the electrode surface where the analyte is oxidized or reduced and finally the products of this reaction are spun away. The resulting current from this redox reaction is measured and can be directly related to the amount of analyte in solution, while the rapid mixing of solution allows for measurement of the whole cell analyte concentration on a scale of milliseconds (Earles 1998). In this study, I used a glassy carbon electrode calibrated to detect serotonin concentration in a 500ul suspension of synaptosomes isolated from the ventral striatum of control and KOR activated mice. Injection of serotonin to the cell produced reliable current, which was continuously measured in real time as the neurotransmitter was transported into synaptosomes and removed from detection by the system. Inhibitors of the norepinepherine and dopamine transporters were used to ensure uptake measured was ocurring through the SERT protein and currents measured in the presence of paroxetine were subtracted to account for uptake through low affinity, high capacity transporters (Hagan 2011).

Using this method, I investigated the effect of pharmacological and behavioral activation of the dynorphin/KOR system on serotonin reuptake kinetics in synaptosomes isolated from the ventral striatum. In mice treated with 10mg/kg i.p. of kappa agonist U50,488, the paroxetine

sensitive serotonin current more than doubled, producing a significant increse in uptake over control samples from mice injected with saline prior to harvesting synaptosomes. I also investigated the effect of repeated forced swim stress on SERT kinetics in the ventral striatum. Here, RDEV currents again showed a doubling of serotonin reuptake and a significanlty increased rate in mice subjected to R-FSS compared to samples collected from stress naïve mice. These data strongly support a role for KOR-mediated increased SERT surface expression in producing a hyposerotonergic state in the ventral striatum. Interestingly, previous studies have demonstrated that substantial KOR activation is necessary for phosphorylation of GRK3 and p38α MAPK, two signaling kinases previously demonstrated to be necessary for stress-induced SERT translocation (McLaughlin 2003; Schindler 2012). p38α MAPK in serotonergic neurons has also been demonstration to mediate the aversive effects of stress (Bruchas 2011). Taken together, these data suggest that chronic stress activation of KOR produces a cascade of signals, including the phosphorylation of p38 MAPK, which culminate with changes in localization of SERT and the rate of serotonin reuptake in the ventral striatum. It is believed that decreased serotonin signaling in this region is responsible for the dysphoric component of stress.

Conclusions

This study demonstrates that repeated forced swim stress promotes translocation of the serotonin transporter from intracellular stores to the plasma membrane in synaptosomes harvested from the ventral striatum, a mechanism previously shown to be KOR-mediated and

p38 MAPK-dependent (Schindler 2012). Further, rotating disk electrode voltammetry demonstrated that repeated forced swim also results in increased serotonin uptake through its transporter in synaptosomes isolated from ventral straital tissue. These data provide strong evidence for a model in which stress activation of KOR produces a hyposerotonergic state in the nucleus accumbens.

Chapter 5

Stress Regulated Gene Expression in Serotonergic Neurons using RiboTag

Preliminary results of this study have been presented at the ACNP and SfN conferences and the full manuscript is currently being prepared for submission.

My contributions to this publication are described in this chapter.

Introduction

Unlike previous chapters, the study in this chapter investigates the effects of stress on the dynorphin/KOR and serotonergic systems using discovery based methods. The previous chapters have detailed studies testing specific hypotheses, based on strong data in order to further describe a particular phenomenon. For example, in chapter 2, I discussed the finding that KOR agonists have demonstrated inconsistent efficacy in female rodents while also demonstrating the strong dysphoric characteristics typical of these drugs. Based on these data, we hypothesized that there were sex hormone regulated differences in the intracellular signaling of the G protein pathway. In testing this hypothesis, we were able to design experiments specifically targeted to discover the differences in analgesic response and G protein signaling between males and females administered KOR agonists. Eventually, we showed that the sexually dimorphic analgesic response was likely due to estrogen-mediated phosphorylation of GRK2, which blunted downstream G protein signaling and analgesia in female mice. In contrast, the discovery based study described in this chapter is focused on

generating and analyzing a large volume of gene expression data in order to discover correlations that will lead to novel hypotheses. Here, we expanded on the general findings that the serotonin and dyn/KOR systems are involved in stress pathways to identify previously unsuspected players in the stress response system. KOR activation of p38 MAPK is required for the dysphoric response (Land 2008). Changes in phosphorylation of cytosolic proteins (e.g. GIRK & SERT) by KOR activated p38 MAPK are already known to mediate physiological responses (Bruchas 2010). p38 regulation of transcription factor function is also likely, but the resulting changes in gene expression have not yet been described.

Currently, the neurobiological basis of depression is unknown, but there is some evidence suggesting that it is due to dysregulation in neurotransmission. The monoamine hypothesis of major depressive disorder (MDD) predicts that this deficiency in neurotransmitter signaling is responsible for the depressed state and may be caused by increases in monoamine oxidase levels (Meyer 2006). Other studies have demonstrated that membrane transporter levels are altered in subjects with MDD (Medina 2013). Clinical studies suggest that neural plasticity plays a central role in depression, with neuronal atrophy in several regions and decreased neurogenesis in the hippocampus observed in depressed patients (Duman 1999). Interestingly, the cAMP response element-binding protein (CREB) is a critical integrator of neural plasticity that may be modulated by monoamines. When CREB signaling is blocked in the nucleus accumbens by overexpression of a dominant-negative CREB mutant, mice demonstrate antidepressive behaviors (Newton 2002). Additionally, expression of the CREB mutant resulted in decreased expression of the stress peptide dynorphin in the nucleus accumbens. While the

majority of studies investigating the connection between genetics and depression have focused on identifying polymorphisms that confer susceptibility, several have focused on alterations in gene expression in models of depression.

Investigating the effect of depression on transcription of genes regulating neurotransmission, Zhao and colleagues found several interesting insights into GABAergic and glutamatergic signaling in the prefrontal cortex. Real time quantitative PCR showed that $GABA_A$ receptor beta 2 (GABRB2) and post synaptic density 95 (PSD95) transcripts were significantly decreased in the anterior cingulate cortex, but not the dorsolateral prefrontal cortex of elderly patients with MDD compared to nonaffected controls (Zhao 2012). Further, the enzymes glutamate-ammonia ligase (GLUL) and aldehyde dehydrogenase (ALDH9A1), critical for regulating glutamate and GABA signaling, were differentially expressed in the brains of depressed patients compared to healthy controls (Kang 2007). These results suggest that depression alters expression of genes regulating neurotransmitter systems and that these may be valid targets for future therapeutics in the treatment of depression.

In addition to changes in neurotransmission, previous studies have also demonstrated altered expression of genes transcribing intracellular signaling proteins in brain tissue derived from depressed patients. As described earlier, CREB has been previously demonstrated to play a role in the transcriptional and behavioral consequences of depression (Chen 2001; Newton 2002). However, the activity of CREB is regulated by its phosphorylation state and there is strong

evidence for differential expression of several kinases and phosphatases in patients with MDD. In a study by Kang et al. (2007), expression of calmodulin 2 and protein kinase C (PKC) were observed to be decreased, while protein phosphatase 2CA (PP2CA) was found to be increased in PFC tissue from subjects with MDD. Interestingly, each of these changes in enzyme transcription results in a decrease in CREB phosphorylation and therefore an increase in dynorphin transcription and prodepressive effects. These studies confirm previous findings demonstrating the importance of CREB activity in promoting depression and suggest several targets for new therapeutics as well as genetic markers for possible depression screening.

Although previous studies have used quantitative PCR and microarray hybridization to probe the transcriptional effects of stress and depression, we used RNA sequencing to produce a discovery based dataset. In this study, we used transgenic floxed RiboTag x Pet1-cre mice to investigate changes in ribosome associated transcripts of serotonergic neurons in response to repeated forced swim stress in both male and female mice. A subset of males and females were pretreated with 10mg/kg norBNI to investigate the KOR-dependent effects of stress on transcription. mRNA sequencing data identified several genes that were significantly modulated by stress, with only a few genes being changed in both sexes and uniquely expressed in the serotonergic neurons of the dorsal raphe nucleus. Additionally, two small groups of genes were identified as being KOR-dependent, one increasing in response to stress and one group decreasing. Sexually dimorphic biochemistry demonstrates a need for investigating all biological processes in male and female models, but the effects of stress and their potential for precipitating MDD are especially important to study in both sexes as major

depressive disorder has been observed to be twice as prevalent in females compared to males (Kuehner 2003).

Methods

Subjects

C57BL/6N (B6) mice ranging from 8-16 weeks of age were used in these experiments. Breeding stocks were maintained in the Harborview Vivarium. All experimental procedures were approved by the University of Washington Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985). All testing was during the light phase of the 14-h light/dark cycle.

Drugs

norBNI (NIDA Drug Delivery program) was dissolved in sterile saline (0.9%) at injected at 10mL/kg.

Forced swim stress

Mice were exposed to a modified Porsolt forced swim stress as described previously (McLaughlin 2003). Briefly, mice were exposed to one 15 min swim, and 24 h later were exposed to four 6 min swims, each separated by a 6 min break in the home cage, 4 hours before decapitation and RiboTag isolation.

Immunoprecipitation and purification of RiboTag associated mRNA

Immunoprecipitation was performed as described in Sanz 2009. Briefly, four hours after swim stress, mice were decapitated and dorsal raphe nucleus was quickly dissected using circular hole punch. Tissues were homogenized by dounce and polysomes immunoprecipitated using antibodies to HA (Covance, Princeton NJ) and protein A/G-coupled magnetic beads (Pierce Biotechnologies, Rockford IL).

cDNA Library prep, RNAseq, and data analysis

These methods were performed by collaborators Atom Lesiak and Kevin Coffey and will be detailed in the publication of this study.

Results

Repeated forced swim stress produces high rates of swim immobility and alters the level of ribosome associated mRNA in serotonergic and nonserotonergic populations of the dorsal raphe nucleus

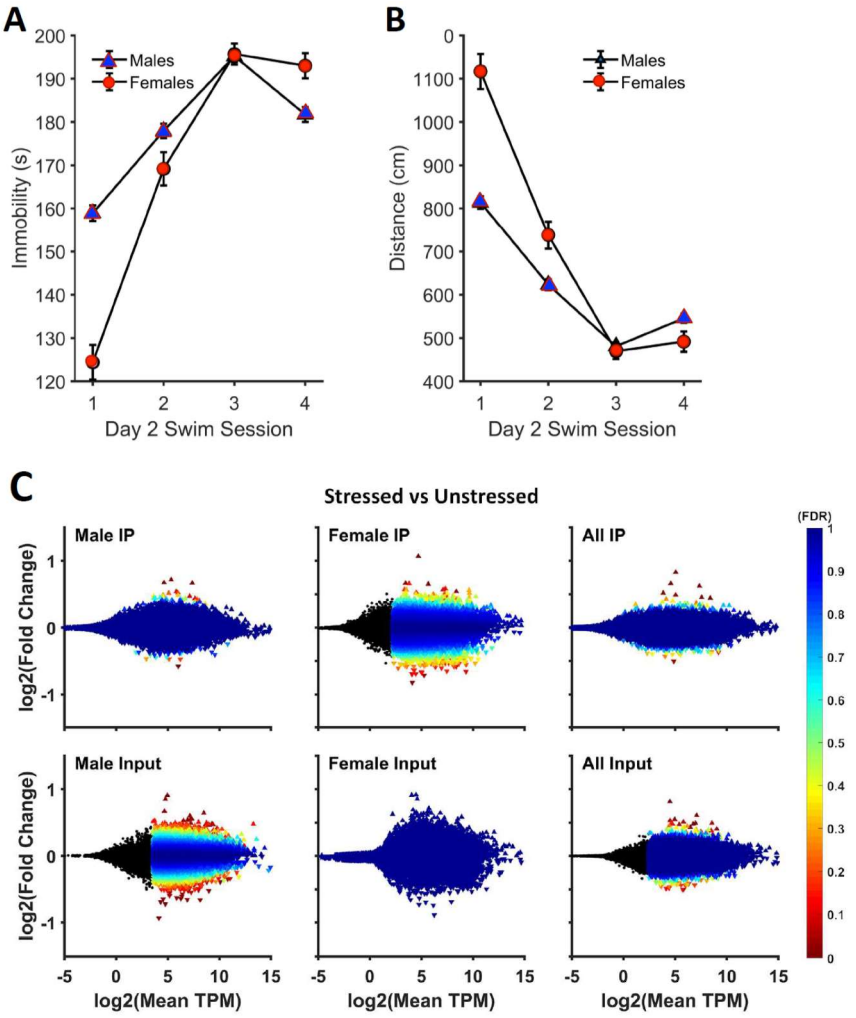
To investigate the transcriptional effects of stress, we began by exposing male and female mice to repeated forced swim stress as previously described (McLaughlin 2003). After two days of repeated swims, both male and female mice displayed a high degree of immobility and decreased distance moved throughout the swims on day two (Fig. 5-1A-B). Immobility and decreased activity in the forced swim test is widely accepted as a behavioral test for depressive-like symptoms and has been and has been demonstrated to be sensitive to the SSRI

antidepressants (Detke 1995; Cryan 2002). These behavioral assays suggest that the animals used in this study were stressed by the repeated forced swim protocol.

Four hours after the last swim, animals were sacrificed and dorsal raphe tissue was quickly harvested for RiboTag mRNA extraction. As previously described by Sanz et al. (2009), RiboTag mRNA was isolated and purified for cDNA library synthesis. Tagging of RPL-22 subunits allowed for analysis of actively transcribing genes from Pet1 neurons expressing Cre recombinase. cDNA libraries were created for RiboTag IP and input samples from each animal and sent for RNA sequencing. Analysis of this data showed significant changes ($q < 0.1$) in transcript levels from both IP and input samples in both sexes. Interestingly, samples from female mice demonstrated more stress induced changes in the IP samples and less in the input samples than male mice (Fig. 5-1C). Considered together, there were several stress-induced transcriptional changes in mice exposed to repeated forced swim stress.

Note: Atom Lesiak and Kevin Coffey performed all cDNA library prep and analysis of RNAseq data comparing stress-mediated changes in gene expression.

Figure 5-1



Repeated forced swim stress produces high rates of swim immobility and alters the level of ribosome associated mRNA in serotonergic and nonserotonergic populations of the dorsal raphe nucleus. Repeated forced swim was used utilized to investigate the effects of stress on gene expression in the DRN. Four hours after stress, DRN was dissected and RiboTag mRNA was isolated. cDNA libraries were delivered to sequencing core for RNAseq analysis. (A-B) Both male and female mice demonstrated stress phenotype during the second day of R-FSS as both groups increased immobility time and decreased distance travelled with successive swims. (C) RNAseq analysis demonstrating stress-induced changes in ribosome associated transcripts in males and females, in both serotonergic and nonserotonergic populations.

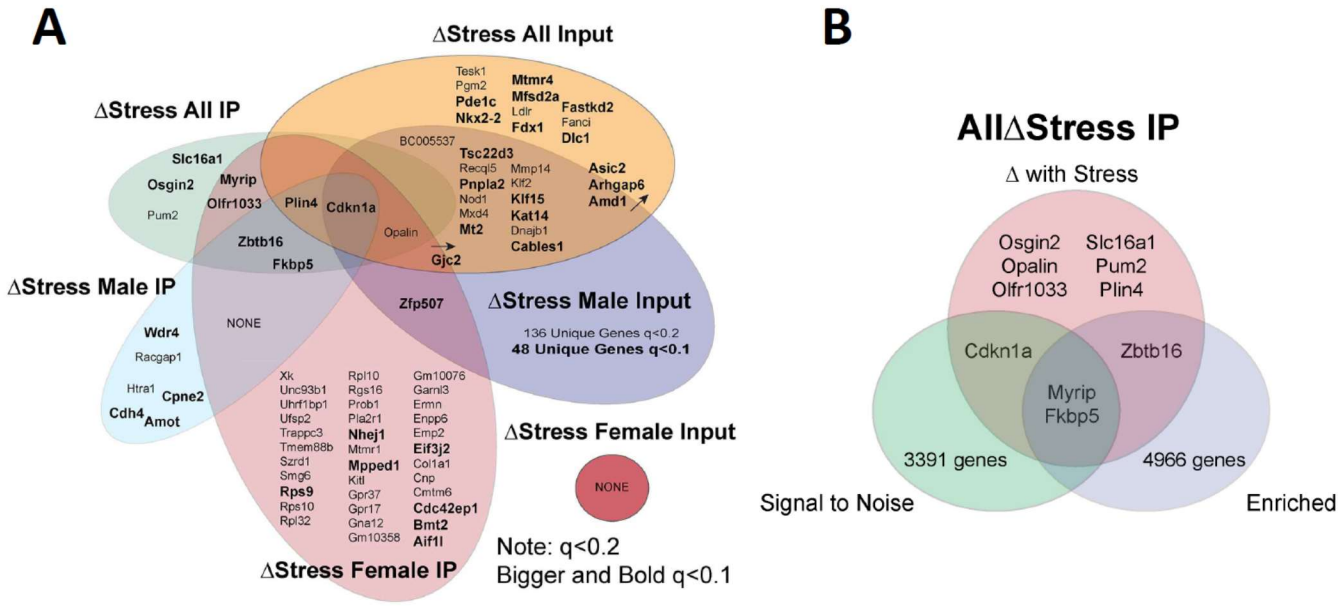
Stress affects ten genes in serotonergic dorsal raphe neurons of male and female mice

The depth of the reads from the RNA sequencing reaction produced a large dataset that will be useful in making several analyses of stress effects on gene expression. Here, we identified specific genes that were significantly changed by stress when comparing across several groups (Fig. 5-2A; $q < 0.2$ or $q < 0.1$). As described above in figure 5-1, the majority of transcriptional changes occurred when comparing stressed and unstressed female IP samples or male input samples. For these groups, R-FSS induced change in 44 transcripts when comparing serotonergic, ribosome associated RNA in females and change in 136 transcripts when comparing total RNA in males. In comparison, expression of only 10 genes was significantly changed when comparing male RiboTag IP samples and no changes in gene expression were found comparing the female input samples. When analyzing the effects of stress across both sexes, 10 RiboTag associated genes and 31 genes from the input samples were significantly changed (Fig 5-2A; $q < 0.2$ or $q < 0.1$).

When comparing the effects of stress on RiboTag RNA in all sexes, of the ten genes whose expression was significantly changed, only three were significantly enriched in the IP samples compared to inputs (Fig 5-2B). Fkbp5, Myrip, and Zbtb16 were all found to be expressed at much higher levels in the IP samples, suggesting that these genes are predominantly expressed in the serotonergic neurons of the dorsal raphe nucleus. The other seven genes that were differentially regulated by stress in the RiboTag IP samples (Osgin2, Slc16a1, Opalin, Pum2, Olfr1033, Plin4, and Cdkn1a) were not enriched, suggesting that these genes are ubiquitously expressed in the dorsal raphe. Significant stress-induced transcriptional changes suggest that

the role of each of these genes is worth examining, but we were particularly interested in genes enriched in the serotonergic neurons. Although all three of the genes Fkbp5, Myrip, and Zbtb16 were enriched, Zbtb16 had low signal to noise expression making these gene changes difficult to interpret. Therefore, this analysis provided two genes whose ribosome association is significantly affected by stress and are significantly enriched in the serotonergic neurons of the dorsal raphe nucleus. Interestingly, the role of Fkbp5 in mood disorders has been extensively studied and a role for this gene has been identified in SSRI treatment outcomes in depressed patients (Zannas 2014; Ellsworth 2013)

Figure 5-2



Stress affects ten genes in serotonergic dorsal raphe neurons of male and female mice. Evaluation of false discovery rate q-values generated from RNAseq analysis identified distinct genetic stress effects. (A) More genes from the IP samples were changed in response to repeated stress in females, whereas more genes from the input samples were changed in males. When considered together, the data suggest that 10 genes expressed in serotonin neurons are affected by R-FSS and 31 genes expressed across cell types in the dorsal raphe nucleus are affected by stress (q<0.2). (B) Of the ten transcripts whose expression changed in

response to stress (q<0.2), three were enriched in IP samples, suggesting a role in regulating the function of serotonergic neurons. FKBP5 is particularly interesting as it has been implicated in several mood disorders including depression, anxiety, bipolar, PTSD and others.

The effect of stress on several genes was blocked by pretreatment with KOR antagonist norBNI

In addition to comparing changes in gene expression across stressed and unstressed animals, we also investigated the role of the kappa opioid receptor in these stress-induced transcriptional changes. Samples from several male and female mice pretreated with 10mg/kg of the KOR antagonist norBNI were included in our RNAseq analysis. Examining the data from these samples, we looked for genes that changed in response to stress in saline treated animals, but not norBNI treated animals. Since the sample size in this experiment was small (n = 4-6), we were more generous in pursuing genes with a q-value over 0.1. To increase our confidence in the trends of these genes we considered the relative change of each sample from each group, checked the log2(normalized counts) for individual samples that may be creating a false trend in the data, and considered the false discovery rate or each of the genes analyzed. After considering all of these factors, we identified a total of six genes that seem to be regulated by stress and sensitive to pretreatment with norBNI (Fig. 5-3).

This group of genes can be separated into a group that is increased in response to stress and a group that is decreased. The increasing genes are phenylalanyl-tRNA synthetase (FARSA), RAB6-interacting golgin (GORAB), and O-linked N-acetylglucosamine transferase (OGT). The decreasing genes are lysyl oxidase homolog 3 (LOXL3), netrin 4 (NTN4), and protocadherin gamma subfamily B, 5 (PCDHGB5). The role of the norBNI-sensitive increasing genes is difficult

to interpret as they all code for proteins required for ubiquitous functions throughout the body. However, the identified genes which decrease in response to stress seem to have similar functions in promoting the development and maintenance of neuronal connections. The negative effects of stress and depression on neuronal proliferation, connectivity, and the maintenance of specific circuits have been well documented (Duman 2014). Interestingly, preliminary data that I have collected in WT mice suggests that R-FSS produces a decrease in spine density in the serotonergic neurons of the dorsal raphe nucleus (not shown). Although previous studies have identified changes in spine density in the hippocampus and cortex of depression models, this phenomenon is novel in the dorsal raphe (Drevets 2008). This suggests that the stress-induced loss of neuronal connectivity may be mediated by activation of the kappa opioid receptor and more studies should be done to confirm these targets.

Figure 5-3

Gene	Gene Function	Stress-induced Change
LOXL3	Lysyl oxidase homolog 3 is required for crosslinking elastin and collagens and is necessary in development of CNS	Decrease
NTN4	Netrin 4 plays a role in axons guidance and neuronal development	Decrease
PCDHGB5	Neuronal protocadherin critical for establishing and maintaining neuronal connections	Decrease
FARSA	Phenylalanyl-TRNA Synthetase charges tRNA with amino acids	Increase
GORAB	RAB6-interacting golgin facilitates vesicular trafficking at the Golgi and regulates secretory pathway	Increase

OGT	O-linked N-acetylglucosamine transferase catalyzes glycosylation of ser and thr residues	Increase

The effect of stress on several genes was blocked by pretreatment with KOR antagonist norBNI. Three genes increased by stress and three genes decreased by stress were identified to be norBNI sensitive in animals administered 10mg/kg norBNI before R-FSS and subsequent RNAseq analysis. These six genes were determined based on false discovery rate, normalized counts, and relative change. Interestingly, the three decreasing genes all play a role in neuronal development and neuronal connectivity. While stress and depression have previously been shown to affect neuronal morphology in other brain regions, this has not been demonstrated in the dorsal raphe nucleus. These results suggest a potential role for the dyn/KOR system in mediating changes in neuronal morphology following stress and several genes that may be involved in this process.

Discussion

These studies elucidate that stress causes a transcriptional change in ribosome associated mRNA in response to repeated forced swim stress in male and female mice. These changes were seen in genes coding for proteins across many cell types and signaling pathways, but a few were found to be specific to serotonergic neurons and the dynorphin/KOR mediated effects of stress. Further, some of these genes have previously been linked to depression and anxiety in clinical studies, while the cellular processes of other gene changes have been implicated in the same. The large dataset produced by this discovery based study provides many avenues for further exploration of the genetic changes associated with stress in both serotonergic neurons and other populations.

Before tissue extraction and RNA sequencing, animals in the stressed groups were exposed to modified Porsolt swim stress (McLaughlin 2003). In addition to acting as a severe, repeated stress event in the transgenic mice, the behaviors exhibited during these swims provided valuable data in determining the stress phenotype of the mice in the study. It has been extensively published that the Porsolt forced swim test provides a model assay for both inducing depressive-like behavior through stress and analyzing the degree of these behaviors through observing immobility (Porsolt 1977; Anisman 1990; Cryan 2004). Tracing of animal movement during the assay using EthoVision software (Noldus) provided immobility data for male and female animals in the stressed groups. These data demonstrate that animals in this study increase their immobility (a depressive-like behavior) with successive swims on the second day of stress. These data confirm the stress phenotype in both sexes and suggest that this assay provides a good model for investigating molecular changes associated with repeated behavioral stress.

Sequencing of the RiboTag RNA and input RNA samples extracted from these animals produced a large dataset with tens of thousands of reads across four experimental groups. Analysis of this dataset revealed enrichment ($q<0.1$) of 4448 genes in the Pet1-cre expressing IP samples including the serotonergic markers tryptophan hydroxylase and serotonin transporter Slc6a4 as well as the RiboTag gene. Interestingly, changes in the ribosome associated transcripts of the serotonergic neurons showed more gene changes in female mice than male mice. Whereas 10 distinct genes were changed in the IP samples in response to stress in male mice, 44 genes were differentially regulated in the IP RiboTag samples from female mice. Perhaps this is a

molecular manifestation of previous observations that depression rates are significantly higher in females (Kuehner 2003). It has been suggested that sex differences in the prevalence of depression may be due to genetic factors, but these studies are incomplete (Kendler 2001). It is possible that the RNA sequencing analysis data from this study could help identify specific genes relating to increased risk of depression in females.

When considering the entire dataset, males and females combined, the field of genes influenced by stress is considerably narrowed. Here, only three genes which demonstrate enrichment in the RiboTag IP samples over input are altered by stress. Additionally, one of these genes demonstrated poor signal to noise, making the sequencing data of this gene difficult to interpret. The two remaining genes are fkbp5 and myrip. Myrip codes for the Myosin VIIA and Rab Interacting Protein. This Rab effector protein can provide a link between melanosome-bound RAB27A-containing vesicles and the motor proteins, and is involved in melanosome transport (El-Amraoui 2002). Additionally, myrip can act as protein kinase A-anchoring protein, serving as a scaffolding protein linking PKA to components of the exocytosis machinery. Although no studies have linked this gene to stress or depression yet, this protein's role in exocytosis suggests it may be able to regulate neurotransmission through the trafficking of proteins to the plasma membrane.

Interestingly, the other gene that was found to be changed by stress in serotonergic neurons of the dorsal raphe nucleus in male and female mice was fkbp5. The fkbp5 gene encodes a heat

shock protein 90 (Hsp90) co-chaperone of the glucocorticoid receptor, the FK506 binding protein 51. Glucocorticoids represent an important class of hormones regulating the stress response. Upon release, cortisol can activate the glucocorticoid receptor (GR) in the cytosol of the cell. Once bound by its ligand, the glucocorticoid receptor translocates to the nucleus where it can act as a transcription factor to upregulate genes such as molecular activators of the hypothalamic-pituitary-adrenal axis (Nicolaides 2010; Russell 2010). Importantly, the function of the unbound, cytosolic GR is regulated by a multiprotein complex including the folding chaperone heat shock proteins and FK506 binding proteins (FKBPs). One of these chaperones is FKBP 51 (FKBP5), named for its 51kDa size (Nair 1997). FKBP5 acts as a co-chaperone of the GR complex, binding to heat shock protein 90, P23 and other chaperones through its tetracopeptide repeat protein (TPR) domain. When the GR is bound by its hormone ligands, the chaperone complex exchanges FKBP5 for other TPR-containing proteins which promotes nuclear translocation and transcription factor-like activity of the receptor (Wochnik 2005). However, increased FKBP5 binding to the GR-cochaperone complex promotes reduced hormone binding and reduced GR-mediated gene regulation, suggesting an inhibitory effect on GR activity (Zhang 2008).

Glucocorticoid secretion can also regulate transcription of FKBP5 directly. Upon binding to steroid hormone response elements, GCs induce transcription of FKBP5 in a number of tissues including the brain (Hubler & Scammell 2004). Interestingly, stress exposure has been found to significantly increase FKBP5 expression in a number of brain regions, ultimately resulting in a reduction of GR activity and an intracellular negative-feedback loop (Kelly 2012). However,

reduced FKBP5 induction and glucocorticoid sensitivity have been implicated in promoting GR resistance in major depressive disorder as individuals with single nucleotide polymorphisms in the gene had increased intracellular expression of the chaperone and less HPA-axis activation during depressive episodes (Binder 2004). It has been proposed that alterations in the FKBP5 gene confer changes in response time to antidepressant drugs and differential rates in the occurrence of depressive episodes in these subjects. The large amount of literature implicating this gene in depression- and anxiety-related disorders validates our methodology for investigating transcriptional changes in response to stress. Additionally, our finding that expression of this gene is altered in the serotonergic neurons of the dorsal raphe nucleus is novel and presents an intriguing insight into potential mechanisms of stress propagation in this region.

Together, these data demonstrate that the development of depressive-like behavior in repeated forced swim stress is accompanied by various transcriptional changes. While the most significant changes discovered by this analysis identified the glucocorticoid co-chaperone FKBP5, many other genes were found to be differentially regulated in both RiboTag IP and input samples and warrant further investigation. Additionally, KOR-sensitive decreases in expression of genes implicated in synaptic plasticity and axonal guidance suggest a role for dynorphin in modulating these functions after repeated stress. The dataset generated by this study reveals genetic risk factors for depression susceptibility and potential targets for treatment depression and depression-related disorders.

Conclusions

This study produced a large dataset describing changes in gene expression in dorsal raphe tissue from male and female mice in response to repeated forced swim stress. Floxed RiboTag transgenic mice allowed for the identification of changes in ribosome associated serotonergic transcripts. Our analysis demonstrated that myrip and fkbp5 were both highly enriched in serotonergic RiboTag samples and significantly changed in response to stress. Additionally, pretreatment with the KOR antagonist norBNI revealed that three genes important for neuronal connectivity were negatively regulated in a KOR-dependent manner. Together, these findings support a role for differential regulations of several genes in response to stress and present a database to guide future studies of stress-mediated changes in gene expression.

Chapter 6

Thesis Summary and Conclusion

The goal of this dissertation research was to enhance our understanding of KOR signaling in response to stress and identify novel target for the treatment of pain, depression, and substance abuse. Overall, the data generated from these experiments have implications for sex differences in the use of opioids to treat of pain, KOR-mediated mechanisms of the stress response, and stress-mediated changes in the translatome of serotonergic neurons of the dorsal raphe nucleus. This concluding chapter aims to integrate these data, discuss future directions suggested by these results, and state the contributions of these studies to the field of stress signaling and therapeutics.

The first chapter began with an introduction to anxiety, depression, and addiction with a focus on how the dynorphin/kappa opioid receptor system promotes these disorders through cellular signaling and its influence on behavior in animal models. It is well documented that the dyn/KOR system plays a critical role in the stress response, especially when that stress is chronic or severe (Land 2008), and several reviews have described the anxiolytic, anti-depressive, and anti-addictive effects of antagonizing this system (Bruchas 2010; Butelman 2012; Carroll and Carlezon 2013). I further presented evidence implicating this system in humans, suggesting this may be an effective target for future therapeutics in the treatment of depression and substance abuse disorders. To date, several KOR antagonists have been

investigated for therapeutic efficacy in clinical trials, but no drugs targeted to KOR have been FDA approved because of issues with low efficacy, low bioavailability, or adverse off target effects. Given the large amount of research implicating KOR in psychiatric disorders and the lack of current therapeutics targeting this system, the goal of my dissertation research was to further identify the signaling and transcriptional changes involved in the KOR-mediated stress response to define novel therapeutic targets to combat depression and addiction.

In order to investigate differences in stress signaling between male and female mice, we measured the abundance of receptor and kinase activation in response to U50,488 across sexes (Chapter 2). A sexually dimorphic analgesic response to opioid receptor activation has been observed in the past and these experiments aimed to identify a specific mechanism this as well. Using quantitative PCR, we determined that KOR message levels in tissue isolated from the ventral striatum were not different between male and female mice, suggesting that any differences in response to agonist administration would be observed downstream. Next we probed activity of kinases downstream from the G protein-mediated and β-arrestin-mediated signaling pathways respectively. Western blotting of ventral striatal tissue demonstrated that administration of 10mg/kg U50,488 significantly increased the phosphorylation of p38 MAPK in both male and female mice. Interestingly, similar activation of the KOR produced a significant increase in phosphorylation of ERK1/2 in male but not female mice, suggesting that females have blunted G protein signaling. This parallels their lack of analgesic response to opioid activation both in this study and in human studies.

Estradiol has previously been demonstrated to modulate the phosphorylation state of Ser670 on G protein-coupled receptor kinase 2 (GRK2) (Dominquez 2009) and GRK2 activity can disrupt signaling upstream from ERK1/2 (Daaka 1997). This suggests that elevated estradiol levels may be responsible for the blunting of ERK1/2 response and analgesia demonstrated in female mice. Quantitative PCR was again used to measure levels of GRK2 message in ventral striatal tissues. No significant difference was found in mRNA between males and females. However, phosphorylation of the estrogen target S670 site of GRK2 was significantly greater in females compared to males suggesting that the hormone was indeed activating GRK2. Ovariectomized females were then used to demonstrate that infusion of estradiol significantly increases GRK2 association with the G_α protein subunit as measured by coimmunoprecipitation of the proteins. Lastly, we pretreated animals with the selective GRK2/3 inhibitor CMPD101 to see if this could facilitate KOR-mediated ERK phosphorylation in intact female mice. Pretreatment with the inhibitor allowed for significantly increased ERK1/2 phosphorylation compared to mice pretreated with saline, suggesting that estradiol phosphorylation of GRK2 is responsible for blunting of downstream G protein-mediated KOR signaling. These data demonstrate a mechanism for the sexually dimorphic response to opioid receptor activation with significant implications for analgesic therapeutics and should influence the future clinical development of opioid drugs for analgesia in males and females.

In addition to targeting opioid receptors for analgesia, much research has demonstrated the utility of antagonizing KOR to prevent p38 MAPK-mediated stress signaling (Carroll and Carlezon, 2013). However, as discussed earlier, many of the current KOR antagonist clinical trials have failed to produce efficacy without adverse side effects. We aimed to describe a novel administration protocol for long-lasting, receptor-inactivating KOR antagonists to improve the therapeutic utility of these drugs. Male and female mice were injected daily with 0.1-10mg/kg of the KOR antagonist norBNI for 1-30 days. Although almost all previous literature has investigated the effects of blocking KOR signaling with a single 10mg/kg injection, we wanted to determine if a prolonged course of administration at smaller doses would produce similar effects in this model. As seen previously, a single injection of 10mg/kg norBNI robustly inhibited U50,488 analgesia measured 24 hours later by the tail withdrawal assay. Interestingly, single norBNI doses of 1mg/kg, 0.5 mg/kg, and 0.1mg/kg did not significantly change the latency to withdraw from 52.5°C water after administration of U50,488. However, repeated daily doses at these lower concentrations were able to significantly block U50,488 analgesia, producing a cumulative dose response curve with a significant linear trend. Further, daily administration of 0.1mg/kg norBNI for 30 days produced a significant blockade of KOR activation that extended at least 7 days after the last injection.

As outlined above, estrogen signaling blunts the analgesic effects of KOR activation. Therefore, we used ovariectomized mice in this study to determine if females show a similar receptor inactivation to repeated, low dose norBNI. These mice demonstrated effects similar to males, as both a single dose of 10mg/kg and 30 days of 0.1mg/kg norBNI significantly blocked KOR-

mediated analgesia. Although there is no evidence suggesting that p38 MAPK/aversion signaling in response to KOR activation is affected by estrogen, this must be investigated further to strengthen the results of this study in ovariectomized animals. These data demonstrate that repeated administration of norBNI at doses 100 times lower than that necessary for acute efficacy can produce significant and long-lasting receptor inactivation as measured by the tail withdrawal assay. This effect is likely due to the long-lasting nature of the drug. As more receptors are inactivated over time, norBNI produces an incremental, cumulative effect until enough receptors are offline to inhibit the behavioral consequences of KOR activation. This suggests that reducing the dose or increasing the time course of receptor-inactivating KOR antagonists may lead to improved outcomes in clinical trials. Further, this study suggests an advantage of receptor-inactivating antagonists over competitive antagonists in the treatment of depression and addiction as lower doses of competitive drugs would not produce cumulative effects on this schedule.

It has been widely demonstrated that the serotonin system influences the emotional and motivational aspect of behavior and has been implicated in anxiety, depression, and impulsivity. Additionally, Land et al. (2008) clearly demonstrated that the dynorphin/KOR system mediates the aversive effects of stress and is able to produce depressive- and addiction-like behaviors in animal models. Chapters 4 and 5 discuss the interactions of these two systems, including new findings in the stress-induced modulation of serotonin signaling and gene transcription in serotonergic neurons of the dorsal raphe nucleus. Previous studies in our lab have demonstrated that p38 MAPK signaling downstream of KOR in serotonergic neurons is

necessary for the pro-depressive and pro-addictive properties of stress (Bruchas 2011). Further, p38 MAPK signaling produces increased trafficking of the serotonin transporter to the cell surface of serotonergic terminals in the ventral striatum (Schindler 2012). The experiments in this dissertation have confirmed that repeated forced swim stress increases surface expression of SERT in the ventral striatum and that this produces a significant increase in serotonin uptake through its transporter, as demonstrated by rotating disk electrode voltammetry. These data support the hypothesis that the dynorphin and serotonin systems interact during stress response to produce a hyposerotonergic state in the nucleus accumbens. The nucleus accumbens is the primary reward center of the brain and altered signaling in this region has been associated with anhedonia and addiction. These results suggest that KOR-mediated kinase activity and alterations in SERT trafficking may be valuable therapeutic targets for the treatment of depression and addiction.

In order to further investigate the signaling involved in the serotonin and KOR stress response systems, we designed a discovery based study to identify changes in gene expression of serotonergic neurons in response to stress using RNAseq. We also pretreated a cohort mice of with norBNI to examine the effect of KOR activation on gene transcription in these neurons. As described previously, KOR activation by the stress peptide dynorphin stimulates kinase activity in serotonergic and nonserotonergic populations (Newton 2002). These kinases are capable of altering the phosphorylation of CREB and influencing gene transcription, a mechanism demonstrated to promote depressive symptoms in animal models and observed in immunostaining of postmortem human tissue (Kang 2007).

We again used repeated forced swim stress to produce male and female cohorts of mice for this study and used video analysis software to show that these mice displayed depressive-like immobility behavior. In order to concentrate our RNAseq results on genetic changes in serotonergic neurons, we used transgenic flox-RiboTag mice expressing Pet1-Cre. After extracting dorsal raphe tissue from the animals, RiboTag mRNA was isolated through affinity tag binding and coimmunoprecipitation with magnetic beads. cDNA libraries were then created from these samples and used in RNAseq. Interestingly, gene expression was largely changed by stress in these mice, with males and females showing differential effects. However, when pooling data from males and females, significant stress-induced changes in ribosome-associated gene expression were found across ten genes. Out of these ten genes, Fkbp5 is particularly interesting as it was enriched in the serotonergic IP sample and has been strongly implicated in mood disorders. This gene encodes a co-chaperone of the glucocorticoid receptor which is bound by the stress hormone cortisol, inducing translocation of the receptor-chaperone complex to the nucleus and gene transcription (Zhang 2008). Glucocorticoids have also been demonstrated to regulate fkbp5 transcription directly and produce a negative-feedback loop inducing glucocorticoid receptor resistance, a phenomenon thought to promote in major depressive disorder in humans (Binder 2004). Our finding that changes in fkbp5 expression were specific to the serotonergic neurons in the dorsal raphe nucleus presents novel insight into the role of this gene in regulating the stress response and strengthens previous research that has suggested polymorphisms in this gene can predict efficacy of SSRI treatment in depressed patients (Zannas 2014)

In addition to stress-mediated changes in gene expression in serotonergic neurons, several changes were shown to be norBNI sensitive. In particular, decreases in lysyl oxidase homolog 3 (LOXL3), netrin 4 (NTN4), and protocadherin gamma subfamily B, 5 (PCDHGB5) were all blocked by pretreatment with norBNI 24 hours before the first swim stress. Interestingly, all three of these genes promote the development and maintenance of neuronal signaling, processes known to be affected by severe stress and observed in models of depression (Duman 2014). However, decreased signaling and altered neuronal morphology in the dorsal raphe nucleus in response to stress has not been published. These data suggest novel KOR-mediated effects of stress on dorsal raphe neuron proliferation and gene transcription. The dataset provided by this RNAseq analysis has provided strong evidence that both fkbp5 and genes encoding proteins involved in neuronal connectivity are significantly affected by stress. Furthermore differential expression of these genes and the processes they are involved in continue to be important considerations in the treatment of anxiety, depression, and addiction.

Taken together, these data identified both sex and dosing schedule as key considerations in the design of future clinical trials. The studies presented also reveal novel roles for hormones, kinases, transporter proteins, and gene targets in the propagation of the kappa opioid receptor stress response. These molecules identify important stress mechanisms and warrant further study as targets for future therapeutics.

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