

KAPPA OPIOID RECEPTOR MODULATION OF NEUROTRANSMISSION IN THE
AMYGDALA

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A dissertation submitted to the faculty of the University of North Carolina at Chapel
Hill in partial fulfillment of the requirements for the degree of Doctorate of
Philosophy in the Department of Neurobiology in the School of Medicine

Chapel Hill
2015

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ABSTRACT

Nicole A. Crowley: Kappa opioid receptor modulation of neurotransmission in the
amygdala
(Under the direction of Thomas L. Kash)

Kappa opioid receptors (KORs) and their endogenous ligand, dynorphin, have been implicated in a variety of neuropsychiatric disorders including anxiety and alcohol addiction. Here, we demonstrate the function and role of KORs in the bed nucleus of the stria terminalis (BNST), a key brain region involved in these diseases.

In the first series of experiments, we show that KORs in the BNST inhibit glutamate release via a presynaptic, p38- and calcium- dependent mechanism. This synaptic inhibition is specific to basolateral amygdala (BLA) inputs, a previously identified key pathway in rodent models of anxiety-related behaviors. Additionally, we identified a frequency-dependent, light-evoked, local dynorphin-induced heterosynaptic plasticity of glutamate inputs to the BNST, allowing for optogenetic control of peptidergic transmission. We found differential KOR modulation of the BLA-BNST input based on the postsynaptic neurochemical identity. Collectively, these results demonstrate a local dynorphin- and KOR- dependent mechanism of inhibiting an anxiolytic pathway, providing a discrete therapeutic target for treatment of anxiety disorders.

In the second series of experiments, we show that following chronic intermittent

ethanol exposure (CIE), a model of alcohol exposure, KORs differentially modulate glutamate and GABA in the BNST. KOR inhibition of electrically-evoked glutamate inputs is decreased, while KOR inhibition of electrically-evoked GABA inputs is increased, despite overall properties of glutamatergic and GABAergic transmission remaining intact. This change in synaptic physiology is complementary to a KOR-dependent behavioral change: mice exposed to ethanol show decreased social preference as compared to air exposed, an effect which is partially rescued by systemic pre-administration of the KOR antagonist JDTic. Taken together, these experiments demonstrate KOR-dependent alterations of synaptic transmission in the BNST following CIE, making the BNST a potential site of action for KOR targeted therapies related to alcohol and anxiety.

Jointly, these experiments expand our understanding of how key peptidergic transmission in the extended amygdala can play a role in anxiety and addiction related diseases.

To my husband, Max,

Supporting a spouse while they are writing a dissertation is incredibly hard – I know, I remember doing it for you. Thank you for all of the work you've done to get us here. Thank you for all the editing you've done, the snacks and dinners you've prepared, the cups of coffee you've made, the phone calls and GChats during late nights in the lab, and the unwavering support you have always provided. Thank you for pushing me and believing in me. Thank you for being my husband, and an amazing father to our little boy. Thank you for believing in my dream of a career and a family. And most importantly, thank you for a decade of memories.

“After all this time?” “Always,” said Snape.

Looking forward to all that follows with you. I love you.

ACKNOWLEDGMENTS

This work was done with the support of many labmates, collaborators, mentors, family, and friends. First and foremost, thank you to my mentor, Tom Kash. I am blessed to call you my advisor and honored to call you my friend. Thank you for the countless hours you've spent advising me, correcting me, and making me a better scientist. I hope you continue to mentor me until you retire, which everyone knows (hopes?) you will never do. To my post-baccalaureate mentor, Dave Lovinger, I have nothing but admiration and thanks for your continual mentorship and support of my career. To all of the members of the Kash lab, both past and present, thank you for all of the support you provided. Thank you for helping with both planning and conducting experiments, and for being both professional colleagues and family. Thank you to former Kash Lab technicians Nora McCall and Alberto Lopez for their tireless assistance in all aspects of lab life. In particular, Nora assisted with much of the work in Chapter 3, which could not have been done without her. Thank you to Kristen Pleil, Lexie Kendra, and Emily Lowery-Gionta; science was always more fun when we were ordering shoes at the same time. To the rest of the members of my committee (Paul, Joyce, Ben, and Garret), I have honestly felt mentored by each of you, and I am thankful to have had your guidance. To the DLAM staff that has supported our work over the years, the many core facilities and collaborators, thank you. Sharon Owens, you rock. Amy Newlin, you were a bright party of my day at

UNC and I miss you dearly. Amanda Applegate, thank you for your voodoo magic copy editing. To my good friends Kristin Sellers, Elliott Robinson, and Zoe McElligott, thank you for the scientific chats, coffee breaks, and personal laughs.

Most importantly, I thank my family, my in-laws, my husband, and my son. Thank you for believing in me, and for your unwavering support, both emotionally and sometimes financially. Max, I couldn't have done it without you. Harrison, you only made completing this work harder, but I love you nonetheless.

This work was supported by an NIH National Research Service Award (F31-AA022280-02) and a Dissertation Completion Award from the UNC Graduate School.

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LIST OF ABBREVIATIONS AND SYMBOLS

ADX	adrenalectomized
aCSF	artificial cerebrospinal fluid
AUD	alcohol use disorder
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CEA	central amygdala
CIE	chronic intermittent ethanol exposure
ChR2	channelrhodopsin
CPP	conditioned place preference
CRF	corticotrophin-releasing factor
DR	dorsal raphe
DYN	dynorphin-expressing neuron
EPM	elevated plus maze
EPSCs	excitatory post synaptic currents
EPSPs	excitatory post-synaptic potentials
FST	forced swim test
GABA	γ -Aminobutyric acid
GIRK	g-protein gated inwardly rectifying potassium channels
GPCR	g-protein coupled receptor
HIPP	hippocampus
HPA	hypothalamic pituitary adrenocortical

ICSS	intracranial self-stimulation
IPSCs	inhibitory post synaptic currents
KO	knock-out
KOR	kappa opioid receptor
LH	lateral hypothalamus
LTD	long-term depression
LTP	long-term potentiation
mEPSC	miniature excitatory post synaptic current
MOR	μ -opioid receptor
mRNA	messenger RNA
NAC	nucleus accumbens
NPY	neuropeptide Y
OFT	open field test
PVN	paraventricular nucleus of the hypothalamus
pDYN	prodynorphin
PFC	prefrontal cortex
RTPA	real-time place aversion
ReaChR	red-shifted channelrhodopsin
VTA	ventral tegmental area

CHAPTER 1: INTRODUCTION¹

INTRODUCTION: KORs IN THE CENTRAL NERVOUS SYSTEM

Kappa opioid receptors (KORs) and their endogenous ligand, the peptide dynorphin (Chavkin & Goldstein, 1981a, Chavkin & Goldstein, 1981b, Chavkin et al., 1982) are at the forefront of potential therapeutic targets for a range of health issues, including anxiety, depression, and drug addiction (Bruchas et al., 2010). Here, we outline current neurobiological research of KORs, focusing on the discrete circuit elements that are regulated by KOR signaling and their role in behavior.

PHARMACOLOGY

Kappa opioid receptors are seven transmembrane g-protein coupled receptors (GPCRs) coupled to Gai/o, and they are known to utilize a variety of signaling cascades (reviewed in detail in Bruchas & Chavkin, 2010). KORs signal through

¹ This chapter has been previously published (Crowley NA, Kash TL (2015). Kappa opioid receptor signaling in the brain: Circuitry and implications for treatment. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 62, 1; 51-60). It has been included with permission from Elsevier, and with additional editing by the author.

both G α and G $\beta\gamma$ subunits, and then activate a host of downstream signaling molecules. Downstream actions include activating g-protein gated inwardly rectifying potassium channels (GIRKs), reducing calcium currents, and decreasing cyclic AMP. KORs have been shown to activate both MAPK and MEK/ERK signaling cascades (Belcheva et al., 2005, Hahn et al., 2010, Kivell et al., 2014a, Li et al., 2012, McLennan et al., 2008, Potter et al., 2011, Yoshizawa et al., 2011), although some groups do not see significant MEK/ERK activation following KOR activation (Asensio et al., 2006). KORs have also been shown to signaling through p38 (Bruchas et al., 2006, Bruchas et al., 2011, Hahn et al., 2010, Yoshizawa et al., 2011). This interaction between KOR and p38 is thought to be mediated by arrestin signaling; co-expression with the dominant-mutant form of β -arrestin prevents human KOR internalization in CHO cells (Li et al., 1999). The interaction between p38 and arrestin may mediate the dysphoria-like side effects of KOR agonists (Bruchas et al., 2007), possibly through a reduction in biogenic amine levels (Chefer et al., 2005, Spanagel et al., 1994) such as serotonin, as shown in Bruchas et al. The ability of KORs to signal through different GPCR signaling cascades may prove useful in creating biased agonists at the KOR, allowing for therapeutic treatments for pain or neuropsychiatric illnesses without the adverse side effects, such as dysphoria and psychomemetic effects observed in humans (Pfeiffer et al., 1986). Interestingly, KORs can utilize different signaling cascades in a single brain region (Hjelmstad & Fields, 2003). Notably, Hjelmstad & Fields demonstrated that while KOR activation inhibits GABA release via a calcium-dependent mechanism, its inhibition of glutamate is calcium-independent. Other groups have similarly

demonstrated KOR-mediated inhibition of GABA (Li et al., 2012), but more in-depth assessments of biased KOR signaling in a single brain region have not been conducted. Potent long-acting inhibitors of KOR include norBNI (Endoh et al., 1992) and JDTic (Bruchas & Chavkin, 2010). More recently, short-acting antagonists have emerged, such as LY2456302 and the tracer 11C-LY2795050 developed by Eli Lilly and Company (Lowe et al., 2014, Zheng et al., 2013). As KORs may utilize different signaling cascades, understanding this divergent pharmacological mechanisms will not only lead to greater understanding of the role KORs play in an assortment of behaviors and conditions, but it will also allow for greater tailoring of pharmacological treatments.

BEHAVIOR

Depression

The forced swim test (FST) is a classic screen for depressive phenotypes, and has been used since the 1970s as a way to screen for novel antidepressive drugs (Porsolt et al., 1977). Porsolt et al. first described the FST as a measurement of behavioral despair, wherein immobility indicates that the animal is no longer attempting to escape the experimental condition; antidepressants typically reverse this behavior (Castagné et al., 2001) (however, although the FST is effective as a screen for anti-depressants, it was not originally intended as a measurement of an actual depressive phenotype in the rodent). KOR antagonists produce antidepressant-like effects in the FST, as measured by a decrease in immobile

behavior (Reindl et al., 2008). A variety of studies have shown that administration of the KOR antagonist norBNI leads to decreased immobility in the FST (Carr et al., 2009, Mague et al., 2003). NorBNI-induced decreases in immobility and increases in swimming are observed in Wistar Kyoto rats, but not in Sprague Dawleys, highlighting important strain differences (Carr et al., 2010). This effect was also seen when Carr et al. administered norBNI directly into the piriform cortex. The KOR antagonist DIPPA prevented the adenosine-mediated decrease in immobility time in the FST, illustrating that the effect of KORs on depressive-like behaviors may involve the moderation of other neurotransmitter systems (Kaster et al., 2007). Therefore, as the KOR system seems to produce a robust phenotype in the FST, this behavioral test may be a useful screen for future KOR antagonist compound development.

In addition to its effects on the FST model of depression, KORs are also involved in depressive like states following drug withdrawal. Work from Chartoff et al. has demonstrated that though norBNI alone had no effect on latency to immobility in the FST, norBNI was able to block the cocaine-withdrawal-induced decrease in time to immobility (Chartoff et al., 2012). Like norBNI, JDTic has been shown to decrease immobility in the FST, and JDTic also decreased stress-induced reinstatement of cocaine responding (Beardsley et al., 2005). In other behavioral assays related to depression, site-specific infusion of the KOR antagonist norBNI into either the hippocampus or nucleus accumbens prevented the depressive phenotype seen in a learned helplessness paradigm (Shirayama et al., 2004). Consistent with these

results, KOR agonists also increase intracranial self-stimulation (ICSS) thresholds, indicating a potential depressive-like phenotype (Todtenkopf et al., 2004).

Anxiety

Much of the animal literature has focused on KOR modulation of anxiety-related behaviors. The elevated plus maze (EPM), a common test for rodent anxiety, involves letting the rodent explore an apparatus with both closed arms and open arms; more time spent exploring the open arms indicates an anxiolytic phenotype (Pellow & File, 1986). Knoll et al. showed that administration of the KOR antagonist norBNI resulted in an anxiolytic phenotype in the EPM (Knoll et al., 2011). KOR antagonists can also reverse the anxiogenic effects of stress in the EPM (Peters et al., 2011), and, similarly, KOR antagonists can reverse the anxiogenic effects of a KOR agonist (Valdez & Harshberger, 2012). These experiments would suggest an anxiogenic effect of KOR agonists and an anxiolytic effect of KOR antagonists. However, some contradictory literature has emerged: administration of the KOR agonist Salvinorin-A increases both open arm time and number of entries into the open arm of the EPM (Braida et al., 2009). In addition, the KOR agonist U50,488 can produce anxiolytic effects in the EPM at low doses (10–100 µg/kg) (Privette & Terrian, 1995). These discrepancies may be driven by two important points: first, in the Braida et al., study, Salvinorin-A was used. Salvinorin-A has been shown to utilize the ERK1/2 signaling cascade (Kivell et al., 2014b), and this biased ligand may produce differential behavioral effects compared to those of other KOR agonists (this hypothesis of differential effects by some KOR agonists is supported by work demonstrating that ICV administration of low, but not high, doses of Salvinorin

produces a robust conditioned place preference (Braidà et al., 2008)). Similarly, the global anxiogenic effect of KOR agonists may be dose dependent; the conditioned place preference seen by Braidà et al., as well as the anxiolytic effect in the EPM seen by Privette & Terrian, were seen at lower doses of KOR agonists that were typically used. This may indicate that KOR agonists do have the potential to be used for therapeutic purposes, but much more research is needed.

Taken together, the literature on KOR interactions with both depression and anxiety provides a mixed and muddled picture at best. Future experiments will need to address the nuances of behavioral effects (e.g., comparing multiple KOR agonists, detailed dose response curves) in order to thoroughly understand the relationship between the dynorphin/KOR signaling system and depression and anxiety. In addition, circuit- and site-specific manipulations, discussed below, provide some clarity as to the convoluted effect seen with systemic administration of KOR agonists. This provides key important information as to how KOR modulation can be used to shift anxiety-related behaviors: both low doses of KOR agonists, as well as KOR antagonists, may prove to be effective.

Addiction

KORs have been shown to be involved in the consumption, withdrawal, and escalation of a variety of drugs of abuse, such as alcohol (Zhou et al., 2013), heroin (Schlosburg et al., 2013 and Sedki et al., 2014), and cocaine (Al-Hasani et al., 2013, Trifilieff & Martinez, 2013b). Despite of an abundance of literature demonstrating the promise of KORs for the treatment of drug addiction (Hasebe et al., 2004, Wee &

Koob, 2010) few drugs impacting the KOR system have been taken to human clinical trials. The KOR antagonist JDTic did reach stage 1 clinical trials for the treatment of cocaine dependence, but the research was terminated due to adverse effects (RTI-International, 2012). The antagonist LY2456302 has upcoming phase 1 and phase 2 clinical trials for treatment resistant depression and anxiety disorders and has completed phase 1 clinical trials for alcohol dependence (Massachusetts General Hospital, 2013). The existing animal literature on KORs and addiction, discussed below, should encourage further clinical investigations.

Work on the KOR system and cocaine has shown that activation of KORs can reduce cocaine self-administration (Glick et al., 1995), and the utility of mixed mu/kappa opioid receptor agonists have been shown for the treatment of cocaine dependence (Bidlack, 2014). Administration of both a KOR agonist and cocaine blocks sensitization to the conditioned rewarding properties of cocaine using a conditioned place preference model (Shippenberg et al., 1996). Freeman and colleagues used experimental manipulations in non-human primates to support the hypothesis that this suppression of self-administration and rewarding properties of cocaine may be due to the ability of KOR agonists to punish responding for cocaine (Freeman et al., 2014). In these experiments, monkeys decreased operant responding for either cocaine or remifentanyl when paired with the KOR agonist Salvinorin-A, highlighting the potential role of the KOR system to curtail drug self-administration. The hypothesized mechanism for KOR-induced changes in cocaine administration and dependence is fairly well established, as KORs are present on dopaminergic terminals and can inhibit dopamine release (Trifilieff & Martinez,

2013a). However, Ehrich, Phillips, & Chavkin (2014) found that KOR activation can potentiate cocaine-induced increases in evoked dopamine release, depending on the timing of KOR activation and cocaine administration. This study emphasizes that while KORs may be a promising target for drug addiction, for cocaine in particular and likely for other drugs as well, timing of the intervention may be crucial. If KOR activation can both increase and decrease drug self-administration based on timing, it is unlikely to be useful for treating addiction (to those particular drugs) in the real world.

KORs have also been implicated in morphine abuse, by interacting with morphine's ability to potentiate dopamine release in the nucleus accumbens via its actions at the mu opioid receptor (MOR) in the ventral tegmental area (Vander Weele et al., 2014). Supporting this hypothesis, MOR and KOR agonists have opposing effects on dopamine release, with the former potentiating and the latter attenuating dopamine release (Di Chiara & Imperato, 1988). Administration of the KOR agonist U50,488 is capable of blocking morphine conditioned place preference (Funada et al., 1993). Dynorphin-A levels are altered during the development of morphine dependence, with levels increased in the hippocampus and hypothalamus (Wan et al., 1998). Interestingly, strains of mice with higher pro-dynorphin expression appear to be morphine insensitive, hinting at a mechanism by which the dynorphin/KOR system may protect against the abuse-potential of drugs activating the MOR system (Gieryk et al., 2010). Taken together, the existing literature on KOR/MOR interactions suggests that the dynorphin system may be a promising target for intervening in MOR-related drugs of abuse, such as morphine.

Research regarding KORs and alcohol addiction has become more prevalent in the last decade, with a greater emphasis being placed on the molecular role of the dynorphin system (Faisal et al., 2014) and its potential therapeutic role in alcoholism (Heilig & Schank, 2014, Nutt, 2014, Walker et al., 2012). Leeman et al. discussed in depth how various animal models can appropriately recapitulate human studies, and importantly, the authors highlight the role rodent alcohol consumption and abstinence (as well as heavy drinking) can play in informing the human literature (Leeman et al., 2010). In the human population, genetic variants in the KOR gene OPRK1 modulate alcohol consumption (Li & Zhang, 2013, Wang et al., 2014) (though note that this effect is not replicated in all populations; see Cupic et al., 2013). KOR antagonists can prevent alcohol self-administration in alcohol preferring rat lines (Cashman & Azar, 2014). Interestingly, administration of KOR agonists can also attenuate responding for alcohol in an operant paradigm (Henderson-Redmond & Czachowski, 2014). KORs are required for alcohol-induced increases in brain-derived neurotrophic factor (BDNF) in the striatum (Logrip et al., 2008). Targeting KORs with U50,488 blocks the rewarding effects of ethanol during conditioning, and importantly, this was seen with sub-anxiogenic doses of the KOR agonist (Logrip et al., 2009). Mice lacking KORs show decreased alcohol self-administration (Kovacs et al., 2005), and alcohol self-administration leads to an upregulation of dynorphin in the central amygdala, a region of the extended amygdala (D'Addario et al., 2013). Mice lacking dynorphin show increased alcohol preference; however, in contrast to control littermates, they do not show increased alcohol consumption following a mild stressor (Racz et al., 2013). Therefore, it seems that both antagonist and agonists of

KORs may play a promising therapeutic role in treating alcohol addiction, but much like the cocaine, depression, and anxiety literature, the timing and nature of intervention and state of the individual may be crucial.

Alcohol consumption, however, is only one part of the spectrum of alcohol addiction. Reinstatement models (as reviewed in detail in Le & Shaham, 2002) allow for critical assessment of another component of addiction, relapse. Though it can be difficult to model alcohol relapse in a rodent (as noted in Leeman et al., 2010), reinstatement provides relevant information on this topic. In addition to effects on alcohol consumption, KORs appear to play an important role in withdrawal from alcohol and further alcohol seeking. Administration of the KOR agonist U50,488 reinstated alcohol seeking in a norBNI-dependent manner (Funk et al., 2014). In addition, Funk and colleagues found that norBNI pretreatment 2 h before the session blocked yohimbine-induced reinstatement of alcohol seeking, further elucidating the KOR/stress interactions in alcohol seeking behaviors. Schank and colleagues similarly demonstrated that the KOR antagonist JDTic attenuates both alcohol seeking behaviors and withdrawal (Schank et al., 2012). Berger et al. has demonstrated that norBNI dose-dependently decreased post-alcohol ultrasonic vocalizations, an indicator of negative affective state; in addition, norBNI altered cue induced alcohol consumption (Berger et al., 2013). However, Morales and colleagues found that the KOR antagonist norBNI increased alcohol self-administration in male rats, while decreasing self-administration in females, highlighting key sex differences in KOR-mediated alcohol phenotypes (Morales et al., 2014).

This relationship between alcohol and KORs may be bi-directional: mice exposed to alcohol prenatally had altered KOR systems, and displayed an appetitive response to KORs (Nizhnikov et al., 2014). Interestingly, prenatal exposure to alcohol increases ethanol intake later, and this effect is partially blocked by KOR antagonists (Diaz-Cenzano et al., 2014). And, despite the abundance of this behavioral evidence, little has been done to investigate the mechanism of interaction between alcohol and KORs, with most of the existing literature focuses in the central amygdala (Gilpin et al., 2014, Kang-Park et al., 2013, Kissler et al., 2014).

Paradoxical effects

The KOR system has been shown to be involved in numerous psychiatric-disease related behaviors, including depression, stress, and addiction. Important studies using modern genetic approaches have highlighted the multiple ways that KORs' effect behavior, and paradoxical effects have emerged when manipulating the dynorphin system. For example, researchers found that when dynorphin was genetically deleted globally, mice showed enhanced cue-dependent fear conditioning (Bilkei-Gorzo et al., 2012) as well as enhanced social partner recognition (Bilkei-Gorzo et al., 2014). In addition, these dynorphin knock out (KO) mice showed decrease c-fos immunoreactivity in key limbic brain regions. In another paper, the authors demonstrated that dynorphin KO mice show subtle alterations in anxiety, most notably increased startle amplitude in the startle response test (Bilkei-Gorzo et al., 2008). These results would conclude that dynorphin KO mice show increased fear and anxiety, and increased pro-social behaviors. In contrast to this finding, administration of norBNI or JDTic increases open arm time in the elevated

plus maze (EPM), with no effect on general locomotor behavior in rats (Knoll et al., 2007), thought to be representative of an anxiolytic phenotype (other examples of behavioral studies consistent with Knoll et al. are outlined above). Though this work by Bilkei-Gorzo et al. may at first seem contradictory to the dogmatic perspective of dynorphin as a stress and anxiety peptide, it importantly highlights the mixed role that dynorphin and KORs may play throughout the brain. While KOR activation in regions such as the hippocampus may have protective effects, activation in regions such as those involved in anxiety-related behaviors (amygdala, prefrontal cortex) may have negative effects, as discussed below. In addition, key differences between the work of the Bilkei-Gorzo et al. and Knoll et al. studies include timepoint of manipulation of the KOR system; importantly, those where dynorphin is knocked out from birth, allowing compensatory mechanisms to come on board, versus those with site-specific or acute dynorphin manipulations. The work from the Zimmer group may highlight that when KORs are globally excised from birth, other compensatory mechanisms may regulate anxiety and fear related behaviors. Equally important, the work by Bilkei-Gorzo et al., 2008, Bilkei-Gorzo et al., 2012 and Bilkei-Gorzo et al., 2014 was conducted in dynorphin KO mice maintained on a C57Bl/6J background; Knoll et al. and others have used Sprague–Dawley rats, highlighting what may be important species differences in mammalian models of anxiety related behaviors. In addition, an important study by Chefer and Shippenberg provided some clarifications to the perplexing effects sometimes seen with dynorphin KO mice (Chefer & Shippenberg, 2006). The authors find that developmental compensations following dynorphin KO may lead to changes in the endogenous dopamine system;

specifically, dynorphin KO mice shown decreased extracellular dopamine in the nucleus accumbens, resulting in a decreased responsiveness to cocaine. Thus, global manipulation of the KOR/dynorphin system may prove to be problematic as a tool to develop treatments for disorders related to the mesolimbic dopamine system, such as anxiety, depression, and addiction, and must be pursued with caution. It may be more fruitful to use pharmacological versus genetic manipulations of the dynorphin KOR system when developing therapeutic lines of research.

CIRCUITRY

Key brain regions have emerged as major players in the actions of KORs — notably, the dorsal raphe (DR) nucleus, the ventral tegmental area (VTA), the nucleus accumbens (NAC), the prefrontal cortex (PFC), the amygdala and extended amygdala, and the hippocampus (HIPP). Novel technological approaches, including genetically modified mice, optogenetics, and other site-specific manipulations, have allowed for a more in-depth analysis of these crucial brain regions, further informing potential therapeutic targets. Importantly, this approach has allowed researchers to parse apart discrepancies seen with classic behavioral pharmacology approaches, and provide a more detailed picture into the roles of the KOR dynorphin system.

Dorsal raphe nucleus

The DR contains the majority of the brain's serotonergic neurons (Lowery-Gionta et al., 2014), and is involved in established KOR mediated behaviors, such as dysphoria (Lemos et al., 2012, Maier & Watkins, 2005), and alcohol addiction (Tomkins et al., 1994). Site-specific administration of the KOR agonist U50,488 into the DR decreased extracellular serotonin by approximately 30% (Tao & Auerbach, 2005). Behaviorally, KORs in the DR have been shown to be necessary for stress related behaviors. In recent years, work from Chavkin and colleagues has thoroughly investigated KORs in the DR and their relationship to stress. They demonstrated (Land et al., 2009) that administration of the KOR antagonist norBNI into the DR blocked not only aversive effects of the KOR agonist U50,488, but also blocked stress-induced reinstatement of CPP. In addition, Land and colleagues further elucidated that this phenotype is dependent on DR projections to the nucleus accumbens. In follow-up studies, they (Bruchas et al., 2011) demonstrated that stress regulates the serotonin transporter via a p38 MAPK dependent mechanism, and that deletion of p38 from serotonin neurons in the DR prevents the development of stress-induced avoidance behavior, as well as a host of other stress-related phenotypes; this p38 mediated translocation of the serotonin transporter appears to be KOR-mediated. Further enhancing our understanding of KORs in the DR, Lemos et al. (2012) also investigated KOR effects using slice electrophysiology. KOR activation in the DR produces an inhibition of evoked glutamatergic transmission onto DR serotonergic neurons, as well a decrease in miniature excitatory post synaptic current frequency (Lemos et al., 2012). Interestingly, Lemos and colleagues express with the dopamine transporter (Svingos et al., 2001), and KORs located on

colleagues did not see changes in GABAergic transmission (evoked and miniature), indicating that KOR regulation of the serotonergic system may be largely through inhibition of excitatory inputs onto serotonin neurons. However, no published work to date has looked at KOR modulation onto other DR neuron subtypes, such as the dopaminergic population; other neurons may also be inhibited by KOR, effectively shutting down DR communication. In addition, Lemos et al. demonstrated that KORs increase post-synaptic G-protein-gated inwardly rectifying potassium channels (GIRK) currents in the DR; this KOR-activated GIRK is reduced following exposure to a two day forced swim stress procedure while the effects of KORs on glutamate transmission remain unaltered following stress. This work was complementary to previous published work (Pinnock, 1992) that demonstrated KOR activation inhibited excitatory post-synaptic potentials (EPSPs) in the DR. However, Pinnock did not see any effects on membrane potential with the KOR agonist CI-977. In contrast to the GIRK-mediated effects of U69593 shown by Lemos et al. Pinnock did not have access to cell-type specific recording techniques; in addition, Pinnock's study was conducted in rats while Lemos et al.'s was conducted in mice, possibly explaining the differences in the two publications.

Ventral tegmental area

Kappa opioid receptors have been studied in the VTA for their role in the inhibitory modulation of dopaminergic neurons (Shippenberg et al., 1993), both on the cell bodies within the VTA and downstream dopamine targets, such as the medial prefrontal cortex (Tejeda et al., 2013). KORs have been demonstrated to co-express with the dopamine transporter (Svingos et al., 2001), and KORs located on

dopaminergic neurons are necessary for conditioned place aversion (Chefer et al., 2013). Chefer et al. demonstrated that when KORs are selectively deleted from dopamine neurons, mice do not display conditioned place aversion to a systemic KOR agonist, as their control counterparts do. This work is consistent with previously published studies which demonstrated that microinjection of a KOR agonist directly into the VTA produce conditioned place aversion (Bals-Kubik et al., 1993), and is consistent with slice electrophysiology experiments demonstrating that postsynaptic KORs directly inhibit dopamine neurons (Margolis et al., 2003). There are likely KORs located on other neurons within the VTA as well, such as on the GABAergic populations synapsing onto dopamine neurons (Graziane et al., 2013). Graziane et al. demonstrated that an acute stressor blocks GABAergic long-term potentiation (LTP) in the VTA, but this LTP is rescued when a KOR antagonist is administered prior to exposure to the stressor. In addition, slice experiments have demonstrated that KORs inhibit glutamate transmission onto VTA neurons (Margolis et al., 2005) (notably, a previous study did not see KOR inhibition of glutamate transmission in the VTA, (Manzoni & Williams, 1999); however, as noted in the Margolis et al., paper, Manzoni & Williams did see a high degree of variability in their KOR agonists effects, and an inhibition may have emerged if parsed apart by cell type characteristics. For example, Margolis et al. (2006) demonstrate that KORs selectively inhibit PFC projecting dopamine neurons in the VTA, and to the amygdala (Margolis et al., 2008), but not those that project to the NAC). Importantly, interactions with other neuropeptides have recently been discovered; dynorphin projections to the VTA also co-express with orexin, and orexin can facilitate reward

in ICSS by blocking KOR-mediated effects (Muschamp et al., 2014). Taken together, work on KORs in the VTA shows a surprisingly consistent and concise effect on dopamine neurons and local circuitry, as well as conditioned place aversion and addiction related behaviors, but much work is left to be done in order to understand how this local circuit interacts with inputs to the VTA. Importantly, KORs also modulate the major downstream target of the VTA, the NAC, discussed below.

Nucleus accumbens

Dynorphin and KORs are known to be important regulators of NAC circuitry; KOR activation inhibits glutamate transmission in the NAC, and is abolished during cocaine withdrawal (Mu et al., 2011). Using microdialysis, Spanagel et al. (1992) demonstrated that activation of KORs by the KOR agonist U69,593 inhibited dopamine release (and complementary, the antagonist norBNI enhanced dopamine release). Interestingly, the main dopaminergic projection to the NAC, arising from the VTA, is also inhibited by KOR activation (Margolis et al., 2003). The Berridge lab (Castro & Berridge, 2014) recently published a comprehensive study of opioids in the NAC: activation of KORs (as well as the μ - and δ -opioid receptors) in the NAC “hotspot”, the rostradorsal quadrant of the medial shell, was found to positively regulate “liking” and “wanting” responses. Castro & Berridge similarly discovered a “coldspot” for hedonic responses in the NAC. This work very importantly, and interestingly, indicates that not just KORs, but the location of KORs, may be integral to their much-researched regulation of stress and anxiety related behaviors.

Stress increased prodynorphin (pDyn) messenger RNA (mRNA) in the NAC, possibly through a CREB dependent mechanism (Chartoff et al., 2009). Following

resident intruder stress, dynorphin mRNA in the NAC dorsal and medial shell is increased, which, in conjunction with other experiments, the authors hypothesize promotes maladaptive behavioral responses following stress (Berube et al., 2013). Similarly, dynorphin mRNA in the NAC was altered following protracted withdrawal from repeated immobilization stress (Lucas et al., 2011). Following a forced swim stress procedure, Bruchas et al. found increased phosphorylation of ERK1/2 in the NAC, which was blocked by the KOR antagonist norBNI and absent in KOR knockout mice (Bruchas et al., 2008). Stress similarly causes a dynorphin-dependent increase in KOR activation in the NAC, as well as the basolateral amygdala, HIPP, DR, and basolateral amygdala, all discussed in detail in other sections of this review (Land et al., 2008).

Much of the work on KORs in the NAC has been done in the context of drug addiction, particularly cocaine, allowing greater clarity as to how KORs modulate the reward system. Gehrke and colleagues (Gehrke et al., 2008) demonstrated that KORs enhance cocaine-evoked dopamine overflow. Similarly, Ehrich and colleagues (Ehrich, Phillips, & Chavkin, 2014) showed that KOR activation can enhance cocaine-induced increases in dopamine release. Erich and colleagues explain the time dependence of KOR agonist/cocaine administration: when KORs are activated in close proximity to cocaine administration, a dysphoric effect occurs, as the KOR activation has blocked cocaine's rewarding properties; however, when they are activated at greater time differences (over 20 min apart), the KOR activation leads to a greater enhancement of cocaine's effects, possibly to overcome the dysphoric effect of the KOR activation. Previously, the Chavkin lab (McLaughlin et al., 2006)

similarly demonstrated that KORs effects on cocaine conditioned place preference (CPP) were time dependent; in addition, forced swim stress induced cocaine CPP was unaltered in KOR(-/-) mice as compared to WT controls, and the effect in WTs was blocked by the KOR antagonist norBNI (also seeMcLaughlin et al., 2003). These findings provide important insight into the time dependents of KOR-mediate stressors and the development of drug addiction; blocking KORs during stress may help prevent the development of further cocaine use.

Modulation of KORs in the NAC has potential therapeutic benefits for other drugs of abuse as well. The Koob lab (Schlosburg et al., 2013) found that pretreatment with norBNI prevented escalation in heroin intake seen in a long-access paradigm in rats, while also reducing anxiety-like behavior seen with withdrawal. This decrease in heroin self-administration was also seen when norBNI was administered site-specifically into the NAC shell (however, administration into the NAC core increased heroin self-administration, but not the escalation of intake).

Prefrontal cortex

Though much work has been done looking at the role of KORs in the main nuclei of the mesolimbic dopamine system (the NAC and VTA), other downstream dopaminergic projections, such as the PFC, are also modulated by KORs. KORs are known to be located presynaptically in the PFC (Svingos & Colago, 2002), and recently Tejeda and colleagues looked at this putative pathway in-depth. Tejeda et al. (2013)demonstrated that systemic administration of the KOR agonist U50,488 decreased extracellular dopamine levels in the medial PFC (without altering reuptake). Interestingly, they also demonstrated that KORs presynaptically inhibit

glutamate release onto pyramidal neurons. This provides a putative circuit for Bals-Kubik et al.'s (1993) findings that administration of a KOR agonist directly into the PFC causes a conditioned place aversion, possibly through inhibition of dopaminergic and glutamateric drive onto PFC neurons.

The amygdala and extended amygdala

The basolateral amygdala (BLA) and its downstream nuclei, the central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST) are enriched with both KOR and dynorphin, and thus are of increasing interest. The Carlezon group (Knoll et al., 2011) demonstrated that fear conditioning caused an upregulation of KOR mRNA in the BLA (but not CeA), and that site-specific administration of the KOR antagonist JDTic decreased conditioned fear (effective fear conditioning was associated with a reduction of KOR mRNA in the BLA). Interestingly, administration of JDTic to the BLA (but not CeA) produced an anxiolytic phenotype in the EPM. Bruchas and colleagues (Bruchas et al., 2009) previously published a similar anxiolytic effect of BLA KORs; they found that site specific administration of norBNI blocked a forced swim- or CRF-induced stress phenotype in the EPM. It has also been found that activation of KORs by U50,488 decreased synaptic local field potentials, as well as blocked high-frequency-induced long term potentiation (Huge et al., 2009). However, the mechanism of KORs in the BLA has not been investigated further.

Dynorphin containing neurons have been demonstrated in the CeA, where they occasionally co-express with CRF (Marchant et al., 2007). Following alcohol intake, sardinian alcohol preferring (SP) rats show increased preprodynorphin levels in the

CeA as compared to both alcohol naïve-SP rats and alcohol naïve-nonpreferring rats (Zhou et al., 2013). Work by Koob et al. and Roberto et al. has focused extensively on characterizing KORs here, and their interactions with alcohol. Gilpin et al. (2014) demonstrated that KOR activation by dynorphin decreased GABAergic transmission in the CeA, while the antagonist norBNI enhanced GABAergic transmission, indicating the presence of a tonic dynorphin tone. In addition, Gilpin et al. showed that KOR activation partially blocked the effects of ethanol at these synapses. This effect was complementary to results published the previous year (Kang-Park et al., 2013) demonstrated that KOR activation decreased GABAergic transmission in the CeA, and also demonstrating tonic activation of KORs, through a presynaptic mechanism. Interestingly, previous studies have shown that KOR activation decreased GABAergic transmission in naïve rats, consistent with Gilpin et al., while it increased GABAergic transmission in rats exposed to a long-access cocaine paradigm (Kallupi et al., 2013). The authors also found that site-specific administration of norBNI blocked cocaine-induced locomotor sensitization, as well as decreased withdrawal related behaviors. KORs in the CeA may prove to be an interesting target for the manipulation and treatment of alcohol consumption.

Though dynorphin and KORs are known to be present throughout the BNST (Poulin et al., 2009), very little work has been conducted in this region. Li and colleagues (Li et al., 2012) demonstrated that activation of KORs produced a robust inhibition of GABA transmission from the CeA, and that this inhibition was presynaptic and MEK/ERK signaling dependent. Despite this promising avenue of

investigation, no systematic work has been conducted to further assess KORs in the BNST, either at the pharmacological or behavioral level.

Hippocampus

KORs play an essential role in HIPP circuitry (Chavkin et al., 1985). Dynorphin has been shown to control glutamate transmission in the hippocampus (Wagner et al., 1993, Weisskopf et al., 1993), as well as increase cell excitability in dentate gyrus granule cells (McDermott & Schrader, 2011). Dynorphin is released from granule cells in the HIPP, and acts as a retrograde transmitter to inhibit excitatory inputs in the HIPP (Drake et al., 1994); for further reading see Chavkin (2000). In addition, dynorphin activates an inwardly rectifying potassium channel as well as voltage gated potassium channels (Wimpey & Chavkin, 1991), including functioning to inhibit M currents, a voltage-dependent potassium current, in CA1 neurons (Madamba et al., 1999). Administration of the KOR antagonist norBNI directly into the HIPP reversed ethanol-induced changes in glutamate transmission (Kuzmin et al., 2013). Bilkei-Gorzo et al. showed that mice lacking dynorphin show increased partner recognition; in addition, when exposed to object or social recognition paradigms, mice showed increased dynorphin-A immunoreactivity in the hippocampus, central amygdala, and basolateral amygdala (Bilkei-Gorzo et al., 2014). They have also demonstrated that mice lacking dynorphin show enhanced fear conditioning, known to involve the hippocampus (Bilkei-Gorzo et al., 2012) (however note differences between the effects seen by the Carlezon group with systemic administration of norBNI, discussed above). This work seems to indicate a potential protective effect of KORs in the HIPP. In addition, stress causes both KOR

and p38 activation in a variety of brain regions, including the hippocampus, and this was not seen in KOR $-/-$ mice (Bruchas et al., 2007). Similar experiments have shown that a single immobilization stress results in increased dynorphin-A immunoreactivity in the hippocampus as well as the nucleus accumbens (Shirayama et al., 2004). Shirayama et al. also demonstrated that infusion of the KOR antagonist norBNI directly into CA3 produced antidepressant effects. Recently, investigators have begun to elucidate the mechanism of KOR induced alterations in the HIPP. Previous work demonstrated that dynorphin was upregulated in the HIPP following inhibition of NMDA receptors, in a pCREB-dependent manner (Rittase et al., 2014), and others have shown that dynorphin inhibition of NMDA receptors appears to be pH dependent (Kanemitsu et al., 2003). Interestingly, KORs and dynorphin in the HIPP have also been heavily investigated for their potential role in epilepsy (Clynen et al., 2014, Dobolyi et al., 2014). Dynorphin levels are elevated in the HIPP following a kainic acid induced seizure in rats (Rocha & Maidment, 2003). Prodynorphin KO mice show decreased seizure threshold and faster seizure onset (Loacker et al., 2007). Administration of a KOR agonist prevents drug-induced seizures in mice, possibly indicating the presence of a regulatory dynorphin tone in the HIPP (Solbrig et al., 2006). In the clinical population, prodynorphin transcription is upregulated in patients with temporal lobe epilepsy (Pirker et al., 2009). In line with this information, researchers have speculated that KOR manipulations may be a potential treatment for seizures, treating abhorrent dynorphin release/KOR activation in the hippocampus (Bortolato & Solbrig, 2007). This exciting line of hippocampal KOR research provides a promising therapeutic outcome for KOR drugs.

INTERACTIONS WITH OTHER SIGNALING MOLECULES

Corticotrophin-releasing factor

It is difficult to discuss the role of KORs in brain functioning without discussing its interactions with the closely related peptide corticotropin-releasing factor (CRF). The interactions between the KOR and CRF systems have been investigated heavily (Koob, 2013, Land et al., 2008, Tejeda et al., 2010). The KOR and CRF systems have long been thought to interact in order to orchestrate anxiety-like responses. Like dynorphin, CRF is a neuropeptide released in response to stress (Koob, 1999). Dynorphin and CRF are co-expressed in the periventricular nucleus of the hypothalamus (Roth et al., 1983) as well as the hypothalamic supraoptic nucleus (Meister et al., 1990). Axon terminals in the locus coeruleus coexpress dynorphin and CRF, most likely arising from the dynorphin–CRF co-expressing neurons in the central amygdala (Reyes et al., 2008). In addition to this anatomical overlay between the two peptidergic systems, behavioral experiments have hinted at their interactions as well. For example, the KOR antagonist JDTic attenuates CRF-mediated performance errors in a five choice serial reaction time task (Van't Veer et al., 2012). Though much of the literature on stress-related effects focuses on the CRF-1R, an emerging importance has been shown for CRF-2R as well (Gysling, 2012), indicating that the two receptors may have differing interactions with the KOR system (CRF-1R and CRF-2R are commonly thought to mediate opposing responses, possibly through different locations in the synapse, see Fu and

Neugebauer, 2008). In the basolateral amygdala, CRF-1R mediated anxiety-like behaviors are dependent on the KOR system (Bruchas et al., 2009). Importantly, CRF-1R antagonists can block KOR-dependent reinstatement of cocaine seeking (Valdez et al., 2007), and antalarmin, a CRF-1R antagonist, blocked KOR-agonist induced reinstatement of alcohol seeking (Funk et al., 2014), highlighting not only how KOR–CRF systems interact in relation to stress and anxiety, but addiction as well. Other work has highlighted the importance of the CRF-2R. Activation of CRF receptors in the CeA increased dynorphin levels as measured by microdialysis; acute alcohol administration also increased dynorphin levels, but this was blocked by a CRF-2R antagonist (a CRF-1R antagonist had no effect) (Lam & Gianoulakis, 2011). In addition, injection of CRF produces a KOR-dependent place aversion, which was blocked by a CRF-2R antagonist but not a CRF-1R antagonist (Land et al., 2008). Supporting this, CRF-1R $-/-$ mice still show KOR-dependent place aversion (Contarino & Papaleo, 2005), although this study did not assess CRF-2R $-/-$ mice.

Taken together, CRF-1Rs may interact with KORs to mediate anxiety (and anxiety related to drug withdrawal and relapse), while CRF-2Rs may interact with KORs in a more nuanced manner, possibly related to aversion. This potential interaction between CRF and KOR systems provides further information for the development of therapeutic targets for stress and addiction related disorders, as the two peptidergic systems can be targeted simultaneously. Further work is necessary to elucidate the causal nature of the KOR–CRF systems (for example, KOR activation may induce CRF release, see Valdez et al., 2007), to better understand

their relationship (KOR–CRF, CRF–KOR, or bidirectional, and the importance of CRF-1Rs versus CRF-2Rs).

Glucocorticoids

A smaller literature has emerged looking at interactions between glucocorticoids and KORs. In non-rodent models, KOR agonists have been shown to bind to glucocorticoid receptors (Evans et al., 2000), and KORs are known to interact with the hypothalamic pituitary adrenocortical (HPA) axis (Iyengar et al., 1986, Iyengar et al., 1987). Prodynorphin mRNA in the hippocampus was decreased in adrenalectomized (ADX) mice, and the effect was rescued with dexamethasone (Thai et al., 1992); similar results were seen with aldosterone (Watanabe et al., 1995). This initial work points towards the interaction between glucocorticoids and the KOR dynorphin system, although much work is left to be done (particularly, studies should address this relationship outside of the hippocampus, in other regions related to the HPA axis, such as the extended amygdala).

CONCLUSION: ROLE OF KORs IN STRESS AND ANXIETY, POTENTIAL AS A NOVEL THERAPEUTIC TARGET

Despite the implication that KORs are involved in a wide range of psychiatric conditions, ranging from maladaptive disorders such as addiction and anxiety, to

basal states such as social interaction and learning and memory, their use as therapeutics in the clinic has thus far been limited.

In addition, though classical behavioral pharmacology literature has focused on the aversive or the anxiogenic properties of KOR agonists, the invention of novel and advanced techniques to probe the site-specific and molecular interactions with KORs may soon add much needed nuance to this global statement. Importantly, we hypothesize that it is unlikely that KORs in any single region work in isolation to modulate any given condition, such as anxiety. More likely, is that KORs throughout the brain modulate interactions between key brain regions involved in anxiogenic phenotypes (such as the interactions between the PFC, HIPPO, and NAC). This point is further supported by differences seen in experiments using global dynorphin KOs and systemic administration of the KOR antagonist norBNI: the dynorphin KOR system may function in discrete brain regions to regulate the anxiogenic–anxiolytic spectrum of behaviors by keeping behavioral responses in an optimal range; neither global deletion nor global blockade of components of the KOR system will function properly to modulate behavior. As KORs modulate many of the major circuits involved in psychiatric behaviors (Fig. 1.1), they may be functioning to dampen or enhance communication between key circuits involved in anxiety, as opposed to just taking offline a single nucleus. As further work probes these questions, it may emerge that dynorphins and KORs, in some cases, serve anxiolytic purposes depending on the functional status of the circuit.

While KOR antagonists have been at the forefront of treatment options for psychiatric illnesses, they are also implicated in a medley of other physiological

disorders, including salt consumption (Nascimento et al., 2012), epilepsy and hypertension (though hypertension is not discussed here, see Wright and Ingenito, 2001, Wright & Ingenito, 2003, Wright et al., 2000 for further reading). This brings together the importance of the “whole body” perspective when considering KOR drugs as therapeutic treatment for brain-related diseases. Drugs targeting KORs, both agonists and antagonists, have profound therapeutic potential, and more research is needed to understand this promising neuropeptide system.

As discussed above, targeting the KOR system is one of the most promising treatments for AUDs. This dissertation attempts to further advance our understanding of KOR involvement in this disease by focusing on KORs in the BNST, a key region of the extended amygdala related to both stress and addiction. The two primary goals were to (1) demonstrate the signaling and function of KORs in a key brain region, the BNST; and (2) demonstrate how functioning of KORs in the BNST is altered following an animal model of alcohol consumption.

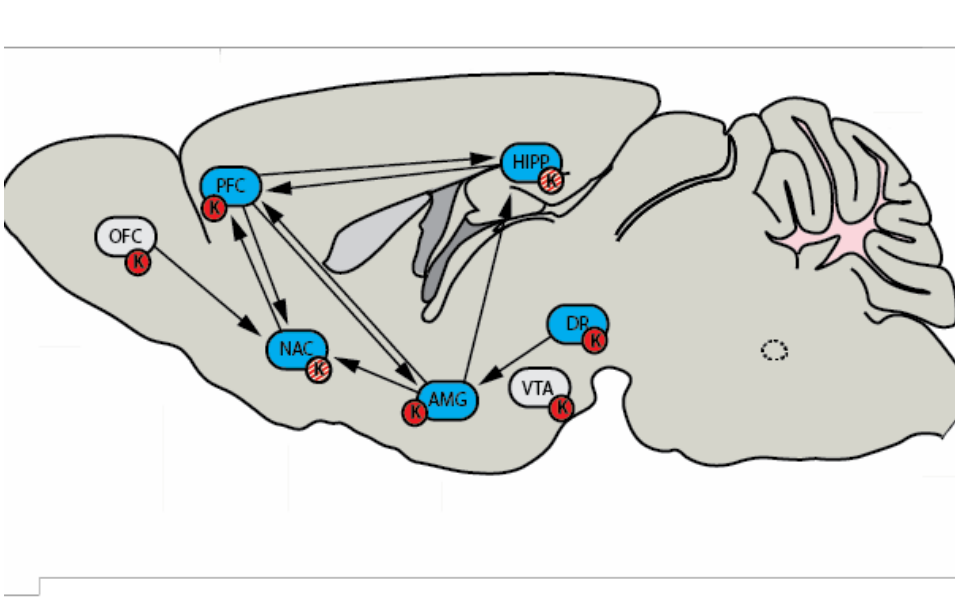


Figure 1.1 - KORs modulate the interactions between major circuits involved in stress responses, anxiety, and addiction. KORs are found in many key brain regions known to be involved in a variety of diseases, such as anxiety and addiction. It has been found that KORs do not only mediate aversive or anxiogenic responses (red circles); literature has shown that KORs (red and white circles) can also be reinforcing, such as in the nucleus accumbens. In addition, dynorphin knockout mice show increased fear conditioning. Key: Orbital frontal cortex (OFC); Pre frontal cortex (PFC); Nucleus accumbens (NAC); Amygdala and extended amygdala (AMG); Hippocampus (HIPP); Ventral Tegmental Area (VTA); Dorsal Raphe Nucleus (DR); Kappa Opioid Receptor (K).

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CHAPTER 2: KAPPA OPIOID RECEPTORS CONTROL THE GAIN OF AN AMYGDALAR ANXIETY CIRCUIT

INTRODUCTION

Anxiety disorders are a major health concern, with 7.3% of the global population suffering from an anxiety disorder at any given time (Lepine, 2002, Baxter et al., 2013). Despite the high societal cost of anxiety disorders (Lepine, 2002) many of the most common treatments, including tricyclic antidepressants, monoamine oxidase inhibitors, benzodiazepines, and selective serotonin reuptake inhibitors, have side effects that limit their utility (Ravindran, 2010). In light of these limitations, there has been a greater effort to discover new modulatory systems for the treatment of anxiety disorders (Holden, 2003, Tye et al., 2011, Johansen, 2013, Deisseroth, 2014). In order to develop new and more efficacious therapeutics, a more thorough understanding of the circuitry underlying anxiety disorders is required.

Kappa opioid receptors (KORs) have been proposed as a potential target for stress and anxiety disorders, as well as substance abuse disorders (Wee and Koob,

2010), and an abundance of behavioral pharmacology experiments support targeting KORs for these disorders. Findings have implicated recruitment of KOR signaling by its endogenous ligand dynorphin (Chavkin et al., 1982) as playing a key role in preclinical and clinical models of anxiety (Knoll et al., 2011); however the mechanism that underlies this effect and the circuitry involved has not yet been defined (Crowley and Kash). KOR modulation has been identified in key anxiety-related regions such as the dorsal raphe nucleus (Lemos et al., 2012), the ventral tegmental area (Spanagel et al., 1992), and the prefrontal cortex (Svingos and Colago, 2002, Tejeda et al., 2013). Despite evidence that all of these regions interact with the bed nucleus of the stria terminalis (BNST), a key region involved in anxiety-related behaviors (Kash, 2012), investigation of KORs in the BNST has so far been lacking. In addition, the BNST is known to express dynorphin (Poulin et al., 2009), making it a promising and important target for neuropsychiatric manipulations. However, to date there has been little investigation into this key dynorphin-rich population due to a lack of available tools to investigate peptidergic release. Therefore, despite the promise of BNST KORs as a therapeutic target, this line of research has attracted little emphasis.

Based on the abundance of evidence implicating KORs in anxiety disorders and the BNST's critical role for regulation of anxiety-like behavior in both humans and rodent models, we hypothesized that KOR signaling in the BNST played an important role in the modulation of glutamate transmission and that dynorphin neurons within the BNST were involved in the microcircuitry of KOR activation. Here, we report that KORs in the BSNT inhibit glutamatergic transmission via a

pharmacologically distinct mechanism, and that this inhibition is pathway-specific; furthermore, we identify a novel technological approach to assessing peptidergic transmission in slice.

MATERIALS AND METHODS

Subjects

All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill, conforming to US National Institutes of Health guidelines. Experiments were performed on adult male C57BL/6J mice and DBA/2J mice, both from Jackson Laboratory (Bar Harbor, ME). *Preprodynorphin-IRES-Cre* mice were generated as described previously, and bred in house at UNC (Krashes et al., 2014). Conditional KOR knock out mice (KOR KO), generated as described below, were bred in house at UNC. All mice were group housed in colony rooms with a 12:12hr light-dark cycle (lights on at 7 a.m.) with ad libitum access to rodent chow and water.

Generation of conditional KOR knockout mice

Mice were generated by Dr. Jennifer Whistler (UCSF) and bred at UNC. To generate these mice, a cassette containing G418 resistance flanked by 2 loxP sites was inserted in the intron downstream of the region to be disrupted for positive

selection of the ES cells. Putative homologous clones were selected and screened by DNA blot. Homologous recombinant ES clones were transfected with a cre-recombinase plasmid. PCR was used to identify Type 1 (non-conditional) alleles, in which loxP sites 1 and 3 were recombined (see **Fig 2.3**) and Type 2 (conditional) alleles, in which loxP sites 2 and 3 were recombined. C57/Bl6 blastocysts were generated from both type 1 and type 2 clones and implanted into C57/Bl6 mice (all resulting mice are therefore purebred C57/Bl6). F1 progeny were genotyped for transmission of the mutant allele by PCR. Disruption of receptor was confirmed by ligand binding (data not shown).

Slice electrophysiology

We performed whole-cell electrophysiology experiments similar to those published (Holmes et al., 2012, Li et al., 2012). All experiments within the BNST were performed in the dorsolateral portion (both oval and non-oval nuclei). Briefly, 300 μ M coronal slices containing the BNST, prefrontal cortex (PFC), paraventricular nucleus of the hypothalamus (PVN), or BLA were prepared on a vibratome (Leica VT1200, Leica, Wetzlar, Germany) from behaviorally naïve mice rapidly decapitated under isoflurane anesthesia. The brains were removed and placed in ice-cold modified high sucrose artificial cerebrospinal fluid (aCSF) containing the following (in mM): 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl₂, 1 MgCl₂, 1.2 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃. Slices were then transferred to normal aCSF maintained at approximately 30 degrees (Warner Instruments, Hamden, Connecticut) containing

the following (in mM): 124 NaCl, 4.4 KCl, 2 CaCl₂, 1.2 MgSO₄, 1 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃. Slices were placed in a holding chamber, were allowed to rest for one hour, and remained there until used. Slices were continuously bubbled with a 95% O₂ / 5% CO₂ mixture throughout slicing and experiments. Thin-walled borosilicate glass capillary recording electrodes (3–6 MΩ) were pulled on a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA). Following rupture of the cell membrane, cells were allowed to rest and equilibrate to the intracellular recording solutions (below). For drug experiments, application bars on Figures indicate when aCSF was switched to aCSF+drug; there is a delay of approximately two minutes between the switch and when the drug reached the slice chamber. Input resistance was monitored continuously throughout the experiment, and when it deviated by more than 20% the experiment was discarded.

Lidocaine *N*-ethyl bromide (1 mg/ml) was included in the intracellular recording solution to prevent postsynaptic sodium spikes for all voltage-clamp experiments. For basal KOR pharmacological effects and characterization, picrotoxin (5 μM) was added to aCSF to isolate excitatory postsynaptic currents (EPSCs). Tetrodotoxin (500nM) and picrotoxin (5 μM) were added to the aCSF to isolate miniature EPSCs. Cells were held at -70mV to isolate AMPAR-mediated current, and EPSCs were recorded with a Cs-gluconate based intracellular recording solution containing the following (in mM): 135 cesium gluconate, 5 NaCl, 10 HEPES, 0.6 EGTA, 4 ATP, 0.4 GTP. For voltage-clamp experiments requiring the simultaneous recording of excitatory and inhibitory events within the same neuron, a cesium-methanesulfonate based intracellular recording solution containing the following (in mM) was used: 135

cesium methanesulfonate, 10 KCl, 1 MgCl₂, 0.2 EGTA, 2 QX-314, 4 MgATP, 0.3 GTP, 20 phosphocreatine. Excitatory events were recorded at -55mV and inhibitory events were recorded at +10mV. For current clamp experiments, cells were recorded using a potassium-gluconate based internal recording solution containing the following (in mM): K-gluc, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.6 EGTA, 4 Na₂ATP, 0.4 Na₂GTP. Experiments were conducted both at resting membrane potential (RMP) and -70mV.

PSCs were electrically-evoked using a twisted bipolar nichrome wire placed dorsal to the recording electrode. For drug experiments, the following concentrations were used: 300nM Dynorphin-A, 1μM U69,593, 100nM norBNI, 5μM RP-Camps, 20μM SB203580, 10μM SL-327. Drugs were applied as indicated in Figures; for experiments that required pre-application of a drug (such as KOR antagonist experiments), the drug was bath applied for at least 30 minutes prior to experiments.

For *in vitro* optogenetic experiments, all brains were checked for light-evoked action potentials in the target region (BLA, PFC, PVN, or BNST) using a potassium-gluconate-based internal recording solution containing in mM: 135 K-gluc, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.6 EGTA, 4 Na₂ATP, 0.4 Na₂GTP. Brains were discarded and not used for further experimentation if action potentials were not obtained or injections were missed. A blue LED (470nm, CoolLed, Hampshire, United Kingdom) was used to optically stimulate release from channelrhodopsin (ChR2)-containing fibers (5 msec pulse for voltage-clamp experiments, 1 msec for current clamp experiments). Following completion of some experiments, NBQX (10μM) was applied to confirm glutamate exclusivity, or 5μM picrotoxin to confirm GABA

exclusivity, of the optically evoked current (data not shown). For experiments isolating the local effects of dynorphin activation, non-ChR2 (putatively DYN-) cells were patched.

Signal acquisition was performed identically to those published previously (Holmes et al., 2012, Li et al., 2012).

Stereotaxic surgery

Mice were anaesthetized in an induction chamber (3-4% isoflurane) and placed into a stereotaxic frame (Leica Angle 2, Leica Biosystems) where they were maintained at 1-2% isoflurane. A craniotomy was performed, and mice were bilaterally injected using a blunt needle (86200 and 65458-01, Hamilton Company, Reno, NV), with 400-500 nl of the vector into the BLA (stereotaxic coordinates from bregma: -1.30 anterior-posterior, \pm 3.15 medial-lateral, -4.95 mm dorsal-ventral), 350 nl of the vector into the BNST (stereotaxic coordinates from bregma: +0.27 anterior-posterior, \pm 0.90 medial-lateral, -4.25 dorsal-ventral), or 400-500 nl of the vector into the PFC (stereotaxic coordinates from bregma: +1.8 anterior-posterior, \pm 0.3-0.5 medial-lateral, -2.5 dorsal-ventral). For floxed KOR experiments, a ChR2/CRE cocktail was made (250nL ChR2, 250nL CRE) and injected into the BLA.

Aseptic techniques were maintained for all surgeries, and mice were allowed to recover for at least six weeks prior to experiments, permitting optimal expression in terminals.

Viral vectors

The viral constructs AAV2-CamKII α -ChR2-EYFP, AAV2-CamKII α -EYFP, AAV5-EF1 α -DIO-ChR2-EYFP, AAV5-EF1 α -DIO-EYFP, AAV5-EF1 α -DIO-ChR2-mCHERRY, and AAV2-CamKII α -CRE-EYFP, described elsewhere (Kim et al., 2013) were obtained from the UNC Viral Vector Core (Chapel Hill, NC).

Data analysis and statistics

Data are expressed as means \pm SEM for all Figures. For all experiments, 2-way ANOVAs, paired *t*-tests, unpaired *t*-tests, and linear regression were used where appropriate, as described in Figure captions. Statistical analyses were conducted using Prism 6.0 (GraphPad, La Jolla, CA), and graphs were made in Illustrator CC 2015 (Adobe, San Jose, CA).

RESULTS

KORs signal via a presynaptic, p38- and calcium-dependent mechanism to inhibit glutamate release in the BNST

Recent work links glutamate signaling in the BNST to both anxiolytic and anxiogenic behavior (Kim et al., 2013, Hubert & Muly, 2014). Therefore, we first

examined whether KOR activation could alter glutamate function in the BNST. We found that multiple KOR-selective agonists (Dynorphin-A and U69,593) inhibited evoked excitatory post synaptic currents (eEPSCs) in the BNST, both of which were blocked by pre-application of the selective KOR antagonist norBNI (**Fig 2.1A-D**). This KOR-mediated inhibition was not reversed by norBNI (**Fig 2.1E-F**), suggesting that this is a form of long-term plasticity. The KOR effect was blocked in the presence of the p38 inhibitor SB203580 (**Fig 2.1G**), but not the MEK/ERK inhibitor SL-327 (**FIG 1G**) or the PKA inhibitor RP-Camps (**Fig 2.1H**). Mechanistically, KORs inhibited function presynaptically, as there was a reduction in miniature EPSC frequency but not amplitude (**Fig 2.1I-K**); in addition, the kinetics of miniature EPSCs remained unaltered (data not shown). This KOR modulation of mEPSCs was absent when recordings were conducted in zero calcium conditions similar to those previously published (**Fig 2.1L-M**). This presynaptic, p38-, and calcium- dependent modulation of neurotransmitter release is consistent with known KOR signaling cascades (Brust et al., 2006, Bruchas, 2011) (**Fig. 2.1N**). In addition, the nature of the inhibition suggests a form of long-term depression (LTD) as has been seen in the striatum with modulation of opioid receptors (Atwood et al., 2014).

KORs inhibit pathway-specific glutamate inputs to the BNST

Glutamatergic innervation to the BNST arises from multiple cortical and subcortical nuclei. We next probed KOR inhibition of two of these known pathways, the prefrontal cortex (PFC) and the basolateral amygdala (BLA) in slice. We injected

ChR2 (AAV2-CamKII α -ChR2-EYFP) in either the BLA or PFC and conducted slice electrophysiology experiments in the BSNT (**Fig 2.2A**). Independent activation of either of these pathways produced a robust, light-evoked EPSC, consistent with other studies examining BLA projections (Felix-Ortiz et al., 2013) (**Fig 2.2B-D**). The light-evoked BLA EPSC was inhibited by KOR activation, while the light-evoked EPSC arising from the PFC was not altered by KOR activation (**Fig 2.2E**).

Optogenetic activation of the BLA input to the BNST produced action potentials in BNST neurons reliably up to 20-40 hz (**Fig 2.2F**). As we have previously shown that KOR activation can inhibit GABA release (Li et al., 2012), we wanted to test whether activation of KOR signaling would lead to a reduction of BLA-induced action potentials. Indeed, consistent with a net inhibitory action of KOR on this pathway, the KOR agonist reduced the fidelity of these BLA-BNST induced action potentials. This robust KOR-mediated inhibition of BLA-BNST circuitry allowed us to further probe the synaptic dynamics of KOR inhibition. KOR-mediated inhibition of the BLA input was absent when KORs were genetically deleted from the BLA, providing converging support for the presynaptic site of action (**Fig 2.3A**). Here, we virally deleted KORs expressed on BLA neurons (AAV2-CamKII α -CRE-EYFP), while simultaneously expressing ChR2 (AAV2-CamKII α -ChR2-EYFP). Though deletion of BLA KORs had no effect on spontaneous EPSC frequency or amplitude in the BNST (**Fig 2.2G-H**), suggesting basal glutamate properties remained intact, there was also no longer inhibition of the optically evoked BLA EPSC, suggesting KORs are exclusively expressed presynaptically at these synapses, and their deletion completely abolishes any KOR-mediated alterations at these synapses (**Fig 2.2I**).

Taken together, our data demonstrate that KORs in the BNST inhibit pathway-specific glutamate release, and that this inhibition has important behavioral implications for the modification of anxiety-related phenotypes.

Dynorphin is a retrograde messenger in the BNST, and dynorphin neurons are preferentially modulated by KORs on BLA to BNST synapses.

We next assessed the dynamics of endogenous dynorphin release in the BNST. In order to probe the activation dynamics of this system, we stereotactically injected ChR2 using a cre-dependent vector (AAV5-EF1 α -DIO-ChR2-EYFP) in the BNST of *Preprodynorphin-IRES-Cre* mice (Krashes et al., 2014) (**Fig 2.4A**).

Dynorphin-positive (DYN+) neurons were located throughout the dorsal-lateral BNST (**Fig 2.4A**). Light activation of these cells reliably produced action potentials at 20 Hz and resulted in a monosynaptic IPSC (mean amplitude = 422.9pA, SEM = 149.0), but no EPSC (mean amplitude = -17.64pA, SEM = 6.414) (**Fig 2.4B**) onto neighboring, putatively non-dynorphin (DYN-) neurons, indicating BNST DYN+ neurons form robust local synapses that are exclusively GABAergic. We next probed whether optogenetic activation of these DYN+ neurons was capable of altering electrically-evoked glutamate release within the BNST. 5 hz activation of DYN+ neurons for 150 seconds produced a transient norBNI-sensitive inhibition of eEPSCs (**Fig 2.4C**), and EPSC amplitude returned to basal amplitudes. When these experiments were conducted with a 20 hz stimulation for 150 seconds, a robust and lasting inhibition of eEPSCs was found (**Fig 2.4D**); this inhibition was blocked by pre-

and continuous- application of the selective KOR antagonist norBNI. This effect was indistinguishable from that seen with KOR agonist application (**Fig 2.1A-D**). This effect was not seen when assessing a GABAergic dynorphin projection to the BNST from the paraventricular nucleus of the hypothalamus (PVN) (**Fig 2.4E**). 20 hz activation of PVN-BNST dynorphin neurons did produce an inhibition of eEPSCs, but this inhibition was not KOR-dependent, implying that another modulatory system may be playing a role in this distal inhibition by dynorphin neurons (Bodnar, 2013). Taken together, these experiments demonstrate that activation of local BNST dynorphin neurons can inhibit electrically-evoked glutamatergic transmission in the BNST. Additionally, these experiments suggest a possible timing-dependent component in the persistence of KOR plasticity, similar to what has been demonstrated with norepinephrine-induced LTD in the BNST (McElligott & Winder, 2008).

We next assessed the relationship between BLA inputs to the BNST and BNST dynorphin neurons. ChR2 (AAV2-CamKII α -ChR2-EYFP) was injected into the BLA and cre-dependent fluorophore (AAV5-EF1 α -DIO-EYFP) was injected into the BNST of *Preprodynorphin-IRES-Cre* mice (**Fig 2.4F**) to assess the BLA inputs onto DYN+ and DYN- neurons. DYN+ neurons had smaller membrane resistance than their DYN- counterparts, and a trend towards a significant difference in capacitance, consistent with known classifications of BNST neuronal types (Hammack et al., 2007) (**Fig 2.5A-B**). DYN- neurons had more action potentials per current injection when held at RMP, indicative of greater intrinsic excitability (**Fig 2.5C-K**). Surprisingly, both DYN+ and DYN- neurons received a glutamate projection from the

BLA, as well as a polysynaptic GABAergic projection, but no basal differences were seen with BLA innervation of these cells as indicated by similar paired pulse ratio and amplitude of evoked responses (**Fig 2.5L-O**). We next examined KOR modulation of BLA inputs to these two discrete cell types. We found that KOR activation more robustly inhibited BLA-induced light-evoked EPSCs in DYN+ neurons as compared to DYN- neurons (**Fig 2.4G**). Taken together, these data suggest that there is both pathway- and cell-type dependent modulation of glutamate function in the BNST by KOR, allowing for important gating of glutamate transmission at DYN+ and DYN- neurons.

DISCUSSION

The BNST has been shown to orchestrate both rewarding and aversive behaviors (Jennings et al., 2013). KORs have historically been thought to modulate aversive (or negatively regulate rewarding) systems (Chefer et al., 2005) or anxiety related behaviors (though some important exceptions exist (Castro & Berridge, 2014)), placing KORs at the interesting and likely position of modulating function in the BNST. First, we demonstrate that KOR activation in the BNST modulates glutamatergic inputs by a presynaptic, p38-, and calcium- dependent mechanism, and that this modulation occurs at BLA-BNST synapses but not PFC-BNST synapses. Previous work has shown p38-dependent effects of KOR modulation in other brain regions, such as the dorsal raphe nucleus (Lemos et al., 2012), and

others have postulated that this intracellular signaling pathway may be critical for the dysphoric effects of KOR agonists (Bruchas et al., 2007). This is, therefore, a pharmacologically distinct mechanism of KOR inhibition of glutamate that is separate from KOR effects on GABAergic transmission in the same region (Li et al., 2012). Differential control by KORs has been demonstrated in the nucleus accumbens (Hjelmstad & Fields, 2003), and these pharmacologically distinct signaling mechanisms may play a key role in the development of biased synapse targeting.

In addition to a pharmacologically distinct mechanism of KOR inhibition, we also demonstrate a pathway-specific inhibition of glutamatergic inputs to the BNST. This mechanism may provide for gating of information flow both to and from the BNST. We also demonstrate a mechanism by which KORs are activated: GABAergic/dynorphin co-expressing neurons in the BNST release dynorphin via a (likely retrograde) mechanism to presynaptically inhibit glutamate inputs to the BNST. This signaling mechanism through which dynorphin regulates transmission may be a common motif throughout the brain: it was originally postulated to be the mechanism of inhibition in the hippocampus (Drake et al., 1994), and, more recently, was shown to be the mechanism of inhibition in the PVN (Iremonger & Bains, 2009). We not only show similar findings in the BNST, but we expand upon this literature by showing a previously unused optogenetic mechanism for activation of such release. This allows for peptidergic and optogenetic coupling, greatly expanding upon the ability to assess peptide release both *in vitro* and *in vivo*.

Importantly, we found this KOR inhibition exhibited both cell-type and pathway-dependent differences in function. Though BLA inputs to the BNST show similar

properties onto both DYN+ and DYN- neurons, there is a greater KOR-mediated inhibition of BLA-DYN+ synapses versus BLA-DYN- synapses. These results suggest a model in which activation of the BNST by the BLA excites DYN+ neurons, and this activation leads to a local retrograde release of dynorphin (**Fig 2.6A-B**). This dynorphin release then gates inputs to the BNST, providing a homeostatic balance within the system during times of heightened activity, suggesting a mechanism by which KOR antagonists exhibit anxiolytic actions. In addition, this further illuminates the micro-circuitry of the BNST and how it may coordinate such a broad range of behavioral states, such as those involved in stress and addiction. Further, this raises a new strategy for development of anxiolytic compounds—blocking endogenous inhibitors of defined circuits that reduce anxiety.

Further work is necessary to elucidate the downstream effects of BNST dynorphin neurons. Specifically, an understanding is needed of their projection and activation patterns. This work provides an important framework to begin that research: though the circuitry of DYN+ and DYN- neurons may be equal, their activation and inhibition may not be.

Taken together, this work demonstrates a pathway specific, p38- and calcium-dependent form of KOR inhibition of glutamate. It also demonstrates a mechanism by which retrograde dynorphin release may mediate this presynaptic effect. Finally, this work shows that KORs may preferentially inhibit glutamatergic inputs onto DYN+ neurons, versus DYN- neurons, allowing for specific gating of information flow. This circuit-based approach has numerous advantages over existing approaches, as it allows unprecedented precision in defining the substrate and mechanism of action.

Given the lack of efficacious and well-tolerated anxiolytic medications currently available, this work introduces a novel, site-specific manipulation of the mammalian anxiety system.

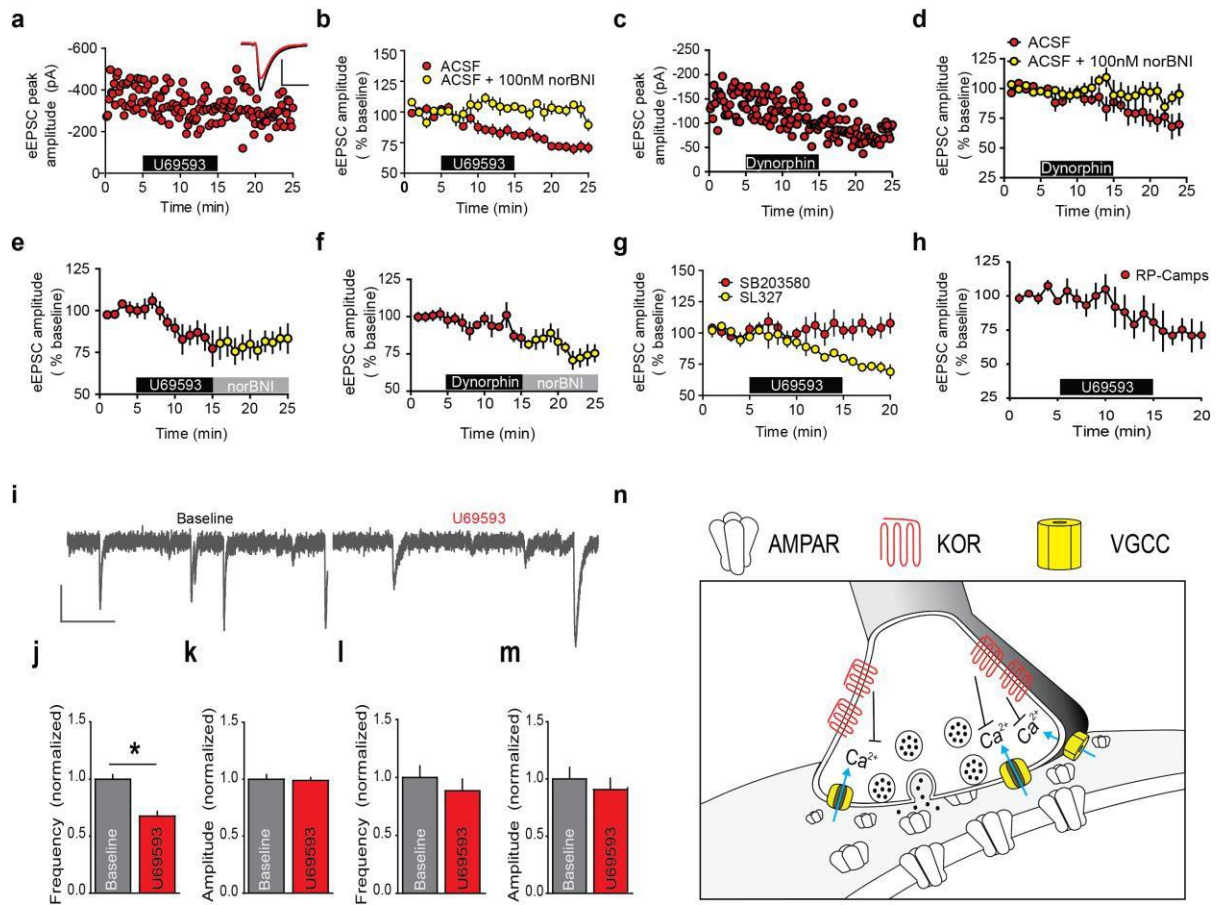


Figure 2.1 - KOR activation inhibits glutamate transmission in the BNST. (A)

Representative experiment demonstrating KOR-mediated inhibition of eEPSC

amplitude. Inset, eEPSC trace from the same neuron showing pre (black) and post

(red) 1μM U69,593 application. Scale bar represents 200pA by 20msec. **(B)** KOR

activation by U69,593 inhibited eEPSC amplitude (red circles, paired *t*-test, baseline

v. min 21-25, $t_4 = 30.70$, $P < 0.001$) and was blocked by continuous application of the

KOR antagonist norBNI, 100nM (yellow circles, paired *t*-test, baseline v. min 21-25,

$t_4 = 0.003$, $P > 0.05$); the KOR agonist U69,593 significantly inhibited eEPSC

amplitude as compared to the norBNI block effect (unpaired *t*-test, acsf v. norBNI,

min 16-20, $t_8 = 10.22$, $P < 0.001$). **(C)** Representative experiment demonstrating

KOR inhibition by 300nM Dynorphin-A. **(D)** KOR activation by 300nM Dynorphin-A produces a robust inhibition of eEPSCs (red circles, paired *t*-test, baseline v. min 21-25, $t_4 = 18.65$, $P < 0.001$) that is blocked by the KOR antagonist norBNI (yellow circles, paired *t*-test, baseline v. min 21-25, $t_4 = 2.783$, $P = 0.05$) mimicking the results seen with U69,593 (Fig. 1). Both U69,593 **(E)** and Dynorphin-A **(F)** activation of KORs are non-reversible forms of inhibition. Post U69,593 application of the KOR antagonist norBNI (100nm) failed to reverse the inhibition by either KOR agonist (U69,593, paired *t*-test, baseline v. min 21-25, $t_4 = 13.88$, $P < 0.001$; Dynorphin-A, paired *t*-test, baseline v. min 21-25, $t_4 = 14.30$, $P < 0.001$). **(G)** The p38 inhibitor SB203580 (10 μ M) but not the MEK/ERK inhibitor SL-327 (20 μ M) blocked KOR-mediated inhibition of eEPSCs (SB203580 effect, baseline v. min 16-20, $t_4 = 2.619$, $P > 0.05$; SL-327 effect, baseline v. min 16-20, $t_4 = 14$, $P < 0.0001$). **(H)** The PKA inhibitor RpCamps (5 μ M) does not alter KOR-mediated inhibition of eEPSCs (paired *t*-test, baseline v. min 16-20, $t_4 = 10$, $P = 0.0004$) **(I)** Representative mEPSC trace pre (left) and post (right) U69,593 application, conducted in 500nM TTX and 5 μ M picrotoxin. No significant changes in mEPSC decay kinetics were seen, (not shown, paired *t*-test, $t_6 = 0.8170$, $P > 0.1$). **(J)** mEPSC frequency (paired *t*-test, $t_5 = 5.567$, $P < 0.001$) but not amplitude **(K)** (paired *t*-test, $t_5 = 0.2141$, $P > 0.1$) was reduced following application of the KOR agonist U69,593 Raw values for mEPSC frequency (not shown, baseline frequency mean = 3.406, SEM = 3.475; post-U69,593 frequency mean = 2.179, SEM = 1.118) and amplitude (not shown, baseline amplitude mean = -29.36, SEM = 1.944, post-U69,593 amplitude mean = -25.71, SEM = 1.465). This inhibition is abolished in zero calcium aCSF, where both

frequency (**L**) (paired t -test, $t_5 = 1.959$, $P > 0.05$) and amplitude (**M**) (paired t -test, $t_5 = 2.017$ $P > 0.05$) of mEPSCs remain unaltered by U69,593. (N), model of KOR signaling at BNST glutamatergic synapses. Activation of KORs inhibits presynaptic glutamate release, likely through a calcium-dependent mechanism.

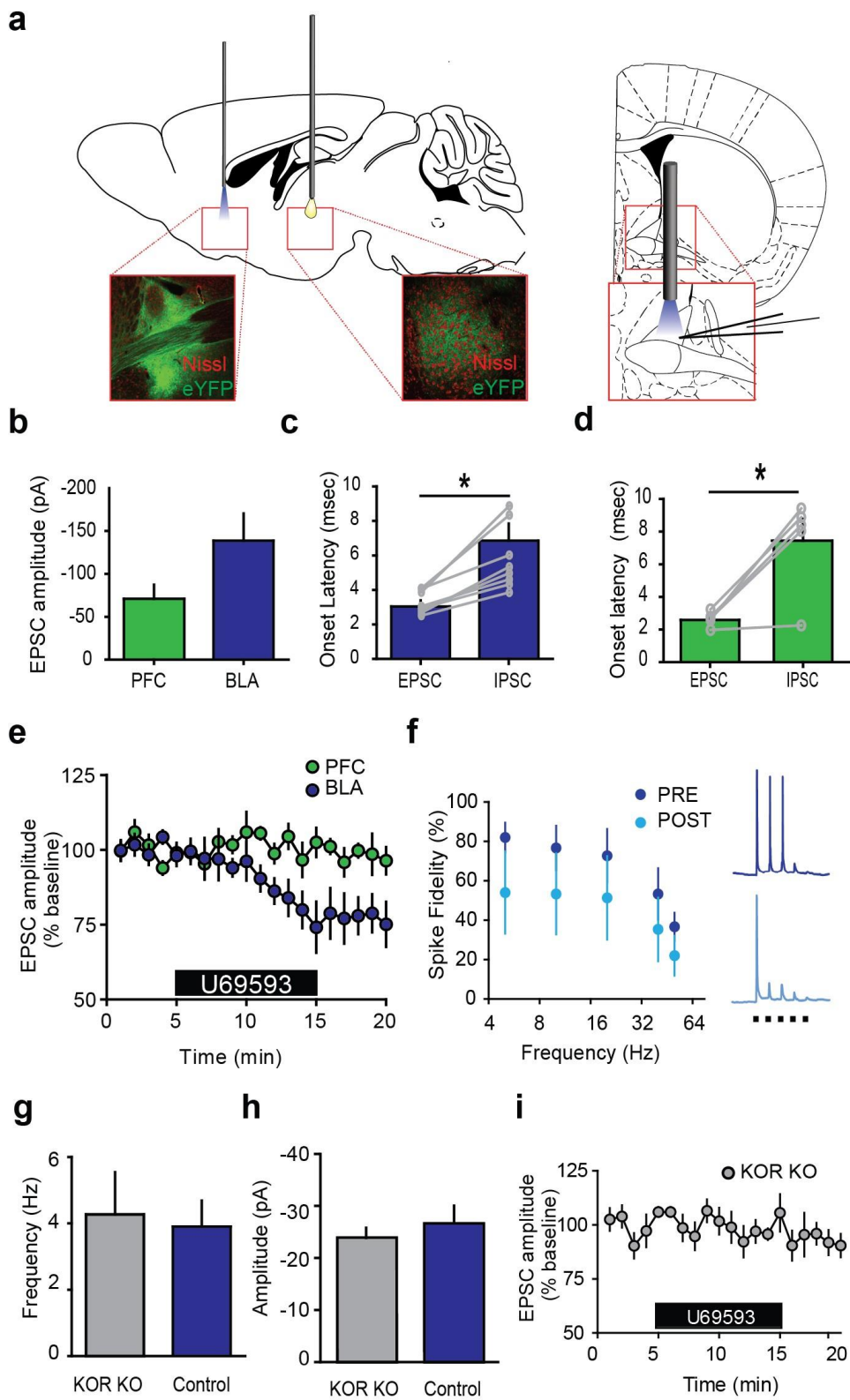


Figure 2.2 - KOR-mediated inhibition of eEPSCs is pathway specific. (A) Left, localization of ChR2 viral injection (BLA) and terminal expression (BNST); right, site of in vitro ChR2 activation and whole-cell recording. **(B)** Both the PFC and BLA send robust glutamatergic projections to the BNST, which do not differ in amplitude (unpaired t -test, $t_8 = 1.901$, $P > 0.05$). **(C)** The BLA sends a monosynaptic EPSC and significantly time-delayed polysynaptic IPSC (paired t -test, $t_7 = 5.232$, $P < 0.01$), comparable to BLA projections to other outputs. **(D)** Similarly, the PFC sends a monosynaptic EPSC and a significantly time-delayed polysynaptic IPSC (paired t -test, $t_4 = 4.138$, $P < 0.05$). **(E)** KOR activation inhibits BLA-BNST light-evoked EPSC amplitude (paired t -test, baseline v. min 16-20, $t_4 = 14.86$, $P < 0.0001$). However, KOR application did not alter PFC-BNST light-evoked EPSC transmission (paired t -test, baseline v. min 16-20, $t_4 = 0.6899$, $P > 0.1$). **(F)** U69,593 reduces the fidelity of light-evoked BLA-BNST action potentials. When fitted with standard linear regression, the slope was significantly non-zero predrug application ($F_{1,28} = 12.86$, $P < 0.01$) but not post ($F_{1,28} = 2.472$, $P > 0.05$). The lines also showed significant different intercept points ($F_{1,57} = 5.51$, $P < 0.05$). Inset, representative traces of action potential fidelity at 20hz 5msec light stimulation pre (dark blue) and post (light blue) U69,593 application. Black boxes indicate light pulses. **(G)** BLA KOR-KO mice show similar spontaneous glutamate transmission to control mice, with no differences in sEPSC frequency (unpaired t -test, $t_9 = 0.2308$, $P > 0.05$) or **(H)** amplitude (unpaired t -test, $t_9 = 0.7782$, $P > 0.05$). **(I)** Deletion of KORs from BLA neurons prevented the KOR-mediated inhibition of BLA-BNST synapses, confirming that KORs act presynaptically to inhibit evoked glutamate release. Mice were

injected with ChR2 (AAV2- CamKII α -ChR2-EYFP) and CRE (AAV2-CamKII-CRE-GRP) into the BLA, and light-evoked EPSCs were recorded in the BNST. U69,593 no longer inhibited light-evoked EPSCs (paired t -test, baseline v. min. 16-20, $t_4 = 1.474$, $P > 0.1$).

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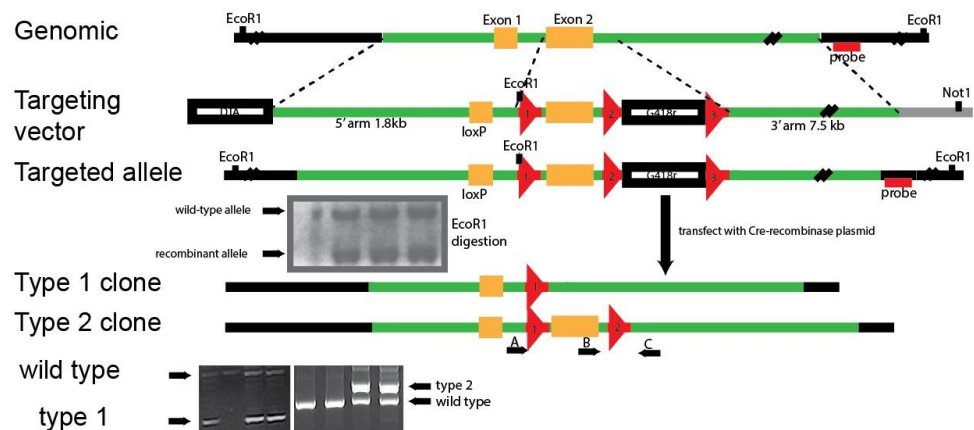


Figure 2.3 - Generation of conditional KOR knockout mice.

(A) Generation of conditional KOR knockout mouse. Mice were generated with non-conditional (type 1) and conditional (type 2) disruption of the KOR gene. The KOR type 1 mice are deleted for exon 1 and type 2 mice have exon 1 flanked by loxP sites. Insets show DNA blots of homologous recombination events. These clones were transfected with Cre recombinase and PCR screening of individual ES clones revealed type 1 (which produces non-conditional mutants) and type 2 (which produces conditional knock-outs) recombination events (lower insets).

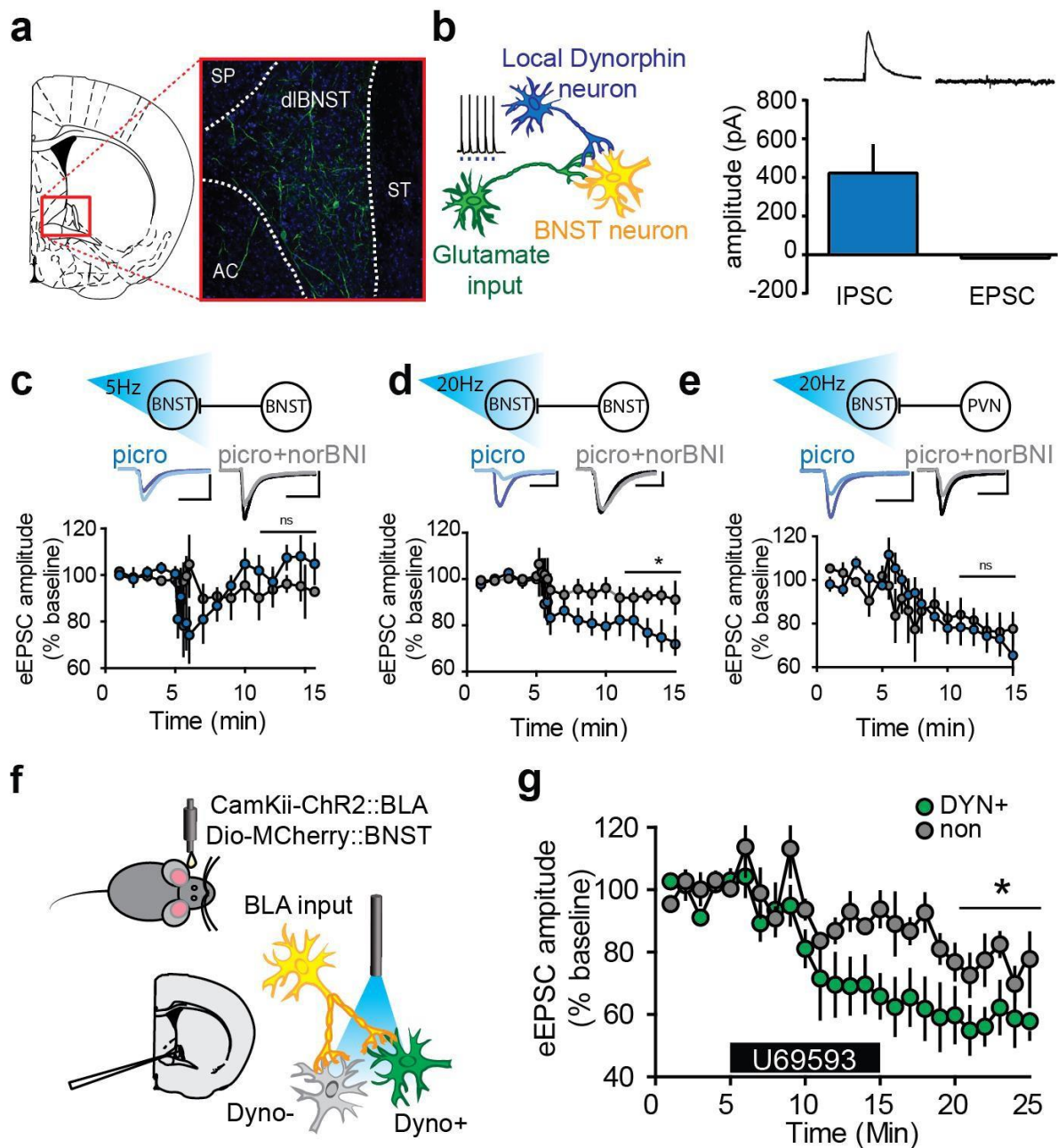


Figure 2.4 - Optogenetic activation of BNST dynorphin neurons inhibits eEPSCs on DYN- neurons, and KORs preferentially inhibit glutamate release onto DYN+ neurons. (A) Localization of cre-driven ChR2 viral injection to the BNST of *Dyno-Cre* mice. DYN+ cells were found throughout the dorsal BNST **(B)** Left, local

dynorphin cells showed reliable light evoked action potentials at 20 hz. These cells synapsed locally, producing a light-evoked IPSC of approximately 423 pA, but no light-evoked EPSC. **(C)** 5 hz stimulation of DYN+ neurons produced a significant transient change in eEPSC amplitude (paired *t*-test, baseline v. min. 5-8, $t_4 = 6.033$, $P < 0.05$), which returned to baseline (paired *t*-test, baseline v. min 11-15, $t_4 = 2.474$, $P > 0.05$). **(D)** 20 hz stimulation of local DYN+ neurons produces a significant and lasting inhibition of eEPSC (paired *t*-test, baseline v. min 11-15, $t_4 = 10.42$, $P < 0.001$). This effect is partially blocked by norBNI (unpaired *t*-test acsf v. norBNI block, min 16-20, $t_8 = 10.59$, $P < 0.0001$). **(E)** 20 hz optogenetic stimulation of PVN to BNST DYN+ neurons produces a significant inhibition of eEPSC (paired *t*-test, baseline v. min 11-15, $t_4 = 8.566$, $P < 0.001$) but this was not significantly different from the inhibition seen when in the presence of the KOR antagonist norBNI (unpaired *t*-test, acsf+picROTOXIN v. acsf+picROTOXIN+norBNI, $t_8 = 1.191$, $P > 0.1$). **(F)** Mice were injected with a cre-inducible mCherry (AAV5-EF1 α -DIO-mCherry) to the BNST, and ChR2 (AAV2-CamKII α -ChR2-EYFP) to the BLA. Optogenetic activation of BLA-BNST DYN+ and DYN- neurons was then assessed. **(G)** Application of the KOR agonist U69,593 significantly reduced the BLA-BNST eEPSC amplitude onto both DYN+ (paired *t*-test, baseline v. min 21-25, $t_4 = 12.14$, $P < 0.001$) and DYN- neurons (paired *t*-test, baseline v. min 21- 25, $t_4 = 9.039$, $P < 0.001$). However, the effect of KOR-mediated inhibition was significantly larger on DYN+ neurons as compared to DYN- neurons (unpaired *t*-test, DYN+ min 21-25 v. DYN- 21-25, $t_8 = 7.071$, $P < 0.001$), indicating KOR activation in the BNST may preferentially inhibit BLA-DYN+ neurons.

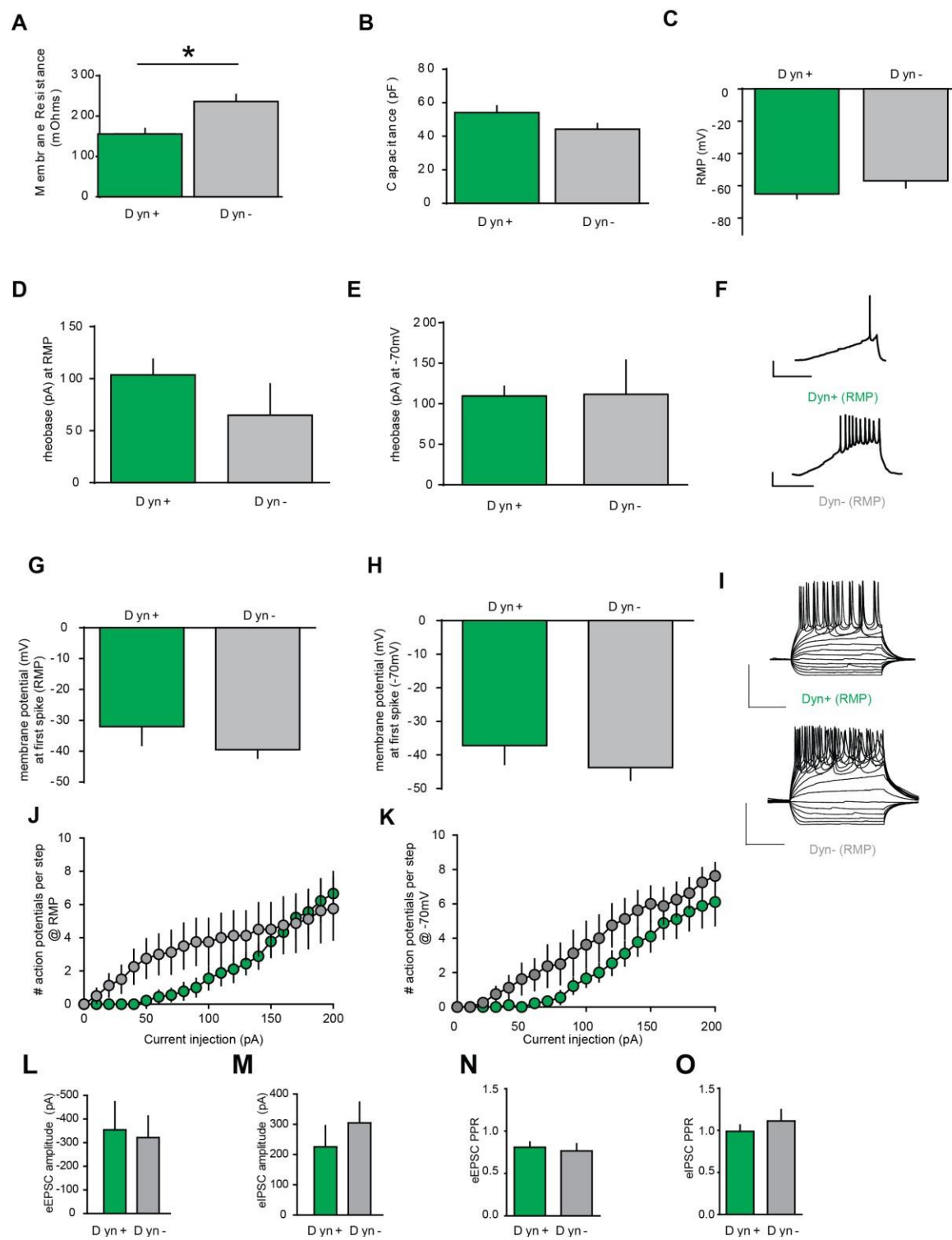


Figure 2.5 - DYN+ and DYN- neurons in the BNST show differences in membrane properties and intrinsic excitability.

(A) Membrane resistance was significant lower in DYN+ neurons as compared to DYN- cells (unpaired t -test, $t_{63} = 3.528$, $P < 0.001$). **(B)** Similarly, differences in cell capacitance approached significance (unpaired t -test, $t_{63} = 1.852$, $P = 0.0687$). There was no significant difference in DYN+ and DYN- neurons in regards to **(C)** resting membrane potential (RMP) (unpaired t -test, $t_{14} = 1.497$, $P > 0.1$), **(D)** rheobase at RMP (unpaired t -test, $t_{14} = 1.147$, $P > 0.1$), and **(E)** rheobase when held at -70mV (unpaired t -test, $t_{14} = 0.04802$, $P > 0.1$), **(F)** representative traces of rheobase recordings at RMP for both cell types. Scale bar represents 20mV by 500 msec. Similarly, there was no difference in **(G)** the membrane potential at first spike (unpaired t -test, $t_{14} = 1.106$, $P > 0.1$) or **(H)** the membrane potential at first spike when held at -70mV (unpaired t -test, $t_{15} = 0.9302$, $P > 0.1$). **(I)** Representative traces of VI plots for both cell types. Scale bar represents 50mV by 100 msec; only every other sweep shown for clarity. **(J)** When recorded at RMP, A 2-way ANOVA revealed a significant interaction between current injection and cell type ($F_{20,300} = 1.936$, $P < 0.05$) and significant effect of current injection ($F_{20,300} = 18.19$, $P < 0.0001$), but no main effect of cell type ($F_{1,15} = 0.9092$, $P > 0.1$). **(K)** At -70mV, a 2-way ANOVA showed a significant effect of current injection ($F_{20,300} = 34.58$, $P < 0.0001$) but no effect of cell type ($F_{1,15} = 2.060$, $P > 0.1$). **(L)** BLA-BNST optogenetic activation produced eEPSCs of equal amplitude onto DYN+ and DYN- neurons ($t_{30} = 0.2233$, $P > 0.05$ **(M)** as well as eIPSC ($t_{35} = 0.8121$, $P > 0.1$). **(N)** There was

also no difference in eEPSC PPR ($t_{32} = 0.7981$, $P > 0.1$) or **(○)** elPSC PPR ($t_{32} = 0.4164$, $P > 0.1$).

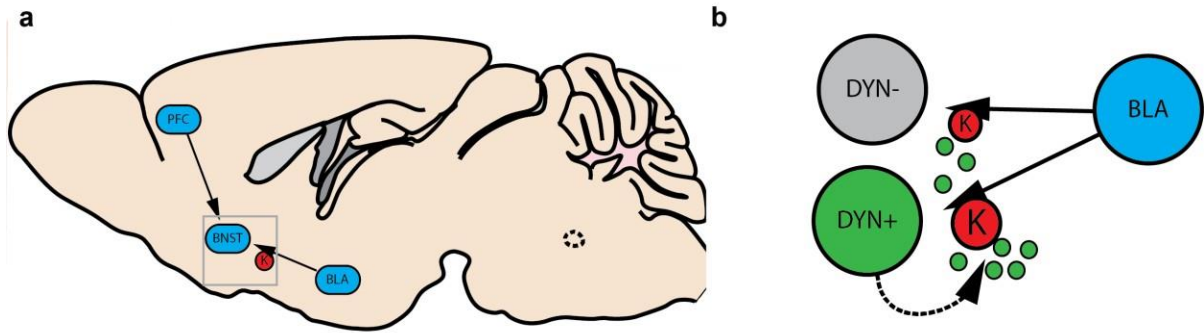


Figure 2.6 - KORs inhibit glutamate release by a pathway specific, presynaptic mechanism, and this inhibition is preferential to DYN+ neurons.

(A) KORs in the BNST presynaptically inhibit glutamate inputs from the BLA, but not PFC. **(B)** This presynaptic inhibition of BLA inputs shows cell type specificity; there is greater KOR modulation of BLA-DYN+ synapses than BLA-DYN- synapses. In addition, this inhibition likely arises from retrograde release of dynorphin from BNST GABA/dynorphin neurons. This allows for dynorphin to preferentially control the strength of BLA-BNST synaptic transmission, likely influencing downstream nuclei.

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CHAPTER 3: CHANGES IN KAPPA OPIOID RECEPTOR FUNCTION FOLLOWING AN ANIMAL MODEL OF ALCOHOL EXPOSURE

INTRODUCTION

Alcohol use disorders (AUDs) comprise a major public health epidemic in the United States, and extant pharmacological treatments have had limited success. In addition, AUDs often have high comorbidity with anxiety disorders, suggesting common pathways and targets in these chronic and potent diseases (Sanchez-Pena et al., 2012). Thus far, attempts to treat comorbid alcoholism and anxiety have had inconclusive effectiveness (for systematic review, see Ipser et al., 2015). Kappa opioid receptors (KORs) have been proposed as a promising therapeutic target for AUDs; KOR antagonists have been shown to reduce alcohol consumption in rodents in free-drinking studies (Cashman & Azar, 2014), operant paradigms (Henderson-Redmond & Czachowski, 2014), and animals that are already alcohol dependent (Walker & Koob, 2008). Complementary work has shown that KOR agonists can reinstate alcohol seeking (Funk et al., 2014). In addition, alcohol has been shown to increase the levels of prodynorphin (pDyn) throughout the brain (Marinelli et al., 2006, D'Addario et al., 2011, D'Addario et al., 2013), suggesting that both the

receptor and ligand may undergo alterations. These findings have led to promising clinical trials using KOR antagonists for the treatment of alcoholism (for detailed review, see Crowley & Kash, 2015). However, the neurobiological locus of this potentially encouraging treatment has not been identified. Most of the existing literature focuses on interactions between alcohol and KOR/pDyn in the amygdala and extended amygdala, most notably the central nucleus of the amygdala (CeA; Zhou et al., 2013). Activation of KORs has been shown to modulate GABA transmission, as well as the interaction between GABA and ethanol in the CeA (Gilpin et al., 2014). The bed nucleus of the stria terminalis (BNST), a region of the extended amygdala involved in integrating cortical and limbic information for behavioral responses, is abundant in both KORs and pDyn (Poulin et al., 2009). Recent work has highlighted the role of KORs in the BNST (Li et al., 2012), but the role of BNST KORs and alcoholism remains unexplored.

Chronic intermittent ethanol (CIE) has been commended as a model that achieves stable and clinically relevant blood ethanol concentrations (BECs) in mice (Lopez et al., 2012). In this model, mice are exposed to vaporized ethanol for 14-16 hours, after which they return to their home cage for 8-10 hours. This exposure is repeated for 4-5 days, which constitutes one cycle. Exposure paradigms use 1-4 cycles, with longer (2-3 day) withdrawal periods in between cycles (i.e. four days of exposure, three days of withdrawal, repeated for two cycles; Melendez et al., 2012). This model has been used to elicit changes in BNST synaptic physiology (Kash et al., 2009) and related behavior (Marcinkiewicz et al., 2015).

In the current study, male DBA/2J mice were exposed to this CIE model for

one cycle. Following a 5 day exposure and 24 hour withdrawal, we assessed changes in pDyn- and KOR-relative gene expression and KOR-mediated synaptic transmission in the BNST. Finally, we assessed changes in social behavior. We found alterations in KOR-relative gene expression, and changes in both KOR-modulation of GABA and glutamate transmission. In addition, we found that mice exposed to CIE displayed a KOR-dependent reduction in social behavior. Taken together, this work provides important evidence for the mechanism of a KOR-mediated treatment of alcohol abuse and alcoholism.

MATERIALS AND METHODS

Subjects

Male DBA/2J mice (6-7 weeks; Jackson Labs, Bar Harbor, ME) were maintained in accordance with the University of North Carolina–Chapel Hill IACUC guidelines. Mice were housed 2-4 littermate mice to a cage and were provided standard rodent chow and water *ad libitum* throughout the duration of the study. All mice within the same cage received the same treatment (i.e., ethanol or air exposure). Mice were housed in the colony housing room for approximately 1-2 weeks to allow for ample acclimation following transport. Lighting in both the vivarium and ethanol chamber room were on a 12hr cycle, with lights on at 7AM. Following acclimation, mice underwent CIE or air exposure, as described below (Fig. 3.1A).

Chronic intermittent ethanol (CIE) exposure

Mice were exposed to 5 consecutive days of CIE or air as previously described (Becker & Lopez, 2004, Lowery-Gionta et al., 2015, Marcinkiewicz et al., 2015). Between 4PM and 5PM daily, both air- and ethanol-exposed mice were taken from the vivarium to the ethanol chamber room. All mice received an intraperitoneal injection with the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg in 0.9% saline) to generate stable BECs throughout ethanol exposure (see Lowery-Gionta et al., 2015 for blood ethanol concentrations). CIE mice were placed with cage mates into the cage compartments of vapor chambers (La Jolla Alcohol Research, Inc., La Jolla, CA). CIE mice were exposed to ethanol vapor from bubbled 95% ethanol and air (15 l/min), to produce BECs of 150-200 mg/dl (approximately 16 hours overnight). Air exposed mice were placed in chambers with the same air flow rate but did not receive vaporized ethanol. All mice were removed from the vapor chamber cages between 8AM and 9AM the next day, returned to their home cages, and taken back to vivarium (approximately 8 hours). This procedure results in 5 x 16 hour-exposures to ethanol vapors and 4 x 8 hour-withdrawal periods. Following the final exposure to ethanol vapor, mice were placed back in their home cage and left for 24 hours without disturbances prior to experiments.

BECs were determined with the Analox Alcohol Analyzer (Analox Instruments) using plasma extracted from ~20ul blood samples taken from the tail identical to those described previously (Lowery-Gionta et al., 2015, Marcinkiewicz et al., 2015; data not shown). BECs were taken from mice not used for other experimental purposes to minimize stress.

Electrophysiology

24 hrs following the final ethanol or air exposure, mice were used for electrophysiology experiments as described previously (Pleil et al., 2015). Briefly, mice were deeply anesthetized under isoflurane and rapidly decapitated. Brains were immediately placed in ice-cold high sucrose cutting solution, containing the following (in mM): 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl₂, 1 MgCl₂, 1.2 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃. 300 μ M coronal hemi-sectioned slices containing the BNST were prepared on a Leica VT1200 Vibratome, and were transferred to normal aCSF maintained at approximately 30 degrees (Warner Instruments, Hamden, Connecticut) containing the following (in mM): 124 NaCl, 4.4 KCl, 2 CaCl₂, 1.2 MgSO₄, 1 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃. Slices were allowed to rest for one hour, and remained there until used. Slices were continuously bubbled with a 95% O₂ / 5% CO₂ mixture throughout slicing and experiments. Thin-walled borosilicate glass capillary recording electrodes (3–6 M Ω) were pulled on a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA). Following rupture of the cell membrane, cells were allowed to rest and equilibrate to the intracellular recording solutions (below). Input resistance was monitored continuously throughout the experiment, and when it deviated by more than 20% the experiment was discarded.

Lidocaine N-ethyl bromide (1 mg/ml) was included in the intracellular recording solution to prevent postsynaptic sodium spikes for all experiments. Electrically evoked excitatory post-synaptic currents (eEPSCs) and electrically evoked inhibitory

post-synaptic currents (eIPSCs) were evoked using a twisted bipolar nichrome wire placed dorsal to the recording electrode.

In order to isolate EPSCs, cells were patched with a cesium-gluconate-based intracellular recording solution, containing the following (in mM): 135 cesium gluconate, 5 NaCl, 10 HEPES, 0.6 EGTA, 4 ATP, 0.4 GTP. 5 μ M picrotoxin was bath-applied to the slices, and cells were voltage-clamped at -70mV. In order to isolate IPSCs, cells were patched with potassium-chloride/potassium-gluconate-based intracellular recording solution, containing the following (in mM): 70 KCl, 65 potassium gluconate, 5 NaCl, 10 HEPES, 2 QX-314, 0.6 EGTA, 4 Na-ATP, 0.4 Na-GTP. 3mM kynurenic acid was bath applied to the slices, and cells were voltage-clamped at -70mV. 1 μ M U69,593 and 100nM norBNI were bath-applied where indicated in Figure legends.

Social Approach Behavior

24 hrs following the final ethanol or air exposure, mice were brought from the vivarium to the testing area. For at least 30 min prior to injections, mice remained in their home cages in a light- and sound-attenuating cabinet. Following this period, 30 min prior to behavioral testing, mice were injected i.p. with either the KOR antagonist JDTic (10 mg/kg dissolved in 0.9% saline; injection volume of 10 μ l/kg) or saline vehicle. Mice were then returned to the light- and sound-attenuating cabinet until testing.

The social approach test took place in a 3-chambered plexiglass apparatus divided by two panels separating the left and right chambers from the middle

chamber, with small openings at the base of each panel to allow movement between chambers (**Fig 3.1B**). Between test phases, mice were confined to the center chamber. The social approach test consists of three 10 min phases: habituation, sociability, and social novelty (habituation and social novelty data not shown). In the habituation phase, mice freely explore all three chambers, which are empty. In the sociability phase, the side chambers each contain a circular plexiglass cage. One cage is empty while the other cage contains a novel mouse, with which the test subject has never interacted (the side of the empty cage and the cage with a novel mouse are randomized). In the social novelty phase, a different novel mouse is placed in the empty cage from the sociability phase (i.e., the novel mouse-paired side remains consistent). The chamber side containing the novel mouse in the sociability phase was counter-balanced within ethanol and air exposed, and JDTic and vehicle treatment groups.

All phases were video recorded and were analyzed using EthoVision (Noldus, Wageningen, The Netherlands). Time (s) spent in the area directly surrounding the cages was quantified using EthoVision. The “sociability ratio” score was calculated by

$$\frac{\text{time with mouse} + \text{time with empty cage}}{\text{time with mouse} + \text{time with empty cage}}$$

where a value of 1 indicates no preference for social approach, values greater than 1 signify a preference for social approach, and values less than 1 indicate an aversion to social approach.

Prior to being used in the social approach behavioral task, the mice used as novel mice were habituated to the circular plexiglass cage for ten minutes per day

for three days. The novel mice reside in a separate vivarium than the CIE and air exposed mice.

RNA detection and quantification

Mice were anesthetized with isoflurane and rapidly decapitated. The brains were blocked into 1mm coronal slices and immediately placed on dry ice. Once the tissue was frozen, punches were collected from all regions of interest. All tools were cleaned between samples with 70% ethanol. Both hemispheres for each region were combined, and tissue samples were stored in RNA later solution until processed by the UNC Animal Clinical Chemistry and Gene Expression Laboratories. RNA isolation and RT-QPCR were conducted identically to methods published previously (Kim et al., 2002). Sequence information of primers and probes is available in **Table 1.1**.

Data analysis and statistics

Data are expressed as means \pm SEM for all Figures. Two-way ANOVAs, paired t-tests, unpaired t-tests, or Mann-Whitney U were used for all experiments, as described in Figure captions. Statistical analyses were conducted using Prism 6.0 (GraphPad, La Jolla, CA), and Figures were made using Prism 6.0 (Graphpad, La Jolla, CA) and Illustrator CC 2015 (Adobe, San Jose, CA).

RESULTS

KOR relative gene expression is reduced following CIE

Levels of pDyn- and KOR-relative gene expression were probed in the BNST following one week of CIE. Though levels of pDyn-relative gene expression were lower, they were not significantly altered (**Fig 3.2A**). However, levels of KOR-relative gene expression were significantly reduced following CIE (**Fig 3.2B**).

Ethanol-exposed mice show differences in KOR-modulation of glutamate and GABA transmission

Ethanol-exposed and air-exposed mice did not show any differences in BNST eEPSC frequency or amplitude (**Fig 3.3A-C**). However, despite the similarities between basal glutamatergic transmission in ethanol-exposed and air-exposed mice, differences emerged when assessing the KOR-mediated alterations of glutamate. The KOR agonist U69,593 produced a significantly smaller inhibition of eEPSCs in ethanol-exposed mice as compared to air-exposed mice (**Fig 3.3D**). In addition, there were small but significant differences in norBNI-mediated alterations in eEPSCs (**Fig 3.3E**).

Similar to glutamate, ethanol-exposed and air-exposed mice did not show differences in BNST sIPSC frequency or sIPSC amplitude (**Fig 3.4A-C**). The KOR agonist U69,593 produced a significantly greater inhibition of eIPSCs in ethanol-exposed mice as compared to air-exposed mice (**Fig 3.5D-E**). In addition, there were significant differences in norBNI-mediated alterations in eIPSCs, with norBNI

application producing robust LTP in ethanol-exposed mice (**Fig 3.5E**).

Ethanol-exposed mice show deficits in social behavior, which is partially rescued by the KOR-antagonist JDtic

Next, we sought to determine whether CIE-exposure-induced changes in sociability were mediated by KORs. The KOR antagonist JDtic had no effect on the social preference score in air-exposed mice (**Fig 5A**). However, systemic administration of the KOR antagonist JDtic was able to significantly increase the social preference score in ethanol-exposed mice (**Fig 5B**).

DISCUSSION

Modulating KOR signaling has been proposed as a promising treatment for both alcohol addiction and anxiety-related disorders. Despite the progression of KOR antagonists to clinical trials, the mechanism of this potential therapy is not well understood. Here, we find that a model of alcohol exposure alters the KOR system in the BNST, a region known to be involved in addiction and anxiety. Activation of KORs at glutamatergic synapses results in an increased inhibition of glutamate in ethanol-exposed mice as compared to air-exposed mice, whereas the opposite effect is seen at GABAergic synapses. This is likely to reflect KOR modulation of different inputs (i.e. the CeA and the BLA). Following CIE, we discovered norBNI-induced potentiation at GABAergic synapses, but not at glutamatergic synapses. This lack of an effect at glutamate synapses is likely due to opioid-mediated long-term depression (LTD), as seen in the striatum (Atwood et al., 2014). Here, the

authors demonstrated that norBNI is unable to reverse U69,593-mediated inhibition at glutamate synapses. Therefore, we hypothesize that following CIE, there is tonic dynorphin activation of KORs at both glutamatergic and GABAergic synapses, but the opioid-induced LTD makes it difficult to unmask. The reduced efficacy of U69,593 at glutamate synapses following CIE is likely due to a combination of opioid-induced LTD and, perhaps, reduced receptor expression. In addition to finding alterations in plasticity, we demonstrate that the KOR antagonist JD1c has the potential to rescue CIE-induced deficits in social behavior. Though the alterations in social behavior were conducted with systemic administration of the antagonist, when coupled with the electrophysiology results, they implicate the BNST as a potential regulator of alcohol-induced changes in social behavior. Indeed, other studies have shown glutamate signaling in the BNST to be essential for aggression (Masugi-Tokita et al., 2015), and serotonergic control of social interaction has been shown to be altered following CIE (Marcinkiewicz et al., 2015). Taken together, this work implicates KOR signaling in the BNST as a further regulator of alcohol-induced changes in social behavior.

<i>TaqMan Primers and Probes</i>		
Gene	Type	Sequence (5'-3')
Pdyn	Forward	CCT GAT TTG CTC CCT GGA GT
	Reverse	ATG AGA AGC CCC GGC ATG TC
	Probe	f AGG ACC TGG TGC CGC CCT CAG Aq
Oprk1	Forward	GGT CAT GTT TGT CAT CAT CCG
	Reverse	CAT CTG CCA AAG CCA GGT TA
	Probe	f CAC GAA GAT GAA GAC CGC AAC CAA C q
β -Actin	Forward	CTG CCT GAC GGC CAG GTC
	Reverse	CAA GAA GGA AGG CTG GAA AAG A
	Probe	t CAC TAT TGG CAA CGA GCG GTT CCG q

f, Reporter dye1 (FAM:6-carboxyfluorescein); t, Reporter dye2 (TET:Tetrachloro-6-carboxyfluorescein); q, Quencher dye (TAMRA: 6-carboxytetramethyl-rhodamine)

Table 3.1 – sequence information for primers and probes.

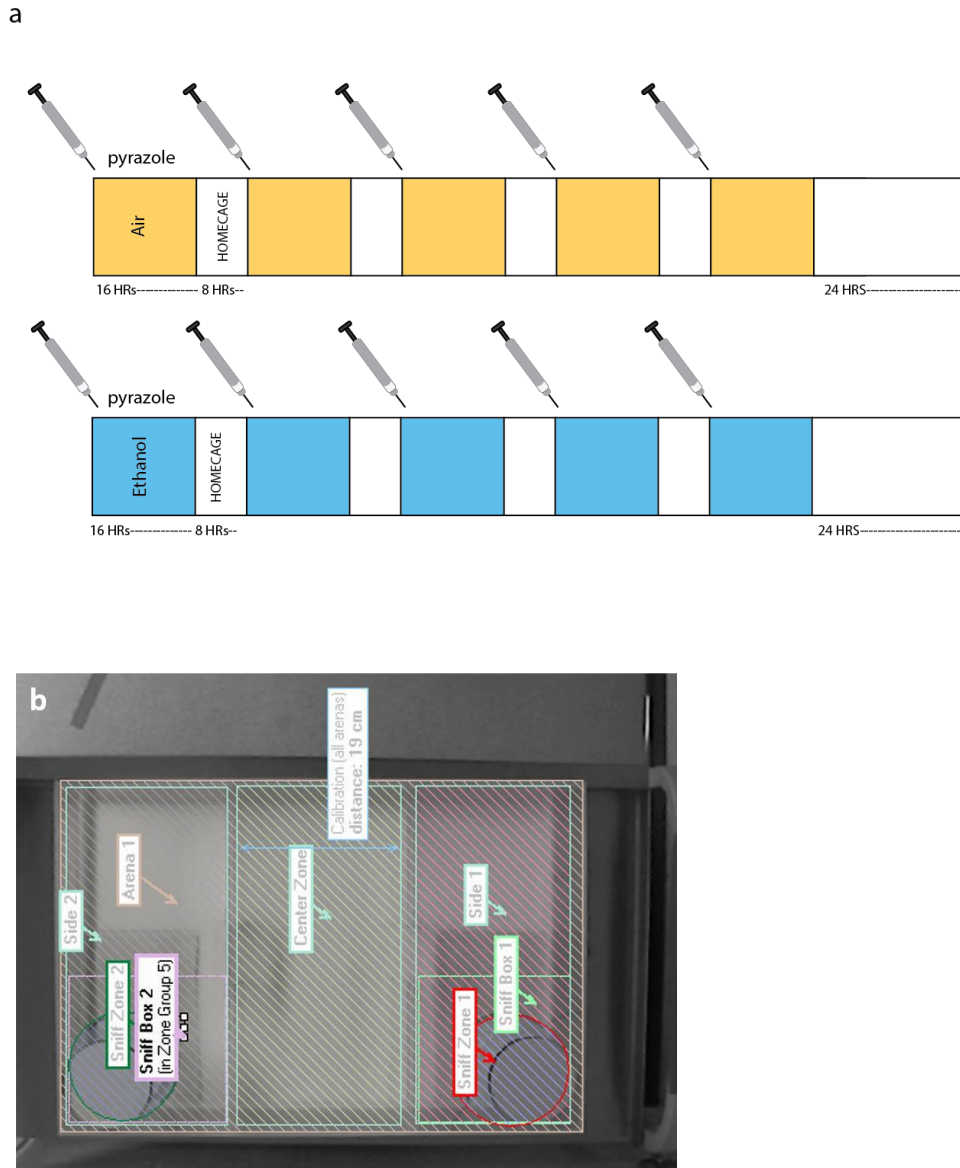


Figure 3.1 - CIE exposure schedule and social approach arena. (A) Mice were exposed to ethanol or air for 16 hrs a day with an 8 hr withdrawal. Prior to each session, both ethanol- and air- exposed mice received priming injections of the alcohol dehydrogenase inhibitor pyrazole (1mmol/kg). Following the last exposure, mice were returned to the vivarium for 24 hrs prior to use for behavioral or electrophysiology experiments. **(B)** Social approach arena as described in methods. Arena contained a center zone and two social approach zones.

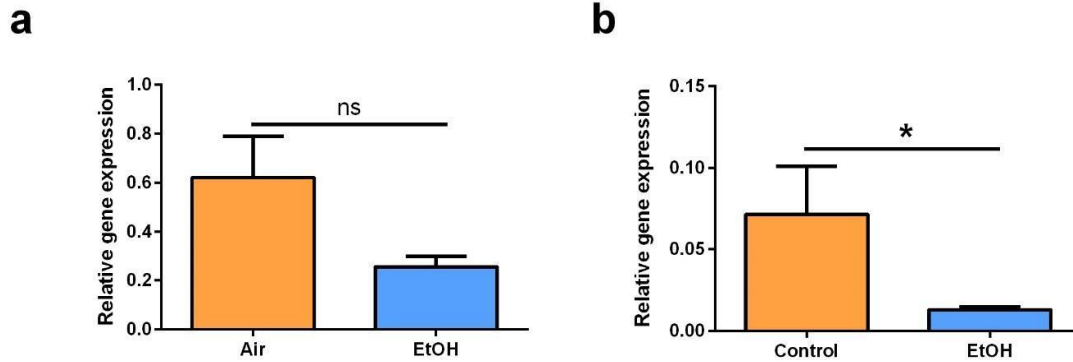


Figure 3.2 – Dynorphin-relative gene expression remains unaltered, while KOR-relative gene expression is reduced following CIE exposure. (A) Dynorphin-relative gene exposure is not significantly altered following CIE exposure (Mann Whitney $U = 20$, $n_1=10$, $n_2=9$, $P > 0.05$ two-tailed). **(B)** KOR-relative gene exposure is significantly reduced following CIE exposure (Mann Whitney $U = 16$, $n_1=10$, $n_2=9$, $P < 0.05$). Both experiments are normalized to β -actin.

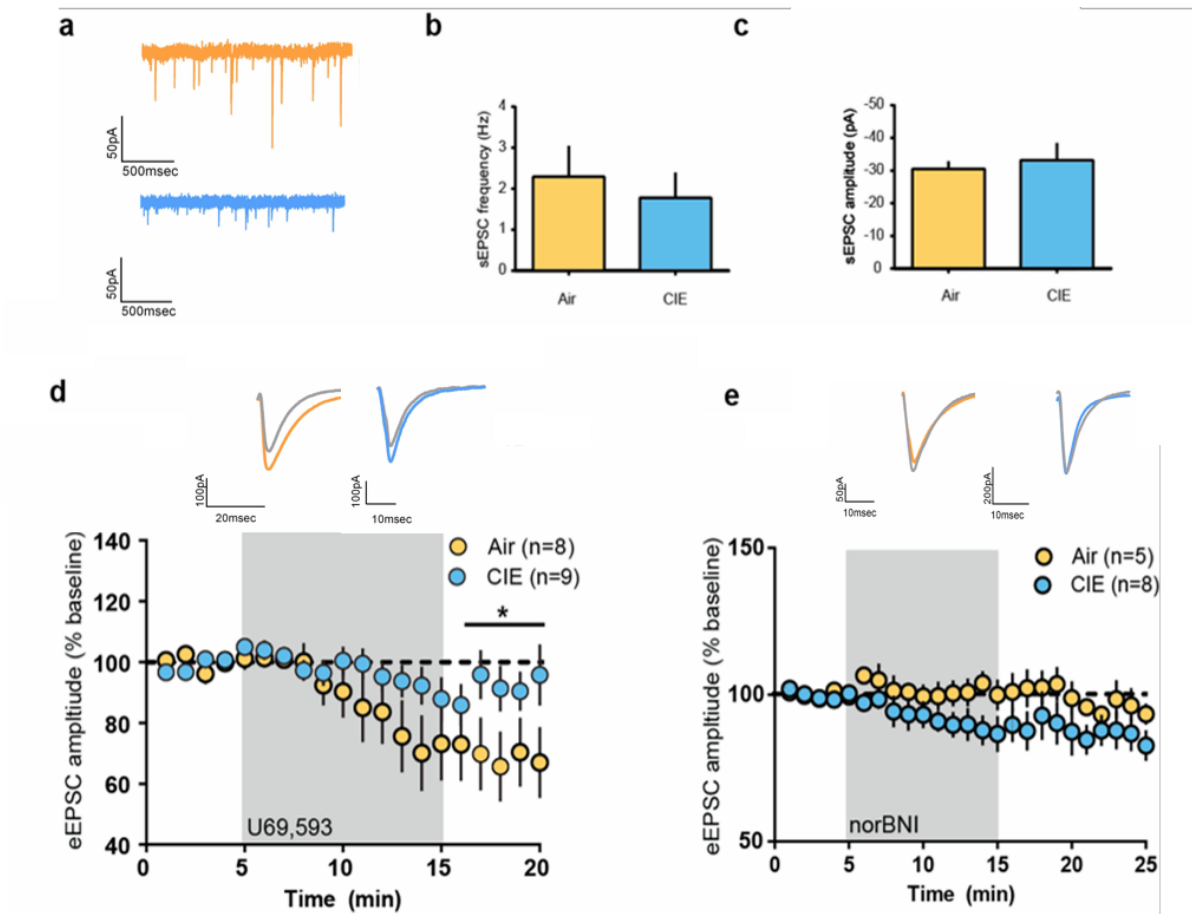


Figure 3.3 - Following CIE, ethanol-exposed mice show a decrease effect of KOR activation on glutamate transmission. (A) Traces showing air-exposed (orange) and ethanol-exposed (blue) sEPSCs. **(B)** Ethanol-exposed and air-exposed mice did not show any significant differences in BNST sEPSC frequency (unpaired t -test, ethanol v. air, $t_{16} = 0.5259$, $P > 0.05$). **(C)** Similarly, no significant differences in sEPSC amplitude were seen (unpaired t -test, ethanol v. air, $t_{16} = 0.4601$, $P > 0.05$). **(D)** Ethanol-exposure resulted in a reduced KOR-mediated inhibition of eEPSCs (unpaired t -test, ethanol v. air min 16-20, $t_8 = 10.63$, $P < 0.0001$). Inset, traces showing baseline (orange and blue) and post-drug (grey). **(E)** A small but significant difference was seen with application of norBNI (unpaired t -test, ethanol v. air min 21-

25, $t_9 = 7.362$, $P < 0.0001$). Inset, traces showing baseline (orange and blue) and post-drug (grey).

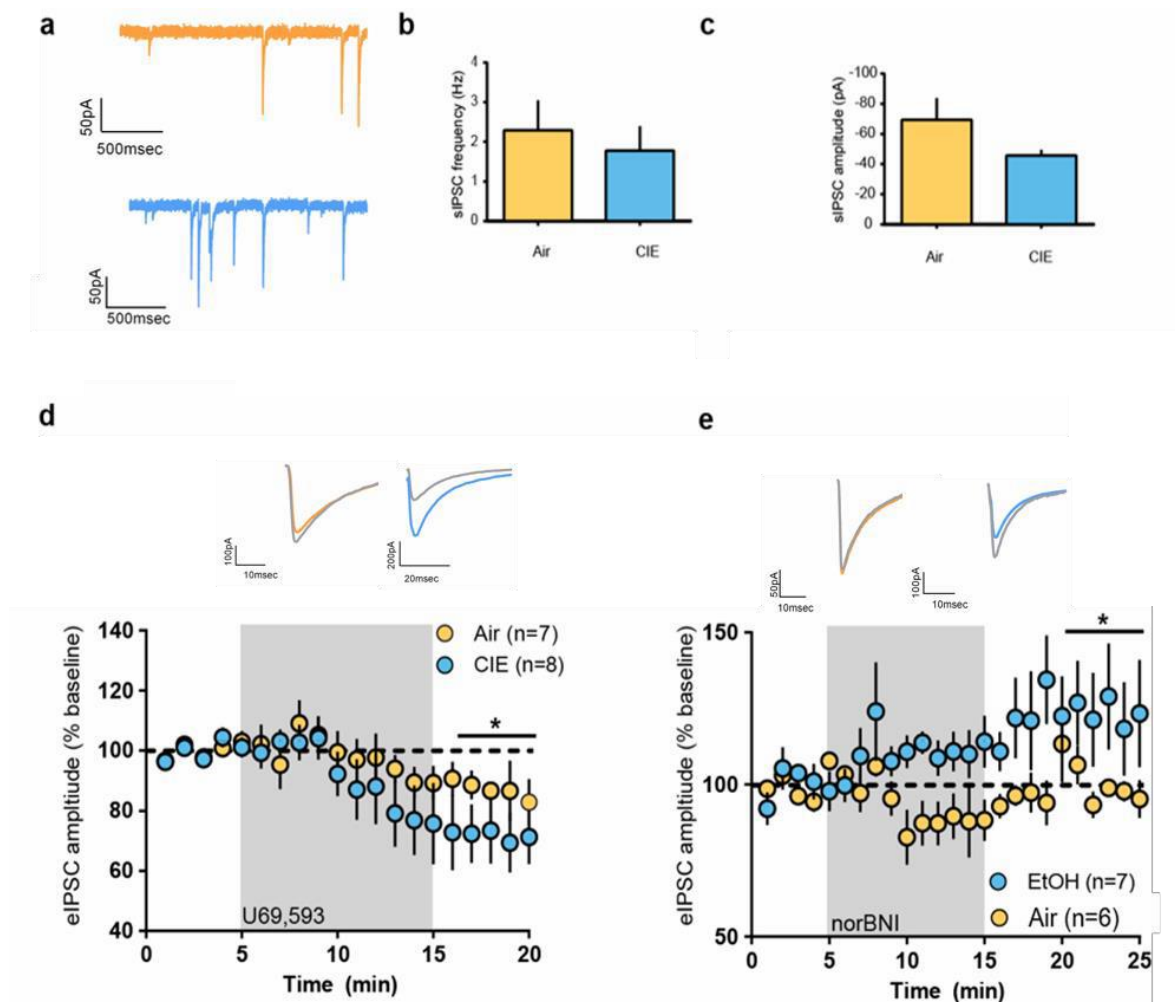


Figure 3.4 - Following CIE, ethanol-exposed mice show a greater KOR-induced inhibition of GABA transmission. (A) Traces showing air-exposed (orange) and ethanol-exposed (blue) sIPSCs. **(B)** Ethanol-exposed and air-exposed mice did not show any differences in BNST sIPSC frequency (unpaired t -test, ethanol v. air, $t_{19} = 0.1141$, $P > 0.05$) Traces showing ethanol-exposed (blue) and air-exposed (orange) sIPSCs. **(C)** and did not show any differences in sIPSC amplitude (unpaired t -test, ethanol v. air, $t_{19} = 1.663$, $P > 0.05$). Inset, traces showing baseline (orange and blue) and post-drug (grey). **(D)** Ethanol-exposure induced a greater KOR-mediated inhibition of eIPSCs (unpaired t -test, ethanol v. air min 16-20, $t_8 = 10.35$, $P <$

0.0001). **(E)** In addition, eIPSCs are potentiated by norBNI in ethanol-exposed mice (unpaired *t*-test, ethanol v. air, min 21-25, $t_8 = 8.610$, $P < 0.001$). Inset, traces showing baseline (orange and blue) and post-drug (grey).

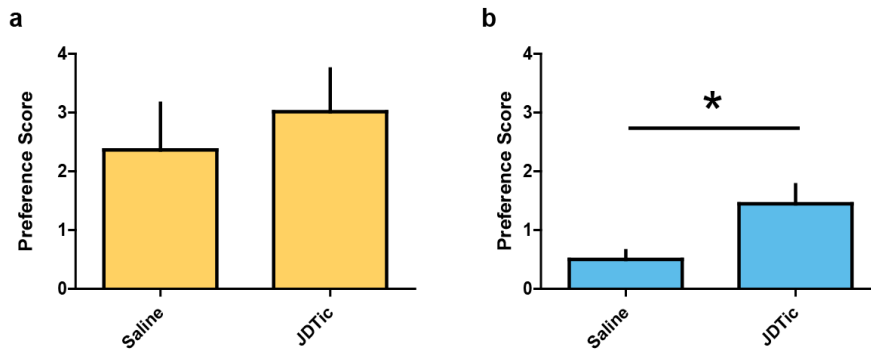


Figure 3.5 - CIE causes a decrease in social preference that is partially rescued by pretreatment with a KOR antagonist. (A) Air-exposed mice do not show any significant changes in social preference following treatment with JDtic (unpaired t -test, saline v. JDtic, $t_{17} = 0.596$, $P > 0.05$). **(B)** However, ethanol-exposed mice show an increase in social preference following treatment with JDtic (unpaired t -test, saline v. JDtic, $t_9 = 2.427$, $P = 0.0350$). Though these levels do not reach those of air exposure mice, they do represent a significant rescue of the phenotype.

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CHAPTER 4: GENERAL DISCUSSION

ALCOHOL USE DISORDERS, ANXIETY, AND KORs

Alcohol use disorders (AUDs) constitute one of the leading causes of preventable death (Hingson, Zha, & Weitzman, 2009), making it a major global public health concern (Room, 2005). In addition AUDs have a strong comorbidity with many psychiatric disorders (Hasin & Grant, 2015), and alcohol dependence is associated with three times greater risk for depression and six times greater risk for anxiety disorders (Klimkiewicz et al., 2015). Though many behavioral and pharmaceutical treatments for AUDs exist, there is no comprehensively effective treatment for either disorder (Witkiewitz et al., 2015). Targeting kappa opioid receptors (KORs) as therapeutic intervention for both anxiety disorders and AUDs is at the forefront of treatment research (Walker et al., 2015). Changes in the KOR/dyn system have been noted in post-mortem brains of alcoholics, further suggesting an interaction between alcohol dependence and this anxiety-related system (Bazov et al., 2013, Taqi et al., 2011). The literature suggests that KOR activation may be a key component of ethanol-withdrawal-induced anxiety, making it a likely candidate

for the treatment of both disorders. Administration of alcohol increases extracellular dynorphin levels in the accumbens (Marinelli et al., 2006), as well as the central amygdala (CeA; (Marinelli et al., 2006). Activation of KORs has been shown to mitigate ethanol-withdrawal induced-anxiety related behaviors (Valdez & Harshberger, 2012), termed “hangover anxiety” (Valdez & Harshberger, 2012). KOR antagonists can block the potentiating effect of stress on alcohol conditioned place preference (CPP; (Sperling et al., 2010), while KOR agonists can induce alcohol CPP in a manner similar to stress-induced alcohol CPP (Walker et al., 2012). Importantly, the role of KORs in ethanol-withdrawal induced anxiety-related behaviors has been noted long after ethanol exposure, up to six weeks in rodents (Gillett, Harshberger, & Valdez, 2013). However, it is unlikely that the interaction between KORs and alcohol is as straightforward as this; others have postulated that KOR-mediated withdrawal-induced anxiety is relieved by further alcohol consumption, implicating a more complex interaction between KORs, anxiety, and alcohol (Walker & Koob, 2008). This complex interaction between KORs and alcohol has led to the testing of various KOR antagonists in multiple clinical trials for the treatment of AUDs, depression, and anxiety (Crowley & Kash, 2015). However, KORs throughout the brain have not been well characterized, limiting their utility as a pharmacological treatment (for review, see (Crowley & Kash, 2015)). It is prudent to better understand how KORs facilitate interactions between anxiety and alcohol use. Here, our overall goals were two-fold: (1) to identify the signaling and function of KORs in the BNST, an alcohol- and anxiety- relevant brain region and (2) to identify how KOR functioning in this region is modulated following a model of alcohol

consumption.

Though both KOR and dynorphin mRNA have been demonstrated to be anatomically present in the BNST (Poulin et al., 2009), few studies exist assessing the role of KORs in altering BNST neurotransmitter signaling or function. Poulin and colleagues hypothesized that either KORs were expressed presynaptically in the BNST or that dynorphin was released locally there; we confirmed both hypotheses with this work. Systemic administration of the KOR agonist Salvinorin-A was found to cause metabolic activation in the BNST (Hooker et al., 2009), producing further evidence of KOR functioning in the BNST. Li, Pleil, et al. (2012) first demonstrated direct functional KOR modulation in this region. They found that activation of KORs inhibited GABAergic transmission, specifically at (but not limited to) GABAergic projections arising from the central amygdala (CeA). In addition, this KOR-induced inhibition was found to be dependent upon ERK signaling. Their paper confirmed previous anatomical work and demonstrated that KORs in the BNST have an important inhibitory control over GABAergic signaling.

KORs IN THE BNST

Building upon the work by Li, Pleil, et al. (2012), we assessed how KORs exert control over glutamatergic signaling in the same region. We found that KORs in the BNST inhibit electrically-evoked glutamate release, albeit through a different mechanism than they employ in GABA release. Though KORs at GABAergic synapses inhibit transmission through ERK signaling, KORs at glutamatergic

synapses inhibit transmission via p38-dependent signaling. This divergent signaling mechanism in a singular region has been demonstrated elsewhere, such as in the nucleus accumbens (Hjelmstad & Fields, 2003). This finding has implications for the development of biased signaling ligands: p38 signaling via KOR activation has been identified as being responsible for the dysorphia-like effects of KOR agonists (Bruchas et al., 2007). Though not addressed in the Li, Pleil, et al. paper or the current study, drugs targeting subsets of synapses or signaling cascades within specific brain regions may be developed for use as therapeutic compounds without side effects. Biased ligands have been developed at other receptors, such as the dopamine D₂ receptor, resulting in greater symptom-specific targeting (Allen et al., 2011). Some attempts have been made to identify biased ligands at the KOR (White et al., 2014), but more work is needed.

We next demonstrated that KORs inhibit specific glutamate inputs to the BNST; though the glutamatergic projection from the basolateral amygdala (BLA) is inhibited by KOR activation, the glutamatergic projection from the prefrontal cortex (PFC) remains unaltered following bath application of the KOR agonist. Interestingly, KORs inhibit other BLA outputs, such as that projecting to the PFC (Tejeda et al., 2015). This result has important implications, suggesting that KORs in the BNST filter specific glutamatergic pathways into the region. Four important questions arise from these results.

(1) *How are other glutamatergic and GABAergic projections to the BNST altered by KOR activation?* Future experiments should address other excitatory projections to the BNST, such those that arising from the hippocampus (HIPP) and

other cortical regions. In addition, though KORs are known to inhibit the GABAergic input from the CeA, other GABAergic projections, such as those arising from the DR, should be investigated as well.

(2) *Do PFC and BLA projections synapse onto the same neurons within the BNST?* The current experiments demonstrated that while KORs inhibit BLA and not PFC projections to the BNST, the experiments were done in separate mice, thus limiting the interpretation. Future experiments should address whether PFC and BLA projections synapse onto the same, overlapping, or entirely different neurons. These experiments could be conducted using a combination of traditional ChR2 and ReaChR, a red-shifted channelrhodopsin (Lin et al., 2013). Though important differences between ChR2 and ReaChR exist, the combination of the two would be useful for basic circuitry identification.

(3) *Do PFC and BLA projections influence different BNST output neurons?* If we learn whether PFC and BLA neurons synapse onto a portion of the same or different BNST neurons, we could then investigate whether these connections have different control over downstream regions. The BNST sends projections to many regions implicated in anxiety, reward, and addiction related behaviors, such as the VTA (Jennings, Sparta, et al., 2013) and the LH (Jennings et al., 2013). Experiments using a combination of anterograde and retrograde viruses, as well as chemogenetic cell-type identification, could help elucidate this.

(4) *Do KORs inhibit all BLA outputs?* KORs inhibit the BLA projections to the BNST and PFC, and it would be interesting to address other outputs including the HIPPO, which is known to regulate a strong anxiety-related behavioral phenotype

(Mamiya et al., 2014). KORs are known to be expressed in the HIPP (Wagner, Terman, & Chavkin, 1993), making this site a likely candidate for a similar circuit.

Previous work has demonstrated the importance of BLA projections, including those to the BNST, in regulating anxiety-related behaviors (Felix-Ortiz et al., 2013; Kim et al., 2013). Indeed, we confirmed the importance of the BLA-BNST circuit as a key mediator of anxiety-related behaviors in both the open field and the elevated plus maze (unpublished data in collaboration with Michael Bruchas, University of Washington-St. Louis), and we demonstrated *ex vivo* that KORs are an important regulator of this circuit. Other neuropeptides within the BNST have robust behavioral effects as well (Pleil et al., 2015), though they have not been assessed in the context of the BLA-BNST circuit. This work provides an important reminder for all optogenetic research that, though light activation of a singular pathway may be sufficient to drive elegant behavioral phenotypes in animal models, these pathways do not exist in a vacuum. It is important to consider the brain's natural checks and balances against hyperstimulation of any one pathway, such as in the current case, where peptidergic activation is capable of overriding a circuitry-driven phenotype. This has important implications for translational research and goals founded on optogenetic studies.

We next demonstrated optogenetic, frequency-dependent control over dynorphin release within the BNST. Previous work demonstrated KOR-dependent inhibition following electrical stimulation (Neumaier & Chavkin, 1989), and to our knowledge, our experiments are the first to demonstrate peptidergic release using optogenetics. These experiments provide an important advancement over the

traditional methodology used to look at neuropeptide effects. Traditionally, peptide-induced effects are assessed either using bath application (*ex vivo*) or systemic administration and site-specific infusion (*in vivo*). In both cases, the physiological relevance of both concentrations and activation patterns is unknown. Here, we find that 20 hz activation of dynorphin/GABAergic local neurons in the BNST is capable of inhibiting electrically-evoked glutamate release in a manner indistinguishable from that seen with *ex vivo* bath application of KOR agonists. Four important questions arise from these experiments.

(1) *Do BNST dynorphin neurons only release dynorphin in the BNST, or are similar effects seen downstream?* Though BNST dynorphin neurons are known to project to other regions such as the LH (Thomas Kash, unpublished data) it is not known whether dynorphin is released downstream. In the current work, we demonstrated that, using the same 20 hz activation protocol, dynorphin-containing PVN projections to the BNST do not inhibit glutamate in the BNST in a KOR-dependent manner. This, coupled with other experiments in the literature, suggests that local, not long-range, release of dynorphin is a common motif throughout the brain. Nonetheless, it would be prudent to thoroughly characterize this effect by demonstrating that the same 20 hz activation of BNST dynorphin neurons does not produce glutamate inhibition in projection sites.

(2) *Does optogenetically-stimulated dynorphin release produce similar effects in vivo?* Preliminary experiments suggest that a 5 minute, 20 hz stimulation of dynorphin/GABA neurons in the BNST can alter anxiety-related phenotypes seen in the elevated plus maze and open field (Nicole Crowley and Jonathan Sugam,

unpublished data). However, these experiments require replication, and further experiments are needed to address any behavioral contribution from the presumed co-release of GABA. Though the slice experiments were conducted in the presence of the GABA_A blocker picrotoxin, behavioral experiments will need to be conducted with site-specific infusion of the GABA_A antagonist as well. And, like the *ex vivo* experiments, it will be important to tease apart the effects of GABA (and putatively, dynorphin) downstream.

(3) *What activates BNST dynorphin neurons?* Though we demonstrated that BLA neurons synapse onto BNST dynorphin neurons, BLA neurons have been shown to fire *in vivo* in ranges under 10 hz (Pelletier et al., 2005), not fast enough to drive the effects seen here. Therefore, either another source of excitatory drive exists, or dynorphin neurons exist in a quiescent state until disinhibited. Understanding how dynorphin neurons are activated will help to place the circuit in greater context.

(4) *Does this paradigm actually mimic in vivo firing patterns of dynorphin neurons?* Though this protocol is sufficient to obtain dynorphin-mediated effects *ex vivo* and is an advancement over traditional pharmacological bath application of KOR agonists, it remains to be seen whether it is truly representative of endogenous firing activity of these neurons. Single-unit recording combined with optogenetic identification of dynorphin-expressing neurons could be used to understand how these neurons fire in different states (i.e., under stressful conditions) and whether a similar high-frequency firing pattern can be observed.

We also demonstrate that activation of KORs results in greater inhibition of

BLA-BNST glutamate onto DYN+ neurons compared to the inhibition seen at DYN- neurons. Three questions arise from these experiments.

(1) *How are DYN+ and DYN- neurons organized within the BNST?* Other work has suggested that neurons within the oval nucleus versus non-oval nucleus neurons can be identified by the presence of D1 receptors (Kim et al., 2012). Though preliminary investigations do not indicate that DYN+ neurons are expressed in any singular sub region of the BNST, a more thorough analysis is warranted. This will have important implications for the next question.

(2) *Do these separate populations interact?* The current set of experiments simply looked at BLA projections onto DYN+ and DYN- neurons separately. Other experiments demonstrate that dynorphin neurons do synapse locally (discussed above) and interestingly, these DYN+ to DYN- synapses are also inhibited by KOR activation (Crowley, unpublished data). This creates an interesting circuit whereby KORs inhibit BLA projections to DYN+ neurons, DYN- neurons, and the local interaction of DYN+ to DYN- (though the percent of inhibition by KORs differs). Another layer on top of this is the CeA projection to the BNST, which is also inhibited by KOR activation (Li et al., 2012). Due to the apparent near ubiquity of KOR inhibition of the BNST, it will be important to tease apart the precise circuitry on both a macro and micro level.

(3) *Do these separate populations represent divisions between other neuropeptide populations?* Other peptidergic populations exist within the BNST, most notably corticotrophin releasing factor (CRF). Interactions between CRF and dynorphin have been proposed (Bruchas et al., 2009), though the direction of this

relationship has been highly debated. Some work has demonstrated potential interactions between CRFRs and KORs (Thomas Kash, Kristen Pleil, and Nicole Crowley, unpublished data) but more work is needed.

KORs, THE BNST, AND ALCOHOL

Alcoholism is a chronic relapsing disorder (Koob, 2013). Alcohol exposure has been shown to activate neurons within the BNST (Chang, Patel, & Romero, 1995) but little is known about the precise interaction between KORs and alcoholism. We identified changes in KOR functioning in the BNST following an animal model of alcohol consumption, chronic intermittent ethanol exposure (CIE). CIE protocols have been shown to produce many changes in synaptic physiology. Previously, we found no changes in BLA intrinsic excitability or current-injected action potential firing, following one week of CIE (Nicole Crowley, unpublished data), though changes in BLA excitability have been seen with anxiety-inducing protocols (Rau et al., 2015). Longer cycles of CIE produced changes in synaptic drive and intrinsic excitability in ventral BNST, with CIE exposed mice having much more excitable neurons than control littermates (Pleil et al., 2015). In addition, CIE-induced changes have been demonstrated elsewhere in the rodent brain (Holmes et al., 2012).

A growing literature identifies alcohol-induced changes in BNST plasticity (Lovinger & Kash, 2015). Alcohol has been shown to activate neurons within the BNST (Chang et al., 1995) and has thus far been demonstrated to modulate glutamate (Kash, Matthews, & Winder, 2008) but not GABA (Weitlauf et al., 2004)

transmission. Kash et al. demonstrated that ethanol modulates NMDA-mediated glutamatergic transmission in the BNST (for extensive review of interactions between NMDA receptors and ethanol, see (Wills & Winder, 2013), but no work has demonstrated similar changes on GABAergic transmission. However, evidence does exist for ethanol-induced changes in peptidergic alteration of both glutamate and GABA transmission, most notably CRF and NPY (Kash & Winder, 2006). For example, in a similar CIE model, researchers demonstrated that alcohol exposure can alter CRF effects on glutamate transmission (Silberman, Matthews, & Winder, 2013).

In our studies, we first demonstrated a decrease in KOR relative gene expression following CIE. These results, unfortunately, are not bound to any specific cell type or synapse. Though most studies (Li et al. and the current study) suggest KORs are exclusively presynaptic in the BNST, a knowledge of where changes in receptor expression are occurring will improve understanding of the current results. We found no changes in spontaneous GABAergic or glutamatergic transmission following CIE. These results are not altogether surprising, as thus far no experiments have identified alcohol-induced changes in GABA transmission in the BNST, and alcohol-induced changes in glutamate transmission may depend on specific postsynaptic chemogenetic cell identities, such as CRF+ neurons (Silberman et al., 2013). However, we did identify a significant reduction in the ability of KOR activation to modulate glutamate transmission in CIE-exposed mice as compared to control mice. Silberman et al. found that following CIE, CRF effects on glutamatergic transmission were occluded. It is likely that a similar phenomenon is occurring with

dynorphin and KORs. Interestingly, we found the opposite effect on GABAergic transmission: KOR-activation-induced alterations of GABAergic transmission were enhanced in CIE-exposed as compared to control mice. We also identified potential tonic activation at GABAergic synapses, which is apparent when the KOR antagonist norBNI is bath applied. Though we do not see this effect at glutamatergic synapses, this is likely due to the irreversibility of KOR activation at these synapses, similar to that seen in the striatum (Atwood, Kupferschmidt, & Lovinger, 2014). Taken together, this suggests that following one cycle of CIE, dynorphin is released (we can assume locally, from the previous studies), and this local dynorphin activates KORs at GABAergic synapses, and likely glutamatergic synapses as well. At some of these synapses, there may be decreased KOR expression. This sets the stage for the change in behavioral phenotype observed.

We next assessed changes in social interaction following CIE. Social interaction has been noted for its simplicity as a measure of anxiety-like behavior (Knapp et al., 2004), and has been used to assess ethanol-withdrawal-induced anxiety (File, Baldwin, & Hitchcott, 1989). We demonstrated that following one week of CIE, social preference in mice was reduced compared to controls, but this deficit can be partially rescued by systemic administration of the KOR antagonist JDTic. This reduction in social preference following various models of alcohol exposure has been described by others (Knapp et al., 2004, Marcinkiewicz et al., 2015, Overstreet et al., 2006, Overstreet et al., 2003).

Coupled with the known GABAergic (CeA) and glutamatergic (BLA) projections to the BNST, this work suggests that alcohol exposure may modulate the peptidergic

control over the balance of inputs from these two regions. CIE has been shown to produce changes in synaptic drive in the ventral BNST (Pleil et al., 2015) suggesting that following alcohol exposure, events that lead to activation of KORs (such as stress or substance withdrawal) may lead to greater changes in synaptic drive. A few interesting questions arise from this information.

(1) *Are the differences in GABA and glutamate modulation actually driven by changes at CeA-BNST and BLA-BNST synapses or other synapses?* Though the CeA is one of the most robust GABA projections to the BNST, there is also an oval to non-oval GABAergic projection within the BNST itself (Kim et al., 2013). Optogenetics should be used to assess whether KOR functioning is altered at both or only one of these synapses following CIE. In addition, similar experiments should be conducted to address whether alterations in KOR functioning are confined to the BLA input, or are present in all glutamate inputs.

(2) *Does a greater alcohol exposure paradigm lead to different changes?* For the current experiments, we used a one week exposure paradigm. Others have used longer periods of exposure (Maldonado-Devicci et al., 2014), as well as protocols involving the combination of CIE and free choice drinking (Lopez, Becker, & Chandler, 2014). These differences may result in important but nuanced differences in neuronal alteration.

(3) *Are KOR changes different at different points of withdrawal?* In the current study, we conducted electrophysiological and behavioral assessments 24 hrs following the final alcohol exposure. In addition to longer exposure periods, other studies have used longer withdrawal sessions, up to 3 days (Becker & Lopez, 2004,

Pleil et al., 2015). KOR changes may be plastic and a more thorough time course analysis of these alterations would enhance our understanding of the therapeutic potential of targeting KORs.

(4) *Are there any protective effects against alcohol-induced KOR changes?* We demonstrate that KOR antagonists administered after alcohol exposure can rescue deficits in social behavior. It would be interesting to assess whether pre-administration of KOR antagonists has similar effects. The drug norBNI is a long-lasting KOR antagonist and can be administered prior to the one-week ethanol exposure. It would also be interesting to identify any protective behavioral or genetic traits prior to ethanol exposure. Correlations can be drawn between pre-ethanol anxiety levels (using assays such as the elevated plus maze) and post-ethanol KOR alterations, which can potentially identify behavioral risk factors for ethanol-induced KOR alterations in synaptic plasticity and behavior.

OVERALL THEMES

Overall, the first major theme of this work is an improved understanding BNST circuitry. Recently, researchers used structural connectivity analysis in humans to identify regions that showed significant likelihood of functional connectivity with the BNST. They identified much of the basal ganglia (including the accumbens), limbic regions such as the hippocampus, and the thalamus as key interconnected regions (Avery et al., 2014), though only a few of these regions have been studied in animal models. Li, Pleil, et al. demonstrated that the CeA sends a GABAergic projection to

the BNST, while Kim et al. demonstrated that the BLA sends a glutamatergic projection to the BNST, both of which suggest the BNST as a key regulating nucleus for anxiety- and addiction- related behaviors. In the current work, we confirm the BLA-BNST circuit, and also identify the PFC-BNST as a glutamatergic circuit. Other glutamatergic projections to the BNST exist, such as that arising from the parabrachial nucleus (Flavin et al., 2014), and functional imaging studies have suggested interconnectivity between the insula cortex and the BNST (Kober et al., 2008). Multiple other neurotransmitter systems project to the BNST as well, including a serotonergic input to the BNST (Lowery-Gionta, unpublished data), as well as a noradrenergic input from the locus coeruleus and nucleus of the solitary tract. There is also dopaminergic innervation from the VTA. Identified outputs of the BNST include reciprocal innervations of some of these nuclei, such as the VTA (Jennings, Sparta, et al., 2013; Silberman et al., 2013), the lateral hypothalamus, the parabrachial nucleus (Kim et al., 2013), the periaqueductal grey, and the dorsal raphe (for detailed review, see (Kash, 2012)). Here, we identify how a neuropeptide system can be a key regulator of some of this circuitry.

In addition to macro-anatomical research, much focus has been placed on micro-connectivity within the BNST itself. The BNST can be divided into multiple sub-nuclei mainly comprised of GABAergic populations (though some glutamatergic neurons exist exclusively in the ventral BNST; see (Jennings, Sparta, et al., 2013)). These sub-nuclei include the oval nucleus, the anterodorsal nucleus, and the juxtacapsular nucleus (in addition to those nuclei in the ventral BNST) (Lowery-Gionta, 2014). Here our work focused on the general circuitry of the dorsolateral

portion of the BNST.

The oval nucleus is comprised of dense GABAergic neurons, expressing a variety of neuropeptides, including dynorphin (Lowery-Gionta & Kash, 2014). Kim et al. demonstrated that GABAergic/dopamine D1 receptor-expressing neurons within the oval nucleus inhibit non-oval neurons, setting up some intraBNST control (however, the oval nucleus also sends projections outside of the BNST, such as to the CeA – see (Dong, Petrovich, & Swanson, 2001)). Kim et al. also assert that BLA-BNST neurons synapse exclusively onto non-oval neurons. Though we refute their finding of exclusive non-oval BNST-BNST circuitry (see image in **Fig 2.2A**), we agree that a complex interplay exists between local circuits in the BNST. Our studies suggest that BLA-BNST projecting neurons, though favorably synapsing onto non-oval regions of the BNST, still show connectivity within the oval itself. We do not find evidence that dynorphin neurons are contained exclusively in either the oval or non-oval portion of the BNST, suggesting that there is not singular control of the oval over non-oval regions. Other neuropeptides, such as CRF and NPY, are similarly not confined to any one nucleus of the BNST (Pleil et al., 2015). So, though some receptors may have discrete expression patterns in the BNST, neuropeptides do not appear to follow this pattern. Taken together, the current work and literature paint a complex overall model of neurotransmitter and neuropeptide interactions in the BNST (**Fig 4.1**).

A second minor theme of this work pertains to how this data relates to our hypothetical understanding of drug targets as therapeutics. KOR agonists and antagonists are in various stages of clinical trials for anxiety, depression, post-

traumatic stress disorder, anorexia, and alcoholism, with mixed results (clinicaltrials.gov). The FDA estimates only 30% of drugs even make it to clinical trials, with the vast majority never seeing the market at all (DiMasi, Hansen, & Grabowski, 2003). Drugs that appear promising in singular brain regions or discrete behavioral studies often do not transfer well to human therapy. Indeed, even the behavioral literature around KOR effects is mixed (as reviewed in detail in the introduction). Here we demonstrate a complex interplay between neuropeptides (dynorphin), receptors (KOR) neurotransmitters (glutamate and GABA), and a model of disease (alcoholism). Great caution should be taken when asserting that any drug, receptor, or pathway has the potential to be the ultimate solution for a neuropsychiatric disorder.

CONCLUSIONS

Taken together, this dissertation expands our understanding of KOR functioning in the BNST. We demonstrate that KOR modulation of glutamate inputs is pharmacologically distinct from KOR modulation of GABA inputs, and that this modulation occurs at some, but not all glutamate inputs to the BNST. The BLA-BNST circuit, an important pathway for anxiety-related behaviors, is heavily modulated by KORs, with greater KOR-induced inhibition of BLA-BNST DYN+ neurons than DYN- neurons. In addition, KORs at glutamate synapses in the BNST show decreased function following CIE exposure, with an enhancement at GABA

synapses. Importantly, the alterations in KOR modulation of glutamate and GABA transmission following CIE are likely related to other key circuits involved in anxiety-related behaviors. The CeA and BLA constitute major GABA and glutamate inputs to the BNST, respectively. Not only are they major nuclei involved in both alcohol consumption and anxiety-related behaviors, but they are likely the site of alcohol-induced changes in presynaptic KOR modulation in the BNST. Our work suggests that not only do KORs modify alcohol-induced changes in an anxiety-related behavior (sociability), but that the BNST is a likely site of this modification. These results will provide important information for the development of KOR-targeted drugs for the treatment of anxiety disorders and alcoholism.

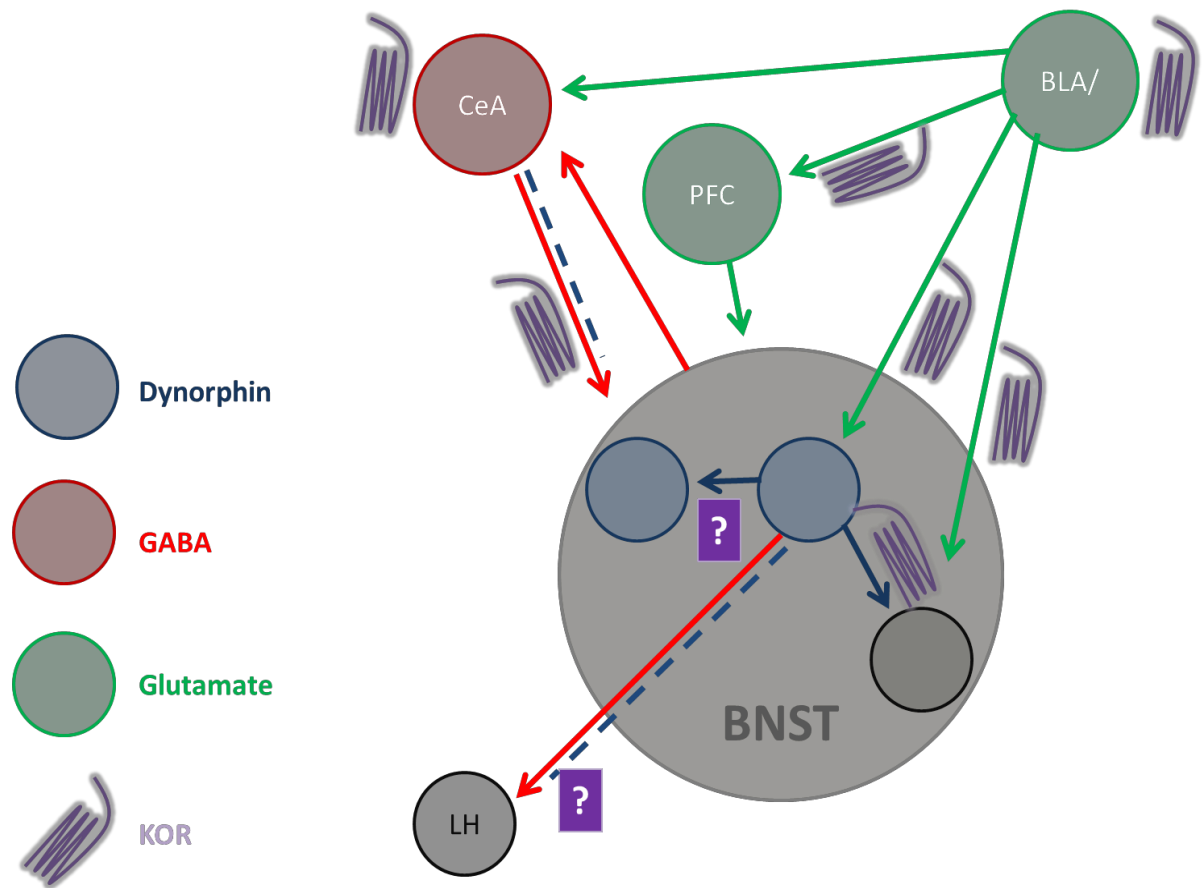


Figure 4.1 - Model of BNST circuitry and KORs. In this depiction of BNST circuitry, the BNST is outlined in grey. Li, Pleil, et al. demonstrated KORs on the CeA-BNST circuit, while the current study demonstrated KORs on the BLA-BNST circuit. Tejeda et al. demonstrated that KORs inhibit other BLA outputs (the PFC) as well. In addition, we demonstrated that KORs inhibit BLA synapses onto both Dyn+ non-Dyn neurons within the BNST. It is not known whether CeA projections the BNST show any specificity in KOR inhibition; in addition, a subset of CeA neurons express dynorphin, and it is unknown whether these neurons specifically project to the BNST. Some questions left unanswered include (1) do dynorphin neurons in the BNST synapse on each other, and are those circuits inhibited by KORs, and (2) are dynorphin outputs, such as the LH, inhibited by KORs.

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