Serotonergic Circuits Mediating Stress Potentiation of Addiction Risk

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Abstract

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Stress-induced release of dynorphins (Dyn) activates kappa opioid receptors (KOR) in monoaminergic neurons to produce dysphoria and potentiate drug reward; however, the circuit mechanisms responsible for this effect are not known. We found that conditional deletion of KOR from *Slc6a4* (SERT)-expressing neurons blocked stress-induced potentiation of cocaine conditioned place preference (CPP). Within the dorsal raphe nucleus (DRN), two overlapping populations of KOR-expressing neurons: *Slc17a8* (VGluT3) and SERT, were distinguished functionally and anatomically. Optogenetic inhibition of these $SERT^+$ neurons potentiated subsequent cocaine CPP, whereas optical inhibition of the $VGluT3^+$ neurons blocked subsequent cocaine CPP. $SERT^+/VGluT3^-$ expressing neurons were concentrated in the lateral aspect of the DRN. SERT projections from the DRN were observed in the medial nucleus accumbens (mNAc), but VGluT3 projections were not present in mNAc. Optical inhibition of $SERT^+$ neurons produced place aversion, whereas optical stimulation of $SERT^+$ terminals in the mNAc attenuated stress-induced increases in forced swim immobility and subsequent cocaine CPP. KOR neurons projecting to mNAc were confined to the lateral aspect of the DRN, and the principal source of dynorphinergic (Pdyn) afferents in the mNAc was from local neurons. Excision of *Pdyn* from the mNAc blocked stress-potentiation of cocaine CPP. Prior studies suggested that stress-induced dynorphin release within the mNAc activates KOR to potentiate cocaine preference by a reduction in 5-HT tone. Consistent with this hypothesis, a transient

pharmacological blockade of mNAc 5-HT_{1B} receptors potentiated subsequent cocaine CPP. 5-HT_{1B} is known to be expressed on 5-HT terminals in NAc, and 5-HT_{1B} transcript was also detected in *Pdyn*$^{+}$, *Adora2a*$^{+}$ and *ChAT*$^{+}$ (markers for direct pathway, indirect pathway, and cholinergic interneurons, respectively). Following stress exposure, 5-HT_{1B} transcript was selectively elevated in *Pdyn*$^{+}$ cells of the mNAc. These findings suggest that Dyn/KOR regulates serotonin activation of $5HT_{1B}$ receptors within the mNAc and dynamically controls stress response, affect, and drug reward.

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Chapter 1. Introduction

Stress Response

The ability of an organism to identify a nocuous stimulus and orchestrate the appropriate constellation of physiological and behavioral responses is a critical to survival. Hans Selye, an endocrinologist attempting to unify several seemingly disparate observations, first proposed a unifying concept to describe an organism's internal responses to a variety of environmental challenges (Selye, 1936). Selye, a modest man, first termed the response 'Selye's syndrome' (Jackson, 2014). It later became known as General Adaptation Syndrome and is now central to the conceptual framework describing stress. General Adaptation Syndrome consisted of three phases: an acute 'alarm reaction' during which the nocuous stimulus is directly addressed (e.g. 'fight or flight reaction'); a subsequent 'stage of resistance' during which internal functions attempt to restore homeostasis; and finally, following chronic exposure to noxious stimuli, a 'stage of exhaustion', in which the ability to restore homeostasis is overrun (i.e. adaptive ability is finite) (Selye, 1950).

Later, Seligman and colleagues described a phenomenon they termed 'learned helplessness' (Maier & Seligman, 1976). In this condition, learning occurs after an animal perceives that its behavioral response to a nocuous is innefective (Maier & Seligman, 1976).This learning ultimately induces the motivational, emotional, and cognitive effects of uncontrollability (Maier & Seligman, 2016). Importantly, learned helplessness has a subjective component: the animal's perception of the stimulus and the effect of this perception on internal state guides behavioral responses. While heretical at the time, this assertion has been supported by more the 50 years of subsequent experiments, solidifying the importance of subjective experience on stimulus processing and behavioral responses (Maier & Seligman, 2016).

Lastly, McEwen and colleagues presented the concept of allostasis to extend Selye's model of stress-mediated homeostasis. Whereas homeostasis refers to stability achieved through a return to baseline, allostasis describes stability through change (McEwen & Wingfield, 2003). Critically, these adaptations are predictive, preventative, and non-linear (McEwen & Wingfield, 2003). When a stress exposure is chronic and repeated, these adaptations can generate an excessive allostatic load that exhausts the system to and leads to a pathological state.

Systemic stress response

The biological adaptations provoked by stress may be described by a both central and peripheral processes. The peripheral effects of stress are primarily mediated by the hypothalamic-pituitary-adrenal (HPA) axis. Stress engages this axis by eliciting release of the neuropeptides corticotropin releasing factor (CRF) and arginine vasopressin (AVP) into portal circulation, which telegraphs this signal to the anterior pituitary (Stephens & Wand, 2012). Binding of CRF and AVP to cognate receptors in the anterior pituitary induces increased production and secretion of the glucocorticoid cortisol (in humans) or corticosterone (in rodents). The systemic effects of glucocorticoids acting at their receptors causes a variety of cardiovascular, immunological, and metabolic responses. For example, cortisol causes both gluconeogenesis in the liver and prevents glucose reuptake from muscles, causing a spike in blood glucose that can facilitate adaptive actions (Ganzel et al., 2010).

In addition to peripheral effects, activation of the HPA axis causes direct and indirect effects on the CNS. Acutely, direct and indirect actions of CRF and cortisol modulate a range of CNS targets, including monoaminergic nuclei implicated in affective regulation, as well regions of the cortex and hippocampus (Ganzel et al., 2010). These actions facilitate adaptive responses such as increased arousal, aversion, and enhanced memory consolidation. Activation of the HPA axis is typically terminated by glucocorticoid receptor-mediated negative feedback. However, when chronically or repeatedly engaged, elevated cortisol levels can engender allostatic overload, resulting in pathological states of insulin resistance, hypertension, hyperarousal, depressed mood, and memory impairment (Stephens & Wand, 2012).

Stress, Mood disorders, and Addiction in Society

The prevalence of stress-related mood disorders in the United States is high. Estimates indicate the lifetime prevalence of anxiety, mood, and substance use disorders (SUD) is high, at 29%, 21% and 15%, respectively (Kessler et al., 2005). Critically, these are often comorbid conditions, and it is estimated that the lifetime prevalence of substance use disorder in major depression is nearly 25%, while comorbidity of SUD with any mood disorder is nearly 50% (Hunt et al., 2020; Kessler, 1996). The fact that these conditions are often comorbid may suggest a common etiology. Mood disorders may be characterized a chronic stress state, suggesting that stress may be a causative or exacerbating factor in both conditions.

Epidemiological studies further support this connection. Factors such as the cumulative lifetime stress as well as present chronic or acute stress have been strongly associated with drug dependence (Turner & Lloyd, 1995). Indeed, past stress exposure is correlated to drug and alcohol dependence in a dose-dependent manner (Sinha, 2008). Recent stress exposure in adolescents (e.g. abuse, parental divorce or death) acutely increases both the likelihood of drug use and drug abuse (Hoyland & Latendresse, 2018; Newcomb & Harlow, 1986).

The COVID-19 pandemic has provided further epidemiological evidence of an intersection between stress and SUD. The CDC reports that nearly 1/7 Americans surveyed self-reported an initiation or increase in substance use to cope with pandemic-related distress (Czeisler, 2020). Additionally, drug overdose deaths per month in the US increased more than 30% during the pandemic compared to the previous year (Ahmad, 2021).

Presently, no pharmacotherapies are approved to prevent these stress-induced increases in initiation or escalation of drug taking. This represents a critical unmet need. In conjunction with robust societal interventions, therapeutics that attenuate the pro-addictive effects of environmental stress will be essential to addressing future addiction epidemics.

Establishing a biological framework addiction risk

While societal factors are a critical component of addiction risk, biological factors strongly influence the probability of developing a substance use disorder. Addiction heritability estimates of substances such as cocaine and opioids are as high 0.72, indicating a strong correlation between genetic factors and substance use (Goldman et al., 2005). These studies may overestimate the relative importance of genetic factors; however, due to confounding environmental factors.

What are the neurobiological factors mediate this interaction between genes and environment to promote addictive behavior? A diverse set of theories have been posited to address this central question. It is likely that instead of one underlying factor, a broad range of neuropsychological components converge to increase addiction risk. Among these are impulsivity and decreased executive function, incentive sensitization, and allostatic dysregulation (Koob & Volkow, 2016; Redish et al., 2008). These factors contribute to different stages of the addiction cycle, generally resulting in a transition from impulsive to

compulsive behavior (Koob & Volkow, 2016). This transition is accompanied by changes in neural circuits that subserve these behavioral changes. I will focus next on the allostatic mechanisms (and the dysregulation of thereof) that mediate the neuropharmacological adaptations to stressors, and their contribution to addictive behavior.

Stress, CRF and dynorphin

Stress evokes the release of the neuropeptides corticotropin releasing factor (CRF) and dynorphin (Rivier & Plotsky, 1986). The cognate receptors of these neuropeptides, CRF_1/CRF_2 and the kappa opioid receptor (KOR), are expressed throughout the CNS and periphery (Bale & Vale, 2004). CRF induces the release of dynorphin (Land et al., 2008). CRF receptors mediate this affect: CRF_2 -induced dynorphin release is associated with the aversive properties of stress, whereas CRF_1 activation is associated with the anxiogenic properties of stress (Bruchas et al., 2009; Land et al., 2008). These neuropeptides also regulate behavior by directly regulating neurotransmitter systems in parallel.

Dynorphin is a member of the endogenous opioid system, which exerts control over emotional and behavioral responses to stress. Dynorphin comprises a family of neuropeptides that are formed from the precursor prodynorphin (Schwarzer, 2009). This family includes six peptides of different lengths that confer different specificity and potency at KOR. Unlike classical neurotransmitters, neuropeptides, which are released from dense core vesicles, require sustained neural activity for release (Drake et al., 1994). Termination of neuropeptide signaling is generally slower than that of classical neurotransmitters. This allows the neuropeptide to diffuse to extra-synaptic sites before peptidase-mediated termination of the signal. These qualities suggest that neuropeptides, as compared to classical neurotransmitters, more broadly regulate neural circuits and may induce distinct processing modes within these circuits (Berridge, 2019).

Intracellular consequences of KOR activation

Activation of the kappa opioid receptor has a variety of intracellular effects that may vary depending on cell type, cellular compartment, brain region, and time relative to activation (Liu et al., 2018)

. Typical activation of KOR engages two intracellular signaling cascades: those dependent on canonical, G-protein mediated signaling, and others dependent on ß-arrestin recruitment. G-protein mediated effects are initiated upon $G_{ßγ}$ dissociation from the $G_α$ subunit of the heterotrimeric G-protein complex (Schattauer et al., 2019). $G_{ßγ}$ induces phosphorylation of extracellular signal-related kinase (ERK) and directly regulates ion channels, resulting in an increase in current through G-protein inwardly rectifying potassium channels (GIRKs) and a decrease in conductance through calcium channels (Bruchas et al., 2010). This decreases neurotransmitter release. Alternatively, sustained activation of KOR induces phosphorylation of the C terminus of the receptor at serine 369 by GRK3 (McLaughlin et al., 2003). ß-arrestin recruitment to the site sterically-inhibits activation of G-protein signaling and serves as a scaffold for phosphorylation of p38α mitogen associated protein kinase (MAPK) and ERK (Schattauer et al., 2019). Substrates of p38α include transporters (serotonin reuptake transporter; SERT), channels (inward rectifier potassium channel 3.1 (Kir3.1)), protein kinases (mammalian target of rapamycin (mTOR)), and transcription factors (zinc finger protein 268 (zif268)) (Ji et al., 2021; Liu et al., 2018).
Additionally, KOR ligands can induce the phosphorylation of c-Jun N-terminal kinas (JNK). Activation of JNK by agonists or collateral antagonist such as norBNI stimulates peroxiredoxin 6 (PRDX6), which induces NADPH oxidase to generate reactive oxygen species (ROS) (Schattauer et al., 2017). This local increase in ROS results in the depalmitoylation of the receptor $G_α$, which irreversibly inactivates G-protein signaling. This form of receptor inactivation mediates the long-lasting effects of JNK-activating KOR antagonists (e.g. norBNI and JDtic) and may be a general feature of G_i signaling.

Cellular and neural pathways mediating KOR effects

The activation of different these intracellular cascades has been implicated in distinct behavioral consequences. Pharmacological inhibition of p38α MAPK blocked stress-induced immobility and kappa agonist-induced aversion but had no effect on conditioned taste aversion, indicating a specific effect on stress-induced aversion (Bruchas et al., 2007). Global deletion of GRK3 and pharmacological inhibition of mTOR cause similar effects (Bruchas et al., 2007; Liu et al., 2018). Additional studies have shown that p38α and GRK3 are required for stress potentiation and reinstatement of cocaine preference (Bruchas et al., 2011; Schindler et al., 2012). KOR agonists that preferentially activate G-protein signaling (e.g.

nalfurafine and 6'GNTI) inhibit chloroquine phosphate-induced scratching and increase tailflick latency in the warm water tailflick assay without aversive effects (Brust et al., 2016). Lastly, 6'GNTI decreases paroxysmal discharges in a mouse model of temporal lobe epilepsy without generating aversion (Zangrandi et al., 2016). These data support the conclusion that KOR-induced activation of p38α signaling promotes dysphoria and sensitization to drug reward, whereas engagement of G-protein signaling has analgesic, antipruritic, and antiepileptic effects.

While widely expressed throughout the CNS, both dynorphin and KOR expression are concentrated within various nuclei. Recent studies conducted by crossing Prodynorphin-Cre and Td-Tomato reporter lines indicated the presence of dynorphin-expressing neurons in the striatum, bed nuclei of the stria terminalis (BNST), central amygdala (CeA), locus coeruleus (LC), and dorsal raphe nucleus (DRN) (Al-Hasani et al., 2015). Similar experiments with KOR-Cre mice indicate particularly dense areas of KOR-expressing neurons in the ventral tegmental area, nucleus accumbens (NAc), prefrontal cortex, anterior cingulate cortex, BNST, CeA/BLA, and DRN (C. Chen et al., 2020). These findings are in line with previous in situ hybridization studies and autoradiography studies in wildtype mice (Chen et al., 2020). While this is not a comprehensive list of Dyn- and KOR-expressing populations, the presence of Dyn/KOR throughout affective circuitry indicates a potential functional role of these populations in mediating the hedonic properties of Dyn/KOR activation.

Stress, CRF and effects on affective circuitry

Stress induces activation centrally-expressed CRF_1 and CRF_2 receptors that is independent of activation of the HPA axis (Bale & Vale, 2004). CRF has a higher affinity for CRF_1 than CRF_2, resulting in preferential activation of CRF_1 at low concentrations (Bale & Vale, 2004). CRF_1 and CRF_2 may mediate opposing behavior effects in response to stressors. It has been posited that CRF_1 overactivation induces stress-related pathophysiology, and that the actions of CRF_2 counteracts this initial stress response to ensure physiological allostasis (Henckens et al., 2016). Alternatively, observations in monoaminergic nuclei including the DRN and locus coeruleus have implicated CRF_1 in mediating active defensive behavior and CRF_2 in passive coping behavior (Valentino & Van Bockstaele, 2008). Changes in the surface expression

of these receptors following stress exposure may mediate a transition from active to passive coping responses.

Actions of CRF in the DRN modulates the behavioral response to swim, restraint, and social defeat stressors (Henckens et al., 2016). Within the DRN, CRF terminals preferentially innervate non-serotonergic (putatively GABAergic) neurons (Waselus et al., 2009). Upon CRF exposure, CRF_1-mediated activation of GABAergic DRN neurons broadly results in decreased 5-HT in the DRN and projection regions (Lukkes et al., 2008). At high concentrations of CRF, however, the activation of CRF_2 expressed in DRN 5-HT neurons results in an increase in 5-HT neuronal activity and release (Kirby et al., 1995; Lukkes et al., 2008). Interestingly, exposure to repeated forced swim stress induces a redistribution of CRF receptors, such that CRF_1 is internalized and CRF_2 surface expression is elevated (Valentino et al., 2010). This change is associated with a change in behavioral patterns in the swim stress assay from active to passive coping (increased immobility). Lastly, acute stress induces increases the activity of tryptophan hydroxylase type II (TPH2), the rate-limiting step of 5-HT synthesis, suggesting possible regulation of serotonin synthesis (Vincent et al., 2018). This effect is potentiated by chronic cortisol (CORT) administration and dependent on the balance between local CRF_1 and CRF_2 actions (Donner et al., 2016). Chronic CORT treatment also increases basal TPH2 expression and activity, which is accompanied by increases in anxiety and depression-like behavior (Donner et al., 2016). Together, these data indicate that CRF exerts potent and dynamic control over the DRN serotonin system.

CRF is also involved in stress-mediated effect on other regions, including the VTA, LC, and amygdala. In the LC, CRF_1 activation induces tonic activation of noradrenergic neurons, which is aversive, anxiogenic, and facilitates behavioral flexibility (McCall et al., 2015; Snyder et al., 2012). CRF has heterogenous effects on VTA dopaminergic neurons, with most reports indicating enhanced dopaminergic neuron firing (Wanat et al., 2008; Zorrilla et al., 2014). VTA CRF_1 receptors mediate stress-induced drug seeking and the aversive properties of drug withdrawal, and CRF_2 activation in this region appear to have a complementary role on these effect (Grieder et al., 2014; Zorrilla et al., 2014). In the extended amygdala, CRF is heavily implicated in regulating basal and stress-induced anxiety like behavior, as well as threat assessment and memory consolidation. CRF in the BLA is associated with increased glutamatergic activity and anxiety-like behavior (Henckens et al., 2016). In the CeA, CRF release is not associated with

changes in basal anxiety levels but rather modulates stress-induced anxiety through CRF_1-induced increases in GABA (Gilpin et al., 2015). Within the BNST, CRF_1 modulates glutamate transmission both directly and indirectly (Henckens et al., 2016). CRF_1 and CRF_2 activation in this region increases anxiety-like behavior (Tran et al., 2014).

Stress, dynorphin and effects on affective circuitry

A variety of genetic and pharmacological studies implicate the Dyn/KOR system in mediating anxiety, dysphoria, and passive coping with stressors. Bals-Kubik and colleagues first established that KOR agonism is aversive and mediated by receptors within the CNS (Bals-Kubik et al., 1989). Subsequently this conclusion has been supported data showing that KOR knockout mice do not form an aversion to a KOR agonist (McLaughlin et al., 2003). KOR antagonism or prodynorphin deletion also blocks stress-induced escalation of passive coping behavior in forced swim stress and social defeat assays (Mague et al., 2003; McLaughlin et al., 2006). The effects of kappa antagonism on stress-induced aversion were assayed by subjecting mice to a repeated forced swim stress in the context of a novel odorant with or without norBNI pretreatment. Subsequently, aversion to the odorant alone was absent in norBNI pretreated animals, indicating that KOR is necessary for stress-induced aversion (Land et al., 2008). KOR also regulates innate and learned fear responses. Blockade of KOR has been shown to decrease anxiety-like behavior in the elevated plus maze and light/dark box assays (Gillett et al., 2013; Knoll et al., 2007). KOR antagonism also decreases fear-potentiated startle and freezing in a foot-shock- paired context (Knoll 2007). While KOR contributes to these behaviors, it is likely that the Dyn/KOR system does not regulate basal anxiety-like behavior in unperturbed animals, indicating low basal dynorphin release in the relevant circuits (Filliol et al., 2000).

Within the DRN, activation of KOR decreases 5-HT efflux (Tao & Auerbach, 2005). Electrophysiological evidence indicates that kappa opioid receptor activation decreases both presynaptic glutamate release and directly inhibits $DRN^{5\text{-}HT}$ by a GIRK-dependent mechanism (Lemos et al., 2012; Pinnock, 1992). Additionally, pharmacological blockade of DRN^{KOR} or selective excision of KOR from SERT-expressing neurons prevents KOR agonist U50,488-induced conditioned place aversion, suggesting that receptor activation in DRN serotonergic neurons is necessary for KOR-mediated aversion (Ehrich et al., 2015).

Viral experiments restoring protein to the DRN of knockout mice prior to U50,488-induced CPA have extended these findings. Viral delivery of KOR to the DRN of KOR KO mice restores U50,488-induced CPA, indicating that the actions of DRN^{KOR} are sufficient to mediate aversion (Land et al., 2009). Further this aversion in DRN^{KOR}:KOR KO mice can be blocked by infusion of norBNI into the NAc (Land et al., 2009). Viral restoration of SERT expression in DRN neurons restored KOR-induced aversion in SERT KO mice (Schindler et al., 2012).

The actions of p38α MAPK are critical to the cellular and behavioral consequences of KOR activation in the DRN. The pro-depressive effects of social defeat stress and forced swim stress are attenuated in mice lacking p38α in SERT neurons, indicating a role for p38α in regulating the aversive actions of stress and KOR activation (Bruchas et al., 2011). Within $DRN^{5\text{-}HT}$ neurons, KOR activation selectively increases surface SERT expression 5-HT reuptake within the ventral striatum in p38α-dependent manner, as measured in synaptosomes from stress mice (Schindler et al., 2012). These effects were blocked by local infusion of norBNI into the NAc, indicating the NAc^{KOR} are necessary for this effect. Interestingly, a recent study found that KOR activation in rat striatal synaptosomes increases SERT phosphorylation, decreases SERT surface expression, and decreases 5-HT reuptake (Sundaramurthy et al., 2017). These effects were not blocked by inhibition of p38α or ERK1/2. The reason for these discrepant findings is not clear but may be due to *in vivo* vs *ex vivo* stimulation of KOR (i.e. stimulation of KOR in striatal synaptosome preparations may not reflect KOR actions in an intact animal). Lastly, as with the CRF system, Dyn/KOR signaling in the DRN is dysregulated following stress exposure. Following repeated stress exposure, direct inhibition of serotonergic neurons is blunted by a p38α and GIRK-dependent mechanism (Lemos et al., 2012). This indicates that stress decreases the subsequent inhibitory effect of KOR on 5-HT neurons. The net circuit effects of inhibition of glutamatergic afferents, decreased postsynaptic inhibition of $DRN^{5\text{-}HT}$, and increased 5-HT reuptake in the NAc following repeated stressors is unclear. However, these changes may reflect a stress-induced shift in DRN serotonin output away from the NAc and towards other serotonergic targets, including the amygdala and PFC.

VTA dopaminergic neurons express somatic and terminal KOR, which inhibit both neuronal firing and dopamine release (Ehrich et al., 2015; Svingos et al., 2001). Conditional removal of KOR from dopaminergic neurons prevents U50,488-induced CPA (Chefer et al., 2013; Ehrich et al., 2015). Re-

expression of wildtype KOR in the VTA of these knockout mice, but not a muted KOR incapable initiating p38α signaling, reinstates aversion to KOR agonism (Ehrich et al., 2015). Pharmacological blockade of VTA^{KOR} also prevents U50,488 aversion and increases in marble-burying, a measure of compulsion (Abraham et al., 2017; Ehrich et al., 2015). As with SERT neurons, conditional removal of KOR effector p38α from DAT-expressing cells prevents KOR-mediated aversion (Ehrich et al., 2015). Surprisingly p38α is not required for KOR-mediated inhibition of DA in the NAc, indicating that somatic effects of KOR are instead responsible for KOR's aversive effects. KOR regulation of DAT surface expression is controversial, with some groups observing an increase following KOR activation and others finding no effect (Ehrich et al., 2014; Kivell et al., 2014). Prior stress exposure induces KOR-mediated plasticity in the VTA. For example, cold swim stress exposure abrogates long term potentiation of GABAergic inputs to VTA^{DA} neurons through a KOR-dependent mechanism (Polter & Kauer, 2014). Additionally, repeated administration of a KOR agonist induces a decrease in VTA^{DA} firing, the persistence of which depends on p38α MAPK (Ehrich et al., 2015).

Kappa opioid receptors are also expressed throughout the NAc. KOR is present on dopaminergic, serotonergic, and glutamatergic afferents, as well as D1- and D2-expressing medium spiny neurons (Tejeda et al., 2017). Local infusion of norBNI in mice with elevated NAc dynorphin levels blocks increases in swim-stress induced immobility, indicating a critical role for the NAc Dyn/KOR system in the aversive property of stressors (Pliakas et al., n.d.; Shirayama et al., 2004). Recently, direct manipulation of prodynorphin-expressing neurons in the NAc has revealed two discrete populations with divergent, KOR-dependent effects on reward-related behavior. Stimulation of dynorphinergic neurons in the ventral shell of the NAc drives aversive responses, while stimulation of dorsal shell dynorphin drives reward (Al-Hasani et al., 2015). The relevance of this finding to Dyn/KOR-mediated stress responses is not yet clear. KOR also mediates stress responses in other regions. In the BLA, anxiogenic effects of CRF_1 activation is blocked by local administration of norBNI (Bruchas et al., 2009). In the BNST, dynorphin release onto presynaptic glutamatergic terminals from the BLA controls the gain on local modulation of anxiety-like behavior (Crowley et al., 2016).

Dyn/KOR system in humans

The effects of KOR agonists are profoundly dysphoric and anxiogenic in humans, and the present literature indicates that the genetic differences in the Dyn/KOR system may increase risk for addiction and that the system is actively dysregulated in subjects with substance use disorders (González et al., 2006; Pfeiffer et al., 1986). Single nucleotide polymorphisms in the dynorphin and the KOR genes have been associated with heroin, cocaine, and alcohol dependence (Gerra et al., 2018; Xuei et al., 2006; Yuanyuan et al., 2018). Additionally, radioligand binding studies have shown higher levels of KOR in subjects with cocaine use disorder that correlates to cocaine preference (Martinez et al., 2019). This binding is significantly reduced following a three-day cocaine binge session, suggesting increased levels of endogenous dynorphin release (Martinez et al., 2019).

Dyn/KOR system in animal models of addiction

Activation of the CRF and Dyn/KOR systems is heavily implicated in regulating the initial hedonic effects of potentially addictive substances, escalation of drug taking behavior, and reinstatement of drug seeking behavior after a period of abstinence. A comprehensive discussion of these data is beyond the scope of this review. I will briefly summarize the role of CRF and dynorphin in escalation and reinstatement of drug taking/seeking before returning to the data relevant to the central focus of this dissertation: stress potentiation of drug reward.

Escalation

Escalation of drug taking is a central feature of the transition from occasional, recreational drug use to habitual, uncontrolled drug taking (Koob & Le Moal, 2008). All drugs of abuse elevate brain reward thresholds during acute withdrawal (Koob & Le Moal, 2008). These allostatic changes typically are highly associated with precede escalation of drug taking (Koob & Kreek, 2007). CRF and KOR antagonists generally attenuate this behavior (Koob & Kreek, 2007). Pharmacological blockade of KOR decreases escalation of cocaine, ethanol, and methamphetamine consumption (Walker & Koob, 2008; Whitfield et al., 2015). Likewise, CRF release in the extended amygdala has been associated with withdrawal-induced anxiety and dysphoria, and CRF antagonists block escalation of cocaine, ethanol, and heroin (Funk et al., 2014; Park et al., 2015; Specio et al., 2008).

Reinstatement

Addiction is a condition defined by chronic relapse, in which compulsive drug taking is reinstated after a period of abstinence (Mantsch et al., 2014). Stress-induced reinstatement of drug seeking and drug taking in animal models has identified dynorphin and CRF actions in reward circuitry as mediators of this behavior (Bruchas et al., 2010; Shaham et al., 2003). Genetic and pharmacological disruption of the Dyn/KOR system prevents stress induced, but not cue or priming induced, reinstatement of cocaine, ethanol, and nicotine seeking (Bruchas et al., 2011; Grella et al., 2014; Land et al., 2008; Redila & Chavkin, 2008). Alternatively, pharmacological activation of CRFRs by ICV administration of CRF reinstates heroin, alcohol, and cocaine seeking (Mantsch et al., 2016).

Potentiation of Preference

Exposure to stress increases subsequent preference for drugs including cocaine, nicotine, ethanol, as measured by conditioned place preference or self-administration (Piazza et al., 1990; Walker & Koob, 2008). This increase in preference is likely mediated by an increase in reward valence and not by other factors, such as associative learning (Groblewski et al., 2015; Schindler et al., 2010). Increase preference facilitates a transition from occasional drug taking to habitual drug taking behavior (Koob & Volkow, 2016). This interaction between an aversive experience a subsequent increase in drug preference was initially attributed to stress-induced desensitization of 'anti-reward' systems (Koob & Le Moal, 2008; McLaughlin, et al., 2003). Desensitization of these systems would, in theory, remove opponent processes that reduce drug preference (Koob and Le Moal, 2008). However, genetic and pharmacological disruption of these systems stress potentiation of reward undermined this hypothesis. The current framework for stress potentiation of drug preference suggests that prior stress induced dysphoria provides hedonic contrast to subsequent euphorigenic effects of drug administration to increase the substance's relative effect on mood (Bruchas et al., 2010).

This model is supported by several studies from our lab and others that indicate the aversive properties of behavioral or pharmacological stressors are critical to potentiation of subsequent drug preference. Systemic administration of norBNI or global knockout of prodynorphin, KOR, or GRK3 blocks stress potentiation of cocaine CPP (McLaughlin, et al., 2003; Schindler et al., 2012). Alternatively, administration of U50,488

potentiates subsequent cocaine preference and does so in a time-dependent manner (McLaughlin et al., 2006). These findings suggest that the activation of the Dyn/KOR system is necessary and sufficient for stress potentiation of cocaine preference. Likewise, pharmacological blockade of CRF_1 (but not CRF_2) systemically blocks stress potentiation of drug preference (Kreibich et al., 2009).

Subsequent work from our group has identified actions of KOR in VTA dopamine and DRN serotonin neurons as central to the stress augmentation of cocaine preference. Recordings of dopamine release in the NAc indicate that KOR activation decreases dopamine tone in the NAc. This KOR-induced decrease in NAc DA prior to cocaine administration is associated with an increased sensitivity to the rewarding effects of cocaine (E. H. Chartoff et al., 2016; Ehrich et al., 2014). Further, this effect is highly time dependent, as KOR activation 24 hours prior to or concurrently with cocaine administration decreases the rewarding effects of cocaine (E. H. Chartoff et al., 2016). In $DRN^{5\text{-}HT}$ neurons, conditional excision of p38α from SERT-expressing cells blocks stress potentiation of cocaine preference (Schindler et al., 2012). This supports a role of KOR-induced activation of p38α within serotonin neurons in stress potentiation of drug reward. Additional work indicates that p38α -dependent signaling and KOR activation in the NAc mediates an increase in the surface expression of SERT in the ventral striatum (Schindler 2012). This KOR-mediated increase in 5-HT reuptake within the NAc may contribute to potentiation of cocaine preference, as the SSRI citalopram blocks U50,488-induced potentiation of cocaine CPP. Together, these data support a role of VTA^{DA} neurons and $DRN^{5\text{-}HT}$ neurons in a hedonic rebound following stress exposure. Conceptually this rebound is the inverse of the hedonic allostasis proposed by Koob and colleagues to mediate escalation and reinstatement of drug taking. In this framework, the aversive properties of a stressor sensitize an animal to the rewarding effects subsequent drug exposure.

Serotonin system in reward and aversion

Although alterations in serotonin signaling regulates affect in humans, the precise nature of the regulation and the mechanisms that mediate this regulation are poorly understood. Historically, manipulation the serotonin system with selective serotonin reuptake inhibitors has led to antidepressant-like effects in animals and humans (Müller & Homberg, 2015). Polymorphisms within the gene encoding the SERT transporter in humans have been associated with an increased risk for stress-induced depression and a

potentiated effect of stress on drug taking, implicating stress dysregulation of 5-HT in these conditions (Caspi et al., 2003; Todkar et al., 2013).

The role of the dorsal raphe nucleus in reward-related behavior was first identified by electrical ICSS mapping experiments (Simon et al., 1976). In the intervening decades, pharmacological, electrical, genetic, and optogenetic manipulations of the DRN serotonin system have yielded a wide, and often conflicting, array of findings concerning function of these neurons in behavior. This is likely due to the complexity of the DRN serotonin system, which projects broadly throughout the brain, innervating virtually all corticolimbic structures involved in the regulation of stress and mood response (Mahar et al., 2014). This complexity is compounded by the variety of serotonin receptors, which includes 14 receptor subtypes that include one ionotropic receptor and others that couple to $G_{i/0}$, G_q, and G_s G-protein signaling. Three competing, but not necessarily mutually exclusive, theories have been posited to address an overarching role of serotonin in behavior. First proposed was the theory that 5-HT mediates behavioral responses to stressful and aversive stimuli by opposing the actions of dopamine (Deakin & Graeff, 1991). A second theory posits that 5-HT promotes behavioral inhibition and patient waiting that is critical to controlling impulsive behavior (Boureau & Dayan, 2011; McDannald, 2015). This facilitates responses to either avoid punishment or receive a delayed reward. Lastly, a ‘mood’ theory suggests that serotonin tracks a cumulative reward and aversion function at slow time scales (Cohen et al., 2015; Luo et al., 2016). The findings of an intersection between stress, aversion, and drug reward outlined above are most consistent with the ‘mood’ theory of serotonin function. Recent findings that have identified genetically and anatomically defined subsystems of serotonin may indicate that different serotonergic subsystems subserve aspects of these functions.

The DRN comprises a heterogenous population of serotonergic, glutamatergic, dopaminergic, and GABAergic neurons. Recently, a wide variety of serotonergic markers an co-markers have been established that allow segregation of genetically similar subpopulations (K. W. Huang et al., 2019; Okaty et al., 2020; Ren et al., 2019). One of these markers, vesicular glutamate transporter type III (VGluT3), encodes for a glutamate transporter within glutamate-releasing serotonegic neurons of the DRN (Qi et al., 2014). Transgenic crosses of VGluT3-cre and SERT-Cre mice has enabled the tracing of common projections regions of these subtypes, which include the insular, entorhinal, and piriform cortex; lateral

hypothalamus; ventral tegmental area; and cortical amygdala (Ren et al., 2019). Interestingly, optogenetic studies have indicated the DRN^{VGluT3} and DRN^{SERT} populations may have distinct effects on reward and motivated (Liu et al., 2014; McDevitt et al., 2014; Qi et al., 2014). However, the role of these neuronal populations in stress modulation of reward behavior has not been evaluated.

These studies indicate that KOR-induced increases in 5-HT reuptake witin the ventral striatum may be critical for stress-induced potentiation of cocaine preference. However, whether a decrease in serotonin tone within the NAc is sufficient to potentiate subsequent cocaine preference, as well as reward sensitivity more generally, is unknown. Additionally, the presynaptic sources of dynorphin and postsynaptic serotonin receptors involved in this phenomenon has not been indentified.

In the following chapter, I will examine the Dyn/KOR regulation of the serotonin system and motivated behavior, with attention to *i)* anatomical subsystems of DRN serotonergic neurons and *ii)* the impact of stress state on reward processing. Integrating these factors may facilitate the dissection of stress modulation $DRN^{5\text{-}HT}$ populations and the consequent effects on reward processing.

Chapter 2. Stress decreases serotonin tone in the nucleus accumbens to promote aversion and potentiate cocaine preference via decreased stimulation of 5-HT$_{1B}$ receptors

Data presented in this section are from the following first-author manuscript:

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Author contributions: *I conducted all behavioral experiments with help from AA and CN with rFSS potentiation of cocaine in cKO experiements. I conducted immunohistochemistry with help from RT, and PRS performed RNAscope. I analyzed all data, with histological analysis help from PRS and RT. I conceived of and designed all experiments with help from BBL, JFN, and CC. CC and I wrote the manuscript.*

Introduction

Stress has profound effects on the risk of substance use disorders and relapse in humans and promotes drug seeking behaviors in animal models of addiction (Mantsch et al., 2014; McLaughlin, Marton-Popovici, et al., 2003; Redila & Chavkin, 2008; Sinha, 2008). Animal studies have shown that the endogenous opioid dynorphin (Dyn) and its cognate receptor, the kappa opioid receptor (KOR), are critical to the enhancement of each stage in the progression towards drug addiction, from initial preference, to escalation, and ultimately reinstatement (McLaughlin et al., 2006; Redila & Chavkin, 2008; Smith et al., 2018; Whitfield et al., 2015). These have been shown to be mediated in part by stress-induced modulatory effects on the serotonin (5-HT) system; however, the contribution of serotonin (5-HT) to hedonic processing remains controversial (Land et al., 2009; Luo et al., 2015; Schindler et al., 2012). In humans, polymorphisms in genes encoding dynorphin, KOR, and the serotonin transporter (SERT) have been linked to stress-induced depression and increased risk for addiction (Caspi et al., 2003; Gerra et al., 2018; Yuanyuan et al., 2018; Yuferov et al., 2010).

Stress-evoked release of neuropeptides including corticotropin-releasing factor (CRF) and the prodynorphin-derived peptides impinge on affective circuitry to orchestrate changes in both

neurophysiological state and observable behavior (Land et al., 2008). CRF-induced release of the dynorphins is necessary for the dysphoric properties of stress, and Dyn action at KOR on dopaminergic and serotonergic neurons is necessary for a stress-induced dysphoric state, which may underlie stress-potentiation of drug-seeking behaviors (Ehrich et al., 2015; Land et al., 2008). KOR activation within serotonergic neurons of the dorsal raphe nucleus (DRN), which is a hedonic hot spot and primary source of forebrain serotonin, results in somatic hyperpolarization and increases the surface expression of serotonin transporter in axon terminals projecting to the nucleus accumbens (NAc) (Bruchas et al., 2011; Lemos et al., 2012; Luo et al., 2015; Schindler et al., 2012). Together, these findings suggest that stress-induced activation of the Dyn-KOR-5-HT axis reduces serotonin tone in the NAc to increase drug reward in mice.

Direct manipulation of serotonergic neuron activity in DRN via optogenetic and chemogenetic techniques, however, has resulted in conflicting conclusions as to the role of 5-HT in mediating responses to rewarding, aversive, and stressful stimuli (Liu et al., 2014; Marcinkiewcz et al., 2016; McDevitt et al., 2014; Qi et al., 2014; Walsh et al., 2018; Wang et al., 2019). These discrepancies may be due to the genetic and anatomical complexity of the DRN as well as the impact of different assay conditions and event timing on stress and reward processing (Cohen et al., 2015; Zhong et al., 2017). In the present study, we resolved a KOR-expressing, serotonergic projection from the lateral aspect of the DRN to the medial NAc (mNAc) that controls 5-HT tone to regulate stress response, aversion, and reward potentiation. We further implicate presynaptic dynorphin and postsynaptic 5-HT_{1B} receptors within the mNAc in mediating these effects.

Materials and Methods

Drugs: Cocaine-HCl, norbinaltorphimine-HCl (norBNI), and ±U50488 were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD) and were dissolved in 0.9% saline. Sodium pentobarbital, Beuthanasia Special-D, and isoflurane were obtained from University of Washington Medical Center Drug Services. CP 94253-HCl, GR 127935-HCl, and GR 125487 were purchased from Tocris Bioscience and dissolved in artificial cerebrospinal fluid (ACSF).

Viral reagents: CAV2-DIO-ZsGreen was provided by Dr. Larry Zweifel (University of Washington). UNC Vector Core or Addgene provided: AAV5-DIO-EYFP (UNC/ Addgene #27056), AAV5-DIO-SwiChR$_{CA}$-EYFP (UNC), AAV5-DIO-ChR2-EYFP (UNC/ Addgene #20298), AAV5-EGFP (#105547), AAV5-Cre-EGFP (Addgene #105545), and AAVrg-DIO-EYFP (Addgene #27056). Viral suspensions were stored at -80°C until use and injected undiluted (2×10^{12} - 3×10^{13} vg/ml).

Animals: Adult (8-20wk) male C57BL/6 mice and transgenic strains on C57BL/6 genetic background were group housed (2-5/cage), given access to food pellets and water *ad libitum*, and maintained on a 12hr light:dark cycle (lights on at 7AM). All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee and conformed to US National Institutes of Health guidelines. We obtained *Slc6a4*-Cre (SERT-Cre) mice from the GENSAT project (MMRRC:017260-UCD), *Oprk1*-Cre (KOR-Cre) mice from Dr. Sarah Ross (University of Pittsburgh) (Cai et al., 2016), *Pdyn*-IRES-Cre (Pdyn-Cre) and *Pdyn*-lox/lox (Pdyn-flx) mice from Dr. Richard Palmiter (University of Washington), *Slc17a8*-Cre (VGluT3-Cre**)** and *Oprk1*-lox/lox (KOR-flx) from Jackson Labs (MGI:5823257, MGI:5316477). KOR^{SERT} conditional knockout (cKO) mice were generated as previously described (Ehrich et al., 2015).

General behavioral methods: Mice were kept in the same housing facility in which behaviors were assayed for at least 1 week prior to experimentation. For all optogenetic experiments, controls were Cre^{+} mice injected with AAV-DIO-EYFP instead of the active opsin. Cage changes were conducted no less than 3 days prior to behavioral testing to minimize confounding effects of environmental stress exposure. Mice were habituated to handling daily for 3 days prior to the initiation of each experiment. All experiments were conducted on mice naïve to prior treatment, except for the optical stimulation during rFSS and social approach assays, which were conducted 2 weeks after the completion of cocaine CPP. EthoVision Software (Version 3.0 & 11.0, Noldus Information Technology) was used to assess movement and generate path heatmap graphics. Experiments were conducted in sound-attenuating behavioral rooms with medium-intensity lighting.

Stereotaxic Surgery: For aseptic surgery, mice were anesthetized in an induction chamber with 4% isoflurane before placement into a stereotaxic frame (David Kopf Instruments Model 1900) where they received 1-2% isoflurane as described previously (Ehrich et al., 2015). Viral injections were performed using Hamilton Neuros syringe (Sigma-Aldrich) at a rate of 100nl/min (500nl for all behavioral studies, 750nl for tracing studies). The syringe was left in place for 5min following the injection. Injection sites were as follows: DRN (AP -4.35, ML 0, DV-2.7; 20° angle) or NAc (AP+1.35, ML +-0.7, DV -4.6) and optic fibers (Doric) were placed 0.5mm above the target site. All NAc viral injections for behavioral studies and optical stimulation were bilateral and unilateral for retrograde tracing. For drug microinfusion, guide cannula (Plastics One #C235G/SPC-1.4mm) were placed above the NAc (AP +1.35, ML +-0.7, DV -4.1), with internal cannula projecting 0.5mm past the guide cannula. Implants were secured using Metabond (Parkell) and dental cement (Stoelting). Following surgeries, mice were given rimadyl for 5 days to reduce inflammation and pain and allowed time for recovery and viral expression (10 days for infusion studies, 4 weeks for somatic optical stimulation, and 5 weeks for terminal optical stimulation and anatomical tracing studies).

Forced Swim Stress: Mice were subjected to a modified Porsolt forced swim stress (rFSS) as described previously (McLaughlin, Marton-Popovici, et al., 2003). All swim sessions were conducted in 31±1ºC water. On day 1, mice received a 15min initial swim, followed 22hr later by four 6min swims, each separated by 6min. After each swim, mice were removed from the water, towel dried, and returned to their home cage.

Optogenetic stimulation during rFSS: Mice were connected to the optical tether 1min prior to swim sessions on day 1 and 2, and they remained tethered throughout the swim session. Mice were visually monitored during swim sessions. Mice that submerged due to impaired swimming were removed from the water and excluded from subsequent analysis (3 EYFP-injected and 2 ChR2-injected mice were excluded). Optical stimulation was delivered 1min prior to the initial swim and 6min prior to the second and fourth swim on the day 2.

Cocaine conditioned place preference: Mice were assayed in a balanced place conditioning apparatus with distinct visual and tactile cues in each chamber as previously described (Ehrich et al., 2015; Schindler et

al., 2012). On day 1, an initial preference test was conducted for place preference bias. Conditioning occurred on days 2 and 3, consisting of cocaine administration and 30min confinement to the drug-paired chamber in the morning (15mg/kg, IP) and saline administration (10ml/kg, IP) and confinement to the other chamber 4hr later. On day 4, mice were allowed to freely explore the apparatus for a postconditioning assessment in the absence of drug. Preference tests and conditioning sessions lasted for 30min and were conducted in sound attenuating chambers.

Manipulations prior to cocaine conditioning: Repeated forced swim stress prior to cocaine conditioning: mice were subjected to rFSS (as described above) 30min after the initial preference test on day 1 and before cocaine conditioning on day 2, terminating 10min prior to cocaine administration.

Optogenetic inhibition of DRN subtypes prior to cocaine conditioning: VGluT3-Cre and SERT-Cre mice expressing EYFP or SwiChR were tethered to fiber optic cables coupled to a 473-nm laser in an empty cage bottom and received optical stimulation (0.33 Hz, 15ms pulse duration) for 30min on each conditioning day. Following optical stimulation, mice were returned to their home-cage 30min prior to each cocaine conditioning session.

Optogenetic excitation of $SERT^{DRN\text{-}NAc}$ terminals during U50488 pretreatment: mice received the selective KOR agonist U50488 (5mg/kg, IP) 1hr prior to cocaine conditioning and were immediately tethered to optical fibers providing 473-nm stimulation (15Hz, 10ms pulse duration) in an empty cage bottom. Mice were untethered 5min before each cocaine conditioning session.

Local infusion of 5-HT receptor antagonists prior to cocaine conditioning: Wild-type (WT) mice with guide cannula placed in the NAc received infusions of the $5\text{-}HT_{1B}$ antagonist GR 127935 or $5\text{-}HT_4$ antagonist GR 125487 (1µg/0.2µl in ACSF, 0.1µl/min) 135min and/or 75min prior to each cocaine conditioning session. Following infusions, mice were returned to their home cage.

Conditioned place aversion: Cannulated SERT-Cre mice expressing EYFP or SwiChR were assayed in a balanced place conditioning apparatus with distinct visual and tactile cues as previously described (Ehrich et al., 2015). An initial preference test was performed on day 1 to assess baseline preference. On days 2 & 3 optogenetic conditioning was performed, comprising a tethering session with confinement to the less-preferred chamber in the morning and optical stimulation (0.33 Hz, 15ms pulse duration) with confinement to the more-preferred chamber 4hr later. On the 4th day, mice were allowed to freely explore the apparatus for a final preference test in the absence optical stimulation.

Social approach: Social interaction was assessed as described previously using a three chambered apparatus with two clear internal partitions (Steger et al., 2020). The day prior to the experiment, age-matched target mice were habituated to confinement in an inverted pencil cup (Spectrum Diversified Designs) for 1hr. WT mice with cannula placed in the NAc received infusions of 5-HT$_{1B}$ antagonist GR 127935 (1µg/0.2µl in ACSF; 0.1 µl/min), and 125min later were allowed to freely explore the social interaction apparatus for a 10min habituation period. The mouse was then briefly removed to a holding cage, and two inverted pencil cups were placed in the far corners of the apparatus, with one cup containing a target mouse. The experimental mouse was then reintroduced and allowed to explore for an additional 10min. Time spent in an interaction zone adjacent to each cup was recorded.

Local infusion of 5-HT$_{1B}$ ligands prior to histology: WT mice with guide cannula placed in the NAc received norBNI (10mg/kg, IP) 24hr prior to the experiment to minimize the effects of infusion-induced stress on pERK-IR (Bruchas et al., 2008). Mice received control infusions (ACSF, 0.2µl) in the right hemisphere and drug infusions (GR 127935 or 5 CP94253; 1µg/0.2µl, 0.1µl/min) in the left hemisphere. Drug infusions consisted of CP 94253 alone or following infusions of GR127935 135min or 75min prior. 15min after infusion of CP 94253, mice were deeply anesthetized, transcardially perfused, and brains were prepared for histology as described below.

Immunohistochemistry: Mice were transcardially perfused with 4% paraformaldehyde in 0.1M phospho-buffered saline (PBS) as reported previously (Lemos et al., 2012). Brains were then dissected,

cryoprotected with 30% sucrose at 4°C overnight, frozen, cut into 40µm sections (Leica microtome, SM200R), and stored in 0.1M PBS w 0.1% sodium azide at 4°C until further processing. Standard immunohistochemical procedures were used to stain NAc sections as described previously (Bruchas et al., 2008). Briefly: floating sections were washed 3x5min in PBS, then blocked for 1hr in 5% normal goat serum (Vector Labs), 0.3% Triton-X in PBS before 24hr at room temperature incubation with primary antibodies: 1:400 rabbit anti-pERK antibody (CS4370, Cell Signaling) for phospho-ERK detection or 1:1000 Chicken anti-GFP (AB12970, Abcam) to enhance detection of anterograde and retrograde tracing. Sections were washed again 4x5min in PBS before incubation with the 1:500 goat anti-rabbit 488 or goat anti-chicken 488 (Life Technologies) for 2hr at room temperature. Lastly, sections were washed 4x5 in PBS, then once with 0.5X PBS before mounting on Fisher Superfrost slides (Sigma-Aldrich) and coverslipped using Vectashield (Vector Laboratories).

Fluorescent in situ hybridization (ISH) using RNAscope: Brains were rapidly dissected and flash frozen on dry ice. For stress experiments, brain dissection was performed either 30min or 24hr following the last swim session of the rFSS protocol or unhandled (no rFSS) controls. Thin (14µm) coronal sections containing the NAc or DRN were collected and mounted onto Superfrost plus slides using a cryostat (Leica CM 1850) maintained at -20ºC. RNAscope ISH was performed according to the Advanced Cell Diagnostics as previously reported (Lesiak et al., 2020). Each set of staining included a negative control, in which probes were omitted from the process. Probes were discriminated using tyramide signal amplification (TSA) fluorophores (NEL744001, NEL745001, NEL741001; Akoya Biosciences).

Characterization of DRN subpopulations: Probes for *Oprk1 (mm-Oprk1), Slc6a4 (mm-Slc6a4), and Slc17a8 (mm-Slc17a8)* were used to label tissue from the central DRN (AP +4.3-4.5) of unstressed mice. *NAc Htr1b distribution:* sections containing the central NAc (AP 1.1-1.3) were obtained from stressed and unstressed mice, and separate sets of tissue were stained with probes to *Htr1b(mm-Htr1b)*/*Chat (mm-Chat)* and *Htr1b/Pdyn (mm-Pdyn)/Adora2a (mm-Adora2a).*

Microscopy and Image Quantification: All images used for quantitation were taken using a confocal microscope (SP8X, Leica Microsystems), except for initial determination of expression in retrograde tracing studies and confirmation of viral expression in behavioral studies, in which a scanning widefield microscope was used (DMI6000, Leica Microsystems).

Anterograde tracing: Two brain sections containing the NAc (AP +1.3 and AP +1.0) were imaged at 10x magnification for each $SERT^{DRN}$ ChR2 and $VGluT3^{DRN}$ ChR2 mouse. Boundaries of the mNAc were determined using the Paxinos atlas (Franklin, 2008). Terminal density was determined using Image J software (NIH) by binarizing the images and calculating the density of positive pixels within the mNAc as described previously (Pinto et al., 2019).

Retrograde tracing: For KOR retrograde tracing from the mNAc, every sixth section within the DRN (AP -4.0 to AP -5.0) was mounted and imaged at 10x magnification following anti-GFP staining to enhance detection of labeled cell bodies. For Pdyn retrograde tracing from the mNAc, every 12^{th} section throughout the collected tissue (AP +2.5 to AP -5.5) was mounted to survey across all brain regions and imaged at 10x on a scanning widefield microscope. These were visually inspected for signal by an observer naïve to treatment. Subsequently, confocal images were taken of the medial NAc at 10x magnification.

Effects of 5-HT_{1B} drug infusion on pERK in the NAc: The left and right medial NAc of two sections (AP +1.3 and AP +1.0) were imaged for each animal. Z-stacks (5µm thick, 7 steps) were taken at 60x magnification and an average projection was generated using LASX software (Leica Microsystems). A detection threshold for each section was set according to the brightest 5% of pixels in the ACSF image and positive cells (more than half of cell above threshold) for each image were quantified manually by an observer blind to treatment using ImageJ software (NIH). Average counts of two sections for the ACSF and drug treated hemispheres were taken for each animal. Percent increase of NAc pERK-IR^+ cells in the drug-treated hemisphere was calculated as $(100*(pERK_{Drug}-pERK_{ACSF}))/ pERK_{ACSF}$.

Fluorescent ISH: DRN subpopulations: The entire DRN was imaged at 20x magnification from one section (AP 4.4) of each subject. Images were taken during the same imaging session, and capture settings were adjusted such that no signal was observed in negative controls and kept constant for all subsequent images. *NAc Htr1b distribution:* a region immediately medial and ventral to the anterior commissure (ac) was imaged at 20x magnification, with capture settings adjusted to ensure no signal in the negative controls. Two bilateral images from two sections were taken. Images were processed using custom MATLAB scripts for positive cells, co-expressing cells, and levels of RNA detected per cell; these values were averaged for each subject animal and then group averages were calculated. For all analyses, total cells were determined by the number of DAPI-stained nuclei.

Data Analysis: Sample sizes were based on prior studies but were not predetermined by statistical methods. All aspects of histology and histological analysis were performed by an experimenter blind to genotype and treatment. Prior to further analysis, outliers in data sets were excluded using Grubb's Test for statistical outliers. The assumption of normal distribution was tested for each data set and was statistically corrected for when this criterion was not met. *Post-hoc* tests used were Sidak's or Dunnett's where appropriate, withα=0.05.

Results

Kappa opioid receptor expression in SERT neurons is required for stress potentiation of cocaine reward

Mice expressing Cre-recombinase under the SERT promoter (*Slc6a4-Cre* or 'SERT-Cre') were crossed with mice in which *Oprk1* was flanked by lox p excision sites (*Oprk1-lox/lox* or 'KOR-flx'), resulting in a conditional knockout of KOR in SERT^{+} cells ('KOR^{SERT}cKO') (Figure 2.1.1A). These mice and their littermate controls (SERT KOR$^{\Delta/+}$ and KOR$^{+/+}$, or 'Ctl') were subjected to repeated forced swim stress (rFSS) prior to cocaine place preference conditioning (CPP) (Figure 2.1.1B). Unstressed controls were handled briefly but were otherwise undisturbed prior to cocaine conditioning.

Consistent with previous reports (McLaughlin, Marton-Popovici, et al., 2003), unstressed control mice developed significant cocaine place preference, and rFSS induced robust potentiation of CPP (Figure

2.1.1C). Cocaine place preference of the unstressed KOR^{SERT}cKO mice was not significantly different from that of littermate controls (two-way ANOVA; $F_{1, 34}$= 0.27, P= 0.604), indicating that excision of KOR in SERT-expressing neurons does not regulate basal cocaine preference (Figure 2.1.1C). There was a significant main effect of stress ($F_{1, 34}$ = 8.87, P=0.005) and a marginal interaction (gene X stress interaction, $F_{1, 34}$ = 3.45, P= 0.072). Comparison within each genotype revealed a significant effect of stress in controls (Sidak *post-hoc*, P= 0.004) that was absent in the KOR^{SERT} cKO group (P= 0.664) (Figure 2.1.1C). To isolate the effect of stress on cocaine preference in these groups, we normalized within each genotype to the average 'No rFSS' preference. Stress increased the preference in controls by more than twofold, significantly more than in KOR^{SERT} cKO group (unpaired, two-tailed t test, welch-corrected, P= 0.036) (Figure 2.1.1D). These data demonstrate that global deletion of KOR in SERT-expressing neurons blocks stress-induced potentiation of cocaine CPP.

Inhibition of SERT neurons in the DRN is aversive

KOR activation by stress-induced release of endogenous dynorphin hyperpolarizes serotonergic neurons in the DRN (Lemos et al., 2012), but stress exposure broadly affects brain physiology. To assess the effects of selective inhibition of DRN neurons, we tested the effects of optogenetically inhibiting $SERT^{DRN}$ neurons using a Cre-dependent inhibitory opsin (AAV5-DIO-SwiChR-EYFP) injected in the DRN of SERT-Cre mice (Figure 2.1E). SwiChR is a channelrhodopsin variant that conducts chloride and has been utilized to generate long-term, reversible inhibition, while avoiding photic damage to tissue (Berndt et al., 2014; C.-C. Chen et al., 2018). We conducted a conditioned place preference assay by inhibiting $SERT^{DRN}$ neurons (15ms pulse duration, 0.33 Hz) during confinement to an optically-paired chamber of a CPP apparatus for 30min on two consecutive days, following and preceding preference tests (Figure 2.1F). Compared to controls (AAV5-DIO-EYFP injected in SERT-Cre^{DRN}), $SERT^{DRN}$ SwiChR-injected mice developed a robust and significant aversion to the optically-paired chamber (SwiChR preference – EYFP preference= -468 ± 93.5 sec; unpaired, two-tailed t test. P< 0.001) (Figure 2.1G). These results support the conclusion that inhibition in $SERT^{DRN}$ neurons either by KOR activation or optogenetic inhibition produces place aversion.

KOR is expressed prominently in SERT and VGluT3 subpopulations of the DRN

DRN serotonin neurons are anatomically and phenotypically heterogenous and have been suggested to form functional subsystems regulating diverse stress-sensitive processes (Okaty et al., 2020; Ren et al., 2018, 2019; Teissier et al., 2015; Valentino et al., 2010), but the distribution of KOR within DRN serotonin neurons has not been evaluated. To characterize KOR expression in the DRN, RNAscope *in situ* hybridization was used to probe for co-expression of transcripts for KOR (*Oprk1)*, SERT (*Slc6a4*), and the vesicular glutamate transporter type III (VGluT3; *Slc17a8*) (Figure 2.1H). $SERT^{DRN}$ and $VGluT3^{DRN}$ neurons are both largely serotonergic neurons that overlap extensively yet may have distinct roles in driving reward-related behaviors (Liu et al., 2014; McDevitt et al., 2014; Wang et al., 2019). We found that KOR transcript was expressed in the majority of DRN cells (84±8.0%) (Figure 2.1I). *Slc6a4* and *Slc17a8* were present in a roughly equal percentage of DRN neurons (35±9.9% and 31±7.5%, respectively). A majority (63.9±2.0%) of *Slc6a4* neurons expressed *Slc17a8,* indicating substantial overlap of $SERT^{DRN}$ and $VGluT3^{DRN}$ populations (Figure 2.1J). Lastly, *Oprk1* was present in nearly all cells that expressed *Slc17a8* or *Slc6a4* (96.6±3.0% and 99.5±0.5%, respectively) (Figure 2.1J). The expression of KOR transcript in the majority of $SERT^{DRN}$ and $VGluT3^{DRN}$ cells indicates a potential for direct regulation of these subsystems by KOR.

To examine how these subpopulations may intersect differentially across subregions in the DRN, the percentage of *Slc6a4*$^+$ neurons in each subregion that co-expressed *Oprk1* (*Oprk1*$^+$/*Slc6a4*$^+$*)* was determined. Across the regions examined, all showed nearly universal (97-100%) expression of *Oprk1* in *Slc6a4*$^+$ cells (Figure 2.1K). In contrast, the percentage of *Slc6a4*$^+$ neurons that co-expressed both *Oprk1* and *Slc7a18 (Oprk1*$^+$/*Slc17a8*$^+$/*Slc6a4*$^+$*)* was significantly higher in the dorsal and ventral DRN than in the lateral DRN (DRL), where *Slc17a8* was nearly absent (two-way ANOVA, region X colocalization interaction $F_{2,8}$= 61.1, P< 0.001). These data indicate that unlike the majority of $SERT^{DRN}$ neurons, which also express the transcripts for KOR and VGluT3, the SERT$^+$ neurons in the lateral DRN are almost exclusively *Oprk1*$^+$*/Slc17a8*$^-$*.*

<u>$SERT^{DRN}$ neurons innervate the medial NAc but $VGluT3^{DRN}$ neurons do not</u>

To assess the projections of these $SERT^{DRN}$ and $VGluT3^{DRN}$ populations to the NAc, an anterogradely trafficked virus with a Cre-dependent fluorophore (AAV5-DIO-ChR2-EYFP) was injected into the DRN of

SERT-Cre and VGluT3 Cre mice (Figure 2.2A). Examination of labeled somata in the DRN showed that both viral constructs were expressed in the DRN (Figure 2.2B). Labeled terminals in the medial NAc (mNAc) revealed that $SERT^{DRN}$ neurons more densely project to mNAc than do the $VGluT3^{DRN}$ neurons (Figure 2.2B, Figure 2.2C) (unpaired, two-tailed t test, welch corrected, P= 0.044). These data are consistent with previous reports of different projection biases of these populations (McDevitt et al., 2014; Ren et al., 2018).

Inhibition of $SERT^{DRN}$ neurons recapitulates KOR-mediated potentiation of cocaine CPP, but $VGluT3^{DRN}$ inhibition does not

Although prior work has demonstrated an association between activation of KOR, increased serotonin reuptake, and potentiation of cocaine preference, a causal link between a prior decrease in serotonin tone and a subsequent increase in cocaine preference has not been established. To test this, we mimicked previous studies of KOR-agonist induced potentiation of cocaine preference but substituted KOR-agonist administration with optogenetic inhibition of DRN subpopulations (Figure 2.2D). SERT-Cre mice received DRN viral injections of the Cre-dependent SwiChR (AAV5-DIO-SwiChR-EYFP) or control virus (AAV5-DIO-EYFP), and an optical fiber was placed above the injection site (Figure 2.2E). Examination of the DRN confirmed robust expression of the opsin (Figure 2.2E). During conditioning days of the cocaine CPP assay, mice received 30min of $SERT^{DRN}$ inhibition (15ms pulse duration, 0.33 Hz) that terminated 30min prior to cocaine conditioning. Comparing cocaine preference scores of mice that received prior inhibition of $SERT^{DRN}$ neurons to EYFP controls revealed a significant potentiation of subsequent cocaine CPP (unpaired, two-tailed t test, welch-corrected, P= 0.037) (Figure 2.2F). These findings indicate that prior inhibition of $SERT^{+}$ neurons in the DRN is sufficient to potentiate cocaine CPP thereafter.

Inhibition of $VGluT3^{DRN}$ neurons attenuates subsequent cocaine reward

In parallel studies, VGluT3-Cre mice were injected with virus containing Cre-dependent SwiChR (AAV5-DIO-SwiChR-EYFP) or an EYFP control (AAV5-DIO-EYFP) in the DRN. Examination of the DRN showed robust expression of the opsin (Figure 2.2G). We performed cocaine conditioning as previously described, with inhibition of $VGluT3^{DRN}$ neurons prior to each cocaine conditioning session. Surprisingly, while analysis of cocaine preference in control animals showed a typical cocaine CPP, the group receiving inhibition of

VGluT3^{DRN} neurons displayed a significant attenuation of cocaine preference (unpaired, two-tailed t test, welch-corrected, P = 0.050) (Figure 2.2H). Expressing cocaine preference scores following SERT^{DRN} or VGluT3^{DRN} inhibition as a fraction of the control preference within each genotype more clearly illustrates the divergent effects of prior inhibition of these populations on subsequent cocaine preference (unpaired, two-tailed t test, welch-corrected, P <0.001) (Figure 2.2I). Thus, inhibition of different populations of DRN neurons exerted bidirectional control of subsequent cocaine preference, and inhibition of SERT^{DRN} neurons (but not VGluT3^{DRN} neurons) was sufficient to replicate the consequences of stress on subsequent cocaine CPP.

DRN-projecting KOR neurons are restricted to the lateral aspect of the DRN

To confirm that KOR is expressed within the DRN-NAc projection, and thus is capable of directly regulating the DRN-NAc projection, a retrograde tracing approach was employed. A retrograde virus containing a Cre-dependent fluorophore (AAVretro-DIO-EYFP) was injected into the NAc of KOR-Cre mice (Figure 2.3A) (Tervo et al., 2016). We examined the DRN for labeled neurons and observed a population of KOR expressing, NAc projecting neurons that was mostly concentrated in the lateral aspect of DRN and almost completely absent in other DRN subregions (Figure 2.3B). This indicates that DRN KOR neurons that project to the NAc define an anatomically segregated subpopulation of DRN neurons. To validate these findings, an anterograde virus with a Cre-dependent fluorophore (AAV5-DIO-ChR2-EYFP) was injected into the DRN of KOR-Cre mice (Figure 2.3C). Examination of the NAc showed robust expression of labeled terminals in the mNAc (Figure 2.3D). Together, these findings indicate that KOR is expressed in a subpopulation of DRN neurons that project to the mNAc and suggest the potential for direct regulation of DRN to NAc projections by Dyn/KOR.

Increased serotonin tone in the NAc blocks KOR-mediated potentiation of cocaine reward

To directly probe the hypothesis that a KOR-mediated decrease in serotonin tone within the mNAc is necessary for potentiation of subsequent cocaine preference, we optogenetically manipulated SERT$^{DRN\text{-}NAc}$ terminals during KOR activation. SERT-Cre mice were injected with a Cre-dependent channelrhodopsin virus (AAV5-DIO-ChR2-EYFP) in the DRN and a bilateral optical fiber was placed above the mNAc (Figure

2.3E). Examination of the NAc confirmed fiber placement proximal to channelrhodopsin-expressing terminals (Figure 2.3E). One hour prior to each cocaine conditioning session, these mice and their EYFP-injected controls ($SERT^{DRN\text{-}NAc}$ EYFP) received pretreatment that included the selective KOR agonist U50488 (5mg/kg) as well as optical stimulation of SERT terminals (to counteract KOR-induced decreases in serotonin tone) that terminated 5min prior to cocaine conditioning (Figure 2.3F). Cocaine preference scores were calculated and compared to unstressed baseline cocaine preference scores (from Figure 2.2F). In EYFP-injected control animals, U50488 pretreatment resulted in a potentiated cocaine preference that was attenuated by concurrent stimulation of serotonergic terminals in the mNAc of the ChR2 group (unpaired, two-tailed t test, P = 0.029) (Figure 2.3G). These results suggest that altered serotonergic terminal activity in the mNAc is required for KOR-mediated potentiation of cocaine CPP.

Increased serotonin tone in the NAc prevents stress-induced immobility

To assess if activation of serotonergic terminals in the NAc mitigates the negative affective consequences of stress, $SERT^{+}$ terminals were optically stimulated during a 2-day rFSS assay. Optical stimulation was provided throughout the day 1 swim and during the second and fourth swims of day 2 (Figure 2.3H). Movements of $SERT^{DRN\text{-}NAc}$ ChR2 and control ($SERT^{DRN\text{-}NAc}$ EYFP) mice were analyzed, showing an escalation of time spent immobile in the control group (two-way ANOVA with Dunnett's *post-hoc*, P=0.038), but not in the group receiving terminal stimulation (Dunnett's *post-hoc*, P=0.997) (Figure 2.3I). Comparing immobility during the final swim shows that the ChR2-stimulated group spent significantly less time immobile (unpaired, two-tailed t test, P = 0.021). To calculate the escalation of immobility in mice of each group, time immobile during first swim was subtracted from the last swim of day 2. The results indicate that stimulation of serotonergic terminals in the NAc prevents escalation of immobility within subjects (unpaired, two-tailed t test, P =0.014). These data are consistent with the hypothesis that stress-induced decreases in serotonin tone within the NAc promote passive coping (Shirayama & Chaki, 2006).

The principal source of dynorphin in the NAc is from $Pdyn^{NAc}$ neurons

The role of endogenous dynorphin acting on KOR expressed in DRN neurons projecting to the NAc has been demonstrated by the effects of global prodynorphin gene deletion and local inactivation of KOR by

the antagonist norBNI (Land et al., 2009; McLaughlin, Marton-Popovici, et al., 2003), but the neuronal source of dynorphin responsible for stress-induced potentiation of cocaine CPP has not been determined. To identify candidate sources of dynorphin to the NAc, two different retrograde viral constructs (AAVretro-DIO-EYFP or CAV2-DIO-Zsgreen) were utilized. Retro-virus was stereotaxically injected into the NAc of Pdyn-Cre mice and sections throughout the brain were surveyed for viral expression (Figure 2.4A). For both retrogradely transported viruses, examining regions for labeled neurons revealed signal only in the mNAc (Figure 2.4B). For each of these retrograde viruses, distinct tropisms have been documented that may cause either virus to undercount input populations (Tervo et al., 2016). However, because both viruses show consistent signal exclusively in the mNAc, we conclude that local neurons within the mNAc likely represent the principal source of endogenous dynorphin for this region.

Dynorphin in the NAc is required for stress potentiation of cocaine preference and regulates basal cocaine preference

To assess the necessity of $Pdyn^{NAc}$ neurons in stress potentiation of cocaine CPP, virus containing either Cre (AAV5-Cre-EGFP) or an EYFP control (AAV5-EGFP) were injected into the NAc of Pdyn-lox/lox ('Pdyn-flx') mice to generate '$Pdyn^{NAc}$ cKO' or 'Ctl' mice. Examining the NAc of these mice demonstrated the successful delivery of the virus and confirmed that expression was confined to the NAc (Figure 2.4C). These mice were subjected to rFSS to determine the role of Pdyn neurons in the mNAc in stress potentiation of cocaine CPP (Figure 2.4D). Cocaine preference scores indicated a main effect of $Pdyn^{NAc}$ excision (two-way ANOVA, $F_{1, 54}$= 4.73, P= 0.034), indicating that dynorphin within the NAc regulates basal cocaine preference. There was also a significant main effect of stress ($F_{1, 54}$= 13.1, P= 0.001) but no significant interaction ($F_{1, 54}$ = 2.44, P= 0.12). There was a significant potentiation of cocaine preference in Cre^{-} controls (Sidak *post-hoc*, P= 0.015) but not in the $Pdyn^{NAc}$cKOs (P=0.90). We have routinely observed cocaine preference scores higher than the ~500 s preference in the unstressed $Pdyn^{NAc}$cKO group, indicating that the lack of stress potentiation is likely not due to a ceiling effect on expressed preference. To isolate the effect of stress on potentiation of cocaine preference, we normalized the rFSS cocaine preference scores in $Pdyn^{NAc}$cKO and control groups to the average preference of their unstressed counterparts (Figure 2.4E). This revealed a stress potentiation of cocaine preference of nearly two-fold in controls, whereas

$Pdyn^{NAc}$cKOs did not show significant stress-potentiation of cocaine CPP (two-tailed t test, welch-corrected, P=0.005). This finding supports a central role of the $Pdyn^{NAc}$ population in regulation of both basal cocaine preference and stress potentiation of that preference.

Pharmacologic blockade of NAc 5-HT_{1B} receptors substitutes for reduced 5-HT in NAc

Together, these results suggest that a reduction in 5-HT tone in NAc is responsible for stress-induced potentiation of cocaine CPP, but the specific type of 5-HT receptors that mediate the consequences of decreased serotonin tone are not known. Of the subset of 5-HT receptors expressed in the NAc and known to regulate cocaine preference, the 5-HT_{1B} receptor was an especially plausible candidate (Ferguson et al., 2009; Neumaier et al., 2002). A possible decrease in serotonin tone at the 5-HT_{1B} receptor caused by stress was pharmacologically mimicked by infusion of a 5-HT_{1B} antagonist into the mNAc. Two time points were selected to evaluate the duration of antagonist-mediated blockade of the receptor. Injection cannula were placed in the mNAc of WT mice and 5-HT_{1B} antagonist GR 127935 was infused unilaterally (Figure 2.5A). This was followed either 75min or 135min later by unilateral infusion of 5-HT_{1B} agonist CP 93129 and perfusion of the brain 15min later. ACSF was infused in the opposite hemisphere during each drug infusion to control for nonspecific effects of the infusion procedure on ERK phosphorylation. Coronal sections containing the mNAc were probed for pERK-immunoreactivity (IR), a consequence of 5-HT_{1B} activation (Figure 2.5B) (Y. Liu et al., 2019). Comparison of treatments showed a significant main effect of agonist treatment on agonist-stimulated pERK-IR and a significant interaction (two-way ANOVA, agonist main effect, $F_{1,25}$ = 7.12, P= 0.013; pretreatment X agonist interaction, $F_{2,25}$= 8.04, P= 0.002). *Post-hoc* comparisons indicated that treatment with CP 93129 induced a significant increase in pERK-IR cells as compared to the ACSF control hemisphere in the absence of antagonist pretreatment (Sidak *post-hoc, P*= 0.002). This result confirmed that local infusion of the 5-HT_{1B} agonist induces pERK-IR within mNAc cell bodies. Pretreatment with the antagonist GR 127935 75 min prior to CP 93129 infusion blocked the agonist-induced increase in pERK-IR (Sidak *post-hoc*, P= 0.293), whereas pretreatment 135min prior did not *(*Sidak *post-hoc*, P= 0.033) (Figure 2.5C). To further interrogate antagonist duration, the number of pERK+ cells were normalized to the ACSF hemisphere for each subject, and this value was expressed as a fraction of the effect from agonist treatment alone (Figure 2.5D). This direct comparison of antagonist pretreatment

timing illustrates the transient block of pERK-IR caused by GR 127935 infused 75min before CP 93129, but not 135min before agonist (unpaired, two-tailed t test, welch-corrected, P= 0.014).

Blockade of 5-HT_{1B} receptors in the mNAc recapitulates KOR-mediated potentiation of cocaine reward

To mimic the transient decrease in serotonin at 5-HT_{1B} receptors caused by stress-induced dynorphin release, GR 12735 was locally infused into the NAc of WT mice prior to cocaine conditioning (Figure 2.5E). Cannula placements were confirmed post-mortem (Figure 2.5E). The 5-HT_{1B} antagonist GR 127935 (1µg/0.2µl) was infused at 135min or 75min prior to each cocaine conditioning session (Figure 2.5F). The resultant raw preference scores revealed a significant effect of pretreatment (one-way ANOVA, welch-corrected, $F_{4, 10.5}$= 7.78, P= 0.004). A *post-hoc* test indicated that only infusion of GR 127935 135min prior to cocaine conditioning was sufficient to potentiate cocaine preference as compared to ACSF controls (Dunnett's T3 *post-hoc,* P=0.001) (Figure 2.5G). As a selectivity control, infusion of a 5-HT_4 antagonist 135min prior to cocaine conditioning was tested separately and failed to potentiate cocaine preference, suggesting this potentiation is not a general consequence of inhibiting serotonin receptors. To further understand the effects of transient 5-HT_{1B} antagonism prior to cocaine administration, we expressed cocaine preference scores as a fraction of ACSF pretreatment preference. These data show a main effect of pretreatment (one-way ANOVA, welch-corrected, $F_{2, 7.5}$= 11.22, P= 0.005) with a significant difference between pretreatment 135min and 75min prior to cocaine (Dunnett's T3 *post-hoc*, P= 0.008) (Figure 2.5H). GR 127935 failed to potentiate cocaine preference in the group that received a second infusion of antagonist 75min prior to cocaine conditioning, suggesting that this potentiation of cocaine CPP is sensitive to 5-HT_{1B} receptor blockade during cocaine administration. These results suggest that a prior decrease in activation of NAc 5-HT_{1B} receptors is sufficient to potentiate subsequent cocaine preference and mimic the effects of rFSS.

Prior blockade of 5-HT_{1B} receptors in the mNAc potentiates social reward

To assess whether this reward potentiation is specific to cocaine or reflective of a broader change in reward processing, mice received infusions of GR 127935 (1µg/0.2µl) or ACSF into the mNAc and 135min later were assayed for social preference, a behavior known to be regulated NAc 5-HT_{1B} receptors (Walsh et al.,

2018). In this assay, an empty, inverted pencil cup was placed in one corner and a pencil cup containing a novel mouse was placed in the opposite corner (Figure 2.5I). Mice were allowed to freely explore the apparatus for 10 min and the time spent in an interaction zone surrounding each cup was determined (Figure 2.5J). Expressing preference for social interaction as time spent in the social zone/time spent in the empty cup zone indicated a significantly greater social preference in mice pretreated with GR 127935 (unpaired, two-tailed t test, P=0.028) (Figure 2.5K). These findings indicate that a prior blockade of 5-HT_{1B} receptors, utilized here to reflect the consequences of decreased serotonin tone in the mNAc, result not only in a potentiation of cocaine reward but social reward as well.

5-HT_{1B} gene transcript is prevalent in the mNAc and equally distributed across NAc subpopulations

5-HT_{1B} receptors are known canonically as autoreceptors that presynaptically inhibit 5-HT release at serotonergic terminals (Stamford et al., 2000). They are also present as heteroreceptors in postsynaptic neurons; however, the distribution of 5-HT_{1B} heteroreceptors across the principal cell types of the nucleus accumbens has not been examined. We employed RNAscope *in situ* hybridization to determine which neurons within the mNAc co-express *Htr1b,* the gene for 5-HT_{1B}. Brain sections from unstressed mice were probed for *Htr1b*, along with markers of the direct pathway (*Pdyn)*, indirect pathway (*Adora2a)*, and cholinergic interneurons (*Chat).* Staining and imaging were performed first to assess the colocalization of *Htr1b* with *Pdyn* and *Adora2a* (Figure 2.5A). Results indicate that nearly half (43±2.7%) of the neurons within the mNAc express *Htr1b* (Figure 2.5B). Staining for other markers show that *Pdyn* and *Adora2a* were detected in a sizeable fraction of neurons (33±2.5% and 45±2.5%, respectively). A second experiment was performed to establish *Htr1b* colocalization with *Chat* (Figure 2.5C). *Chat* was expressed sparsely, in line with prior findings (2.5±0.5%) (Figure 2.5D)(Virk et al., 2016). Co-expression of *Htr1b* with *Pdyn, Adora2a,* and *Chat* markers was nearly uniform and detected in a slight majority of each of these cells (67%, 62%, and 70%, respectively) (Figure 2.5E). This prevalence across the subtypes of the NAc supports a potential role of 5-HT_{1B} receptor-mediated regulation of these NAc subpopulations by serotonin. Additionally, equal distribution of the transcript across subtypes indicates that instead of predominant effects on one subpopulation, 5-HT_{1B} regulation of the NAc may occur by effects on multiple NAc cell types.

rFSS increases 5-HT_{1B} transcript expression in $Pdyn^{NAc}$ neurons

Chronic stress or chronic exposure to psychostimulants increases 5-HT_{1B} transcript in the NAc, but regulation by sub-chronic stress exposure has not been detected (Furay et al., 2011; Hoplight et al., 2007; Neumaier et al., 2009). This may be attributed to relatively low sensitivity of prior techniques to changes in transcript levels and difficulty assessing cell-type specific changes. RNAscope was used to evaluate the effect of rFSS on *Htr1b*, *Pdyn*, and *Adora2a* in the NAc. Brains were extracted from mice 30min and 24hr after rFSS and compared to brains from unstressed mice (Figure 2.6F). Both the overall levels of these transcripts and the levels of *Htr1b* with $Pdyn^+$ and $Adora2a^+$ cells in the NAc were examined (Figure 2.6G). No significant changes were observed in the overall expression levels of these transcripts 30min or 24hr after stress (two-way ANOVA, P> 0.05) (Figure 2.6H). In contrast, examining the levels of *Htr1b* in *Pdyn* and *Adora2a* subpopulations revealed a significant increase in *Htr1b* expression in $Pdyn^+$ cells 30min after stress (two-way ANOVA, stress timing X transcript level interaction, $F_{2,36}$= 3.53, P= 0.040; Dunnett's *Post-hoc,* P= 0.042) (Figure 2.6I). *Htr1b* expression in $Adora2a^+$ cells was not affected by stress, and levels in both subpopulations were not significantly different than the no stress condition 24hr after stressor completion (Dunnett's *Post-hoc*, P>0.05). Changes in *Htr1b* expression in ChAT cells were not assessed because of their low abundance. These results indicate that the sub-chronic stressor (rFSS) able to potentiate cocaine CPP induces a selective and transient increase in 5-HT_{1B} transcript in $Pdyn^+$ cells of the mNAc.

Discussion

The principal findings of the present study provide insight into the mechanisms by which stress impinges on the serotonin system to sensitize animals to subsequent reward. KOR expression within serotonergic neurons and dynorphin expression within the mNAc were critical to stress potentiation of cocaine reward (Figure 2.6J). Temporally precise manipulations of DRN circuitry showed that acute inhibition of DRN serotonin neurons drives negative affect and can potentiate subsequent cocaine preference. Additional experiments indicated that KOR-induced decreases in serotonin within the NAc control both behavioral coping and increased cocaine preference. We isolated the effect of decreased serotonin tone at the 5-HT_{1B} receptor by transient blockade of 5-HT_{1B} receptors, which was sufficient to recapitulate the potentiation of

cocaine preference observed following KOR activation (Figure 2.6J). Lastly, we show that prior to stress, 5-HT_{1B} transcript is evenly distributed across cell types of the NAc, but stress selectively increases expression of 5-HT_{1B} transcript in *Pdyn*-expressing neurons (Figure 2.6J). Together, this evidence details a potential Dyn-KOR-5-HT-$5HT_{1B}$ axis contained within the NAc, in which stress provokes a transient decrease of serotonin tone in the NAc that is central to passive coping, aversion, and increased cocaine preference.

Stress and KOR activation potentiate cocaine preference through interactions of KOR and serotonin terminals in the ventral striatum, but the circuitry involved has not been fully characterized (Bruchas et al., 2011; Land et al., 2009; McLaughlin et al., 2006; McLaughlin, Marton-Popovici, et al., 2003). We indicate that the KOR-expressing neurons in the lateral aspect of the DRN that project to the mNAc and Pdyn-expressing neurons located within the mNAc regulate stress potentiation of cocaine preference. Interestingly, only selective excision of the Pdyn in the NAc increased basal preference for cocaine. This may be due to effects of $Pdyn^{NAc}$ on other KOR-expressing populations in the NAc, or potential effects of $Pdyn^{NAc}$ subpopulations that drive opposing affective states (Al-Hasani et al., 2015; Chartoff et al., 2016; Ehrich et al., 2014; Mu et al., 2011; Yang et al., 2018). Retrograde tracing of dynorphin inputs to the medial NAc revealed only inputs from the mNAc itself, implying that $Pdyn^{NAc}$ neurons may provide the sole source of dynorphin to this region. This indicates that in addition to a critical role in stress-potentiation of cocaine preference, this population may also regulate other behaviors that are dependent on KOR activation in the NAc, including escalation of drug taking, learned helplessness, and pain-induced negative affect and anhedonia (Liu et al., 2014; Massaly et al., 2019; Nealey et al., 2011; Newton et al., 2002; Shirayama et al., 2004; Whitfield et al., 2015).

We observed robust aversion to optogenetic inhibition of $SERT^{DRN}$ in the present study. Although place preference is not a direct measure of affect, this finding supports theories of serotonin function that assert a central role of decreased serotonin tone in negative affect (Cohen et al., 2015; Luo et al., 2015). Together with data indicating the necessity of KOR^{SERT} in stress potentiation of cocaine CPP, these results suggest that a decrease in serotonin tone may mediate stress-induced dysphoria, thereby increasing subsequent

preference for cocaine. During stress potentiation of cocaine preference, stress likely decreases NAc serotonin prior to cocaine administration, but this effect is unlikely persistent, as cocaine inhibition of SERT function dramatically increases NAc serotonin tone (Andrews & Lucki, 2001; Schindler et al., 2012). Thus, our temporally precise manipulation of DRN serotonin was crucial to evaluating our central hypothesis that a *prior* stress-induced decrease in serotonin tone drives subsequent increased sensitivity to drug reward. Our findings support this model and may support a general framework by which KOR and 5-HT-mediated dysphoria increases the relative impact of reward on affect.

Recently, an integral role of $SERT^{DRN}$ neurons in regulating response to stress and reward has begun to come into focus, but how $SERT^{DRN}$ neurons mediate the impact of stress on responses to natural and drug reward remains opaque (Ren et al., 2018; Seo et al., 2019; Teissier et al., 2015; Zhong et al., 2017). We isolated the potential role of decreased serotonin tone in stress potentiation of cocaine preference first by selective inhibition of $SERT^{DRN}$ neurons prior to cocaine conditioning and demonstrated this that a prior reduction in serotonin was sufficient to potentiate subsequent cocaine preference. Surprisingly, we found that inhibition of $VGluT3^{DRN}$ neurons, which overlap extensively with $SERT^{DRN}$, strongly attenuated subsequent cocaine preference. These findings further demonstrate that the DRN is a critical regulator of reward behavior, and we reason that these divergent effects may be mediated by the non-overlapping fraction of these DRN populations (i.e., $SERT^{+}/VGluT3^{-}$ neurons drive reward potentiation) and their projections. The population of overlapping $VGluT3^{+}/SERT^{+}$ DRN neurons project to a myriad of target regions, including the lateral hypothalamus, ventral tegmental area and many aspects of the cortex (Ren et al., 2019). Based on our DRN *in-situ* hybridization and projection tracing studies, we suggest the population responsible for stress potentiation of cocaine preference may comprise $Slc6a4^{+}/Slc17a8^{-}/Oprk1^{+}$ neurons of lateral DRN that project to the mNAc. Prior work has indicated that the lateral DRN is highly responsive to stress and is anatomically segregated from regions known to innervate other regions involved in reward processing, including the amygdala and ventral tegmental area (Bang & Commons, 2012; Valentino et al., 2010).

Historically, the effect of stress on serotonin in the NAc has been controversial, with evidence for increases, decreases, and no effect on serotonin tone (Carlezon et al., 2006; De La Garza & Mahoney, 2004; Kirby et al., 1995; Price et al., 2002; Schindler et al., 2012). While we did not directly measure 5-HT tone, this study leveraged the neurochemically and anatomically precise nature of terminal optogenetic stimulation during stress to manipulate the $SERT^{DRN\text{-}NAc}$ projection and indicates that a decrease in 5-HT tone within the mNAc promotes stress-induced immobility. These findings contribute to recent findings of parallel DRN serotonin subsystems innervating distinct targets to regulate reward and response to stressors (Ren et al., 2018; Teissier et al., 2015), which indicated a role for $SERT^{DRN}$ neurons that project to the orbitofrontal cortex, but not the central amygdala, in promoting active coping in a repeated forced swim test. Thus, while stress-induced decrease in serotonin tone within the mNAc is a critical regulator of passive coping behavior, 5-HT tone in other regions may also regulate passive coping, possibly in coordination with the projection we detail.

In this study, we examined potential contributions of the $5\text{-}HT_{1B}$ receptor to stress potentiation of reward. $5\text{-}HT_{1B}$ receptors are G_i-coupled GPCRs that inhibit neurotransmitter release and have been implicated in regulating the response to stressors and psychostimulants. However, the direction of these effects is dependent on brain region, involvement of autoreceptors or heteroreceptors, and stage of addiction cycle (González et al., 2006; Pfeiffer et al., 1986, Filip et al., 2002; Fletcher et al., 2002; Pentkowski et al., 2009, 2014). In the mNAc, transient overexpression of $5\text{-}HT_{1B}$ heteroreceptors during mild stress enhances stress-induced potentiation of the psychomotor effects of amphetamine (Ferguson et al., 2009). We observed that transient antagonism of $5\text{-}HT_{1B}$ receptors in the NAc was sufficient to recapitulate potentiation of cocaine preference induced by stress or decreased serotonin tone. Transient prior antagonism of NAc $5\text{-}HT_{1B}$ receptors also potentiated social preference, a behavior mediated by NAc $5\text{-}HT_{1B}$ receptors (Walsh et al., 2018). Together, these data suggest that $5\text{-}HT_{1B}$ receptors in the NAc act as a critical signal transducer, sensing a decrease in 5-HT tone and initiating postsynaptic consequences that result in potentiation of reward. Our findings also indicate that the mechanism by which decreased $5\text{-}HT_{1B}$ activation result in potentiation of subsequent cocaine reward may generalize to processing of other rewarding stimuli, including natural rewards. Lastly, we showed that potentiation of cocaine preference induced by transient

prior blockade of NAc 5-HT_{1B} was attenuated by an additional antagonist infusion that blocked 5-HT_{1B} receptors during cocaine conditioning. This supports the conclusion that the potentiation of reward elicited by 5-HT_{1B} antagonism is primarily due to direct consequences of 5-HT_{1B} receptor blockade and not indirect effects on other neurotransmitter systems. These findings further indicate that the 5-HT_{1B} receptor is not only involved in initiating reward potentiation but is involved in mediating the expression of increased reward as well.

5-HT_{1B} transcript is highly expressed in the NAc, and prior work indicates that chronic exposure to stressors or psychostimulants may regulate its expression within the accumbens (Furay et al., 2011; Hoplight et al., 2007). However, the precise distribution of 5-HT_{1B} transcripts across the populations of the NAc and whether these populations express functional receptor protein at their cell bodies has not been established. We detected an increase in pERK-IR, a proxy for 5-HT_{1B} receptor activation (Liu et al., 2019), in NAc cell bodies following local infusion of a 5-HT_{1B} agonist. This supports the presence of functional receptors on the somata or local axon collaterals of neurons within the mNAc; however, we cannot exclude the possibility of polysynaptic effects. Colocalization of the 5-HT_{1B} transcript *(Htr1b)* with markers of the direct pathway (*Pdyn*), indirect pathway (*Adora2a),* and cholinergic interneurons (*Chat)* showed uniform distribution across these cell types, indicating that serotonin actions through 5-HT_{1B} receptors may regulate these populations in concert to modulate processing in the NAc. Following stress, 5-HT_{1B} mRNA increased within *Pdyn*$^{+}$*,* but not *Adora2a*$^{+}$ neurons, suggesting that stress selectively increases the expression of 5-HT_{1B} transcript in cells of the direct pathway. Whether this increase in transcript is reflected by an increase of functional receptors remains to be tested. Prior work has also shown that overexpression of 5-HT_{1B} receptors in the NAc neurons that project to the ventral tegmental area sensitizes mice to the hedonic properties of cocaine (Barot et al., 2007; Neumaier et al., 2002), which intimates that stress-induced increases of postsynaptic 5-HT_{1B} receptors expressed in direct pathway neurons may mediate stress potentiation of reward.

Chronic stress induces hedonic and motivational deficits that contribute to depression like behavior, but sub-chronic stress can provoke coping responses. Exposure to sub-chronic stress may induce a proadaptive hedonic allostasis by increasing sensitivity to reward that is reflected by changes in 5-HT_{1B}

transcript expression within the mNAc. We indicate this coping response is maladaptive in the context of drug exposure, resulting in increased drug preference and enhanced addiction risk. While our data are consistent with this interpretation, future work is required to assess the behavioral and cellular tenets of this theory. In human studies, polymorphisms of the 5-HT_{1B} receptor have been associated with major depression and substance use disorder (Cao et al., 2013; Y. Huang et al., 2003; Sun et al., 2002), and receptor binding studies show altered 5-HT_{1B} binding in the NAc of subjects with major depression and alcohol dependence (Hu et al., 2010; Murrough & Neumeister, 2011). Such findings suggest a central role of NAc 5-HT_{1B} receptors in regulation of affect and substance use, but a potential connection to the Dyn/KOR system and stress potentiation of addiction risk has not been previously evaluated. The insights gleaned from this study support a functional Dyn-KOR-5-HT-5-HT_{1B} axis in which decreased 5-HT is a central regulator of drug preference, affect, and response to stressors. Future studies will be required to directly evaluate the consequences and kinetics of the stress and dynorphin-mediated effects on functional 5-HT_{1B} receptors and evaluate the therapeutic potential of this dynamic circuit.

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Figure 2.1

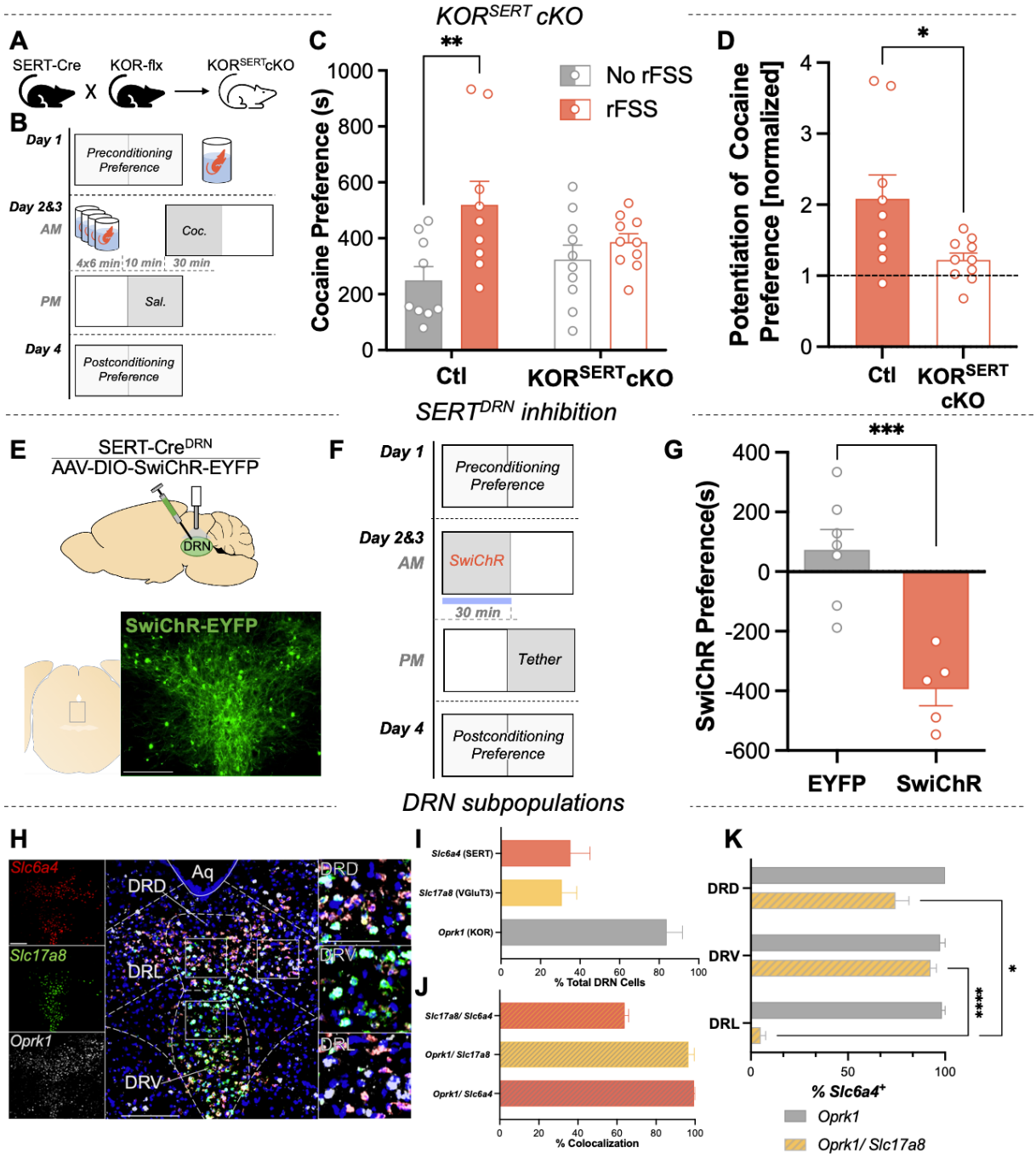

Figure 2.1. Serotonin neuron kappa opioid receptors mediate stress potentiation of cocaine reward and can be mimicked by optogenetic inhibition. (A) Breeding scheme used to excise KOR gene from SERT expressing neurons. (B) Schematic of rFSS potentiation of cocaine CPP protocol. Mice were subjected to rFSS on day 1 and day 2 prior to cocaine conditioning. (C) Cocaine preference scores

(postconditioning preference- preconditioning preference for drug-paired chamber) for control and KOR^{SERT} cKO mice with or without prior stress (n=9-10). (D) Preference scores from panel C with each genotype normalized to its unstressed control (n=9-10). (E) Cartoon depicting DRN injection of inhibitory opsin (AAV5-DIO-SwiChR-EYFP) and cannula placement in a SERT-cre mouse. Below: image showing expression in the DRN. Scale bar= 50 μm. (F) Schematic of optogenetic CPA protocol. During optogenetic conditioning, the mouse was confined to one chamber where it received inhibition of $SERT^{DRN}$ neurons. (G) Serotonin inhibition preference scores (postconditioning-preconditioning preference for laser-paired chamber) for control and SwiChR-inhibition conditioned groups (n=5-7). (H) Representative image showing expression of transcripts for SERT (*Slc6a4)*, VGluT3 (*Slc17a8*), and KOR (*Oprk1*) in the medial DRN (n=3). Right inset: higher magnification of rectangular regions showing colocalization in cells of the dorsal, ventral, and lateral aspects of the DRN (DRD, DRV, DRL, respectively). Scale bar= 200μm, 200μm, 25 μm. (I) Quantitation of cells expressing transcripts for SERT, VGluT3, KOR, expressed as percentage of total DRN cells. (J) Quantitation of cells co-expressing two transcripts, expressed as percentage cells in the denominator indicated. (J) Quantitation of *Slc6a4*$^+$ cells co-expressing *Oprk1* or both *Oprk1* and *Slc17a8* in each subregion, expressed as percentage of *Slc6a4*$^+$ cells per subregion.

Figure 2.2

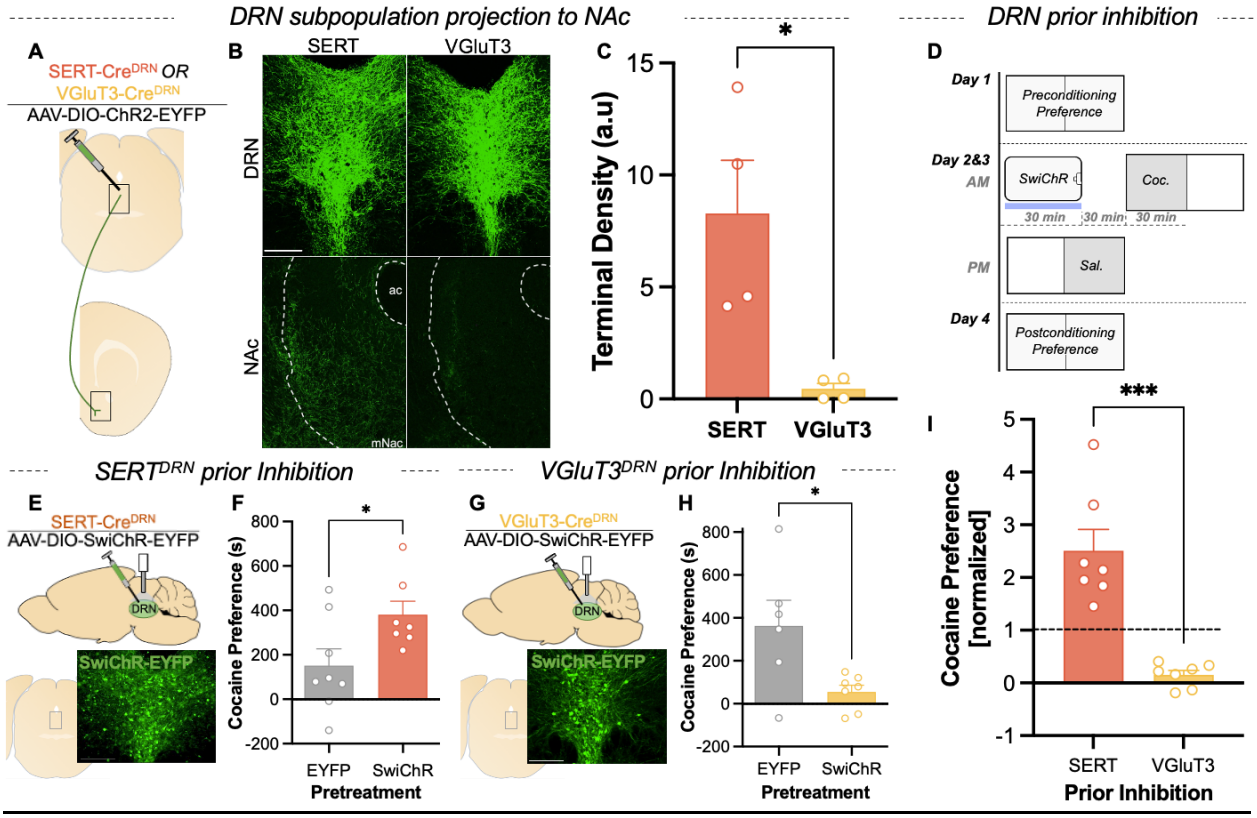

Figure 2.2. Prior inhibition of dorsal raphe nucleus serotonin subpopulations with distinct projection bias has divergent effects on cocaine preference. (A) Cartoon depicting DRN injection of fluorescently-tagged ChR2 (AAV5-DIO-ChR2-EYFP) and rectangular regions showing areas imaged. (B) DRN (cell body) and NAc (terminal) expression of EYFP-tagged ChR2 in a SERT-Cre and VGluT3-Cre mouse (n=4). Scale bar=200μm. (B) Quantification of terminal density (arbitrary units) in the NAc of SERT-cre and VGluT3-cre mice (n=4). (C) Schematic showing assay of optogenetic replication of KOR-mediated cocaine CPP potentiation. Mice received optogenetic inhibition of specified DRN subpopulations prior to cocaine conditioning. (D) Cartoon depicting injection of AAV5-DIO-SwiChR-eYFP into the DRN and placement of cannula above injection site. Below: native expression of EYFP-tagged SwiChR in the DRN of a SERT-Cre mouse. Scale bar=50μm (E) Cocaine preference scores (postconditioning preference-preconditioning preference) for groups subject to control treatment and SERT inhibition prior to conditioning (n=7-8). (F) Cartoon depicting injection of AAV5-DIO-SwiChR-eYFP into the DRN and placement of the

cannula above the injection site. Below: native expression of EYFP-tagged SwiChR in the DRN of a VGluT3-Cre mouse. Scale bar=50μm. (G) Cocaine preference scores for groups subjected to control treatment and VGluT3 inhibition prior to conditioning (n=6-7). (H) Comparison of cocaine preference scores (normalized to respective EYFP controls) following inhibition of $SERT^{DRN}$ or $VGluT3^{DRN}$ neurons (n=7).

Figure 2.3

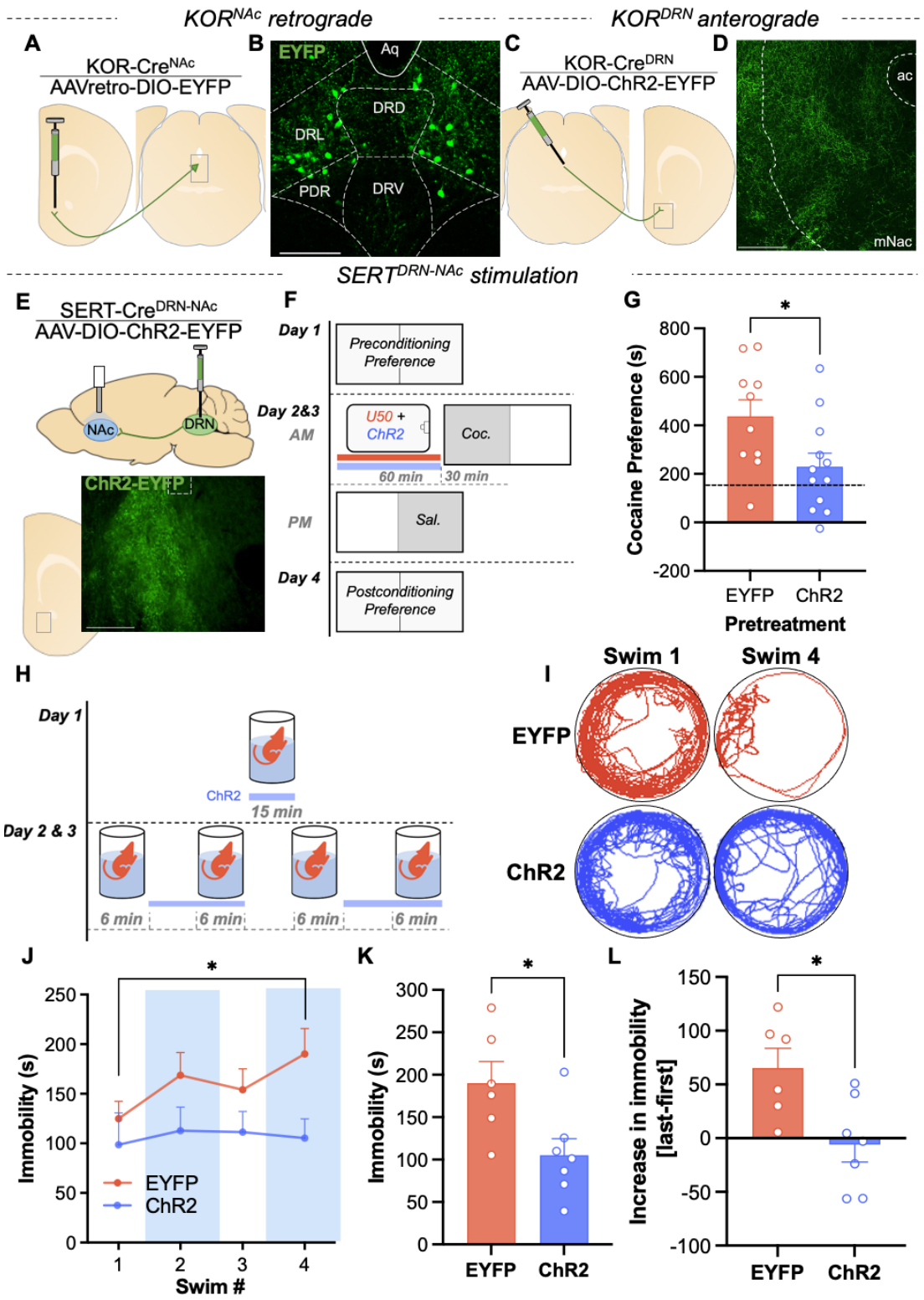
KOR^NAc retrograde
KOR^DRN anterograde
A
KOR-Cre^NAc
AAVretro-DIO-EYFP
B
EYFP
Aq
DRD
DRL
PDR
DRV
C
KOR-Cre^DRN
AAV-DIO-ChR2-EYFP
D
ac
mNac
SERT^DRN-NAc stimulation
E
SERT-Cre^DRN-NAc
AAV-DIO-ChR2-EYFP
NAc
DRN
ChR2-EYFP
F
Day 1
Preconditioning Preference
Day 2&3
AM
U50 + ChR2
Coc.
60 min
30 min
PM
Sal.
Day 4
Postconditioning Preference
G
Cocaine Preference (s)
800
600
400
200
0
-200
EYFP
ChR2
Pretreatment
H
Day 1
ChR2
15 min
Day 2 & 3
6 min
6 min
6 min
6 min
I
Swim 1
Swim 4
EYFP
ChR2
J
Immobility (s)
250
200
150
100
50
0
EYFP
ChR2
1
2
3
4
Swim #
K
Immobility (s)
300
250
200
150
100
50
0
EYFP
ChR2
L
Increase in immobility [last-first]
150
100
50
0
-50
-100
EYFP
ChR2

Figure 2.3. Stimulation of DRN-NAc serotonin terminals attenuates KOR-mediated increases in cocaine preference and stress-induced immobility. (A) Cartoon depicting NAc injection of retrograde virus (AAVretro-DIO-EYFP) in KOR-Cre animal and rectangle showing imaging field. (B) Native expression of EYFP in subregions of medial DRN of KOR-Cre mouse. Scale bar=200μm. (C) Cartoon depicting DRN injection of fluorescently-tagged ChR2 (AAV5-DIO-ChR2-EYFP) in KOR-Cre animal and rectangle showing imaging field. (D) Native expression of $EYFP^+$ terminals in the NAc of KOR-Cre mice. (E) Cartoon showing DRN injection of AAV5-DIO-ChR2-EYFP and placement of optic cannula above the NAc in a SERT-Cre mouse. (F) Expression of $EYFP^+$ terminals in NAc of SERT-Cre mouse. (G) Schematic of cocaine CPP with pretreatment of KOR agonist and stimulation of serotonin terminals prior to cocaine conditioning. Prior to each cocaine conditioning session, mice were pretreated with KOR agonist (U50,488; 5mg/kg I.P) and received concurrent optic stimulation of serotonin terminals in the NAc or control treatment. (H) Cocaine preference scores of mice receiving KOR agonist prior to cocaine conditioning with or without concurrent ChR2 stimulation of $SERT^+$ terminals in the NAc (Dashed line shows typical unstressed cocaine preference) (n=10-12). (I) Schematic of optical stimulation of SERT terminals in the NAc during rFSS. Mice received optical stimulation during the swim on day 1 and prior+during swim bouts 2 and 4 on day 2. (J) Example track traces showing movement during swims 1 and 4 on day 2 for mice with optic stimulation of SERT terminals in the NAc (ChR2) or controls. (K) Time immobile during swim bouts on day 2 of rFSS for mice with stimulation of NAc SERT terminals and increase in immobility across day 2 of rFSS (swim 4 - swim 1) shown in panel J. controls (n=6-7). (L) Time immobile during last swim bout of rFSS in panel J.

Figure 2.4

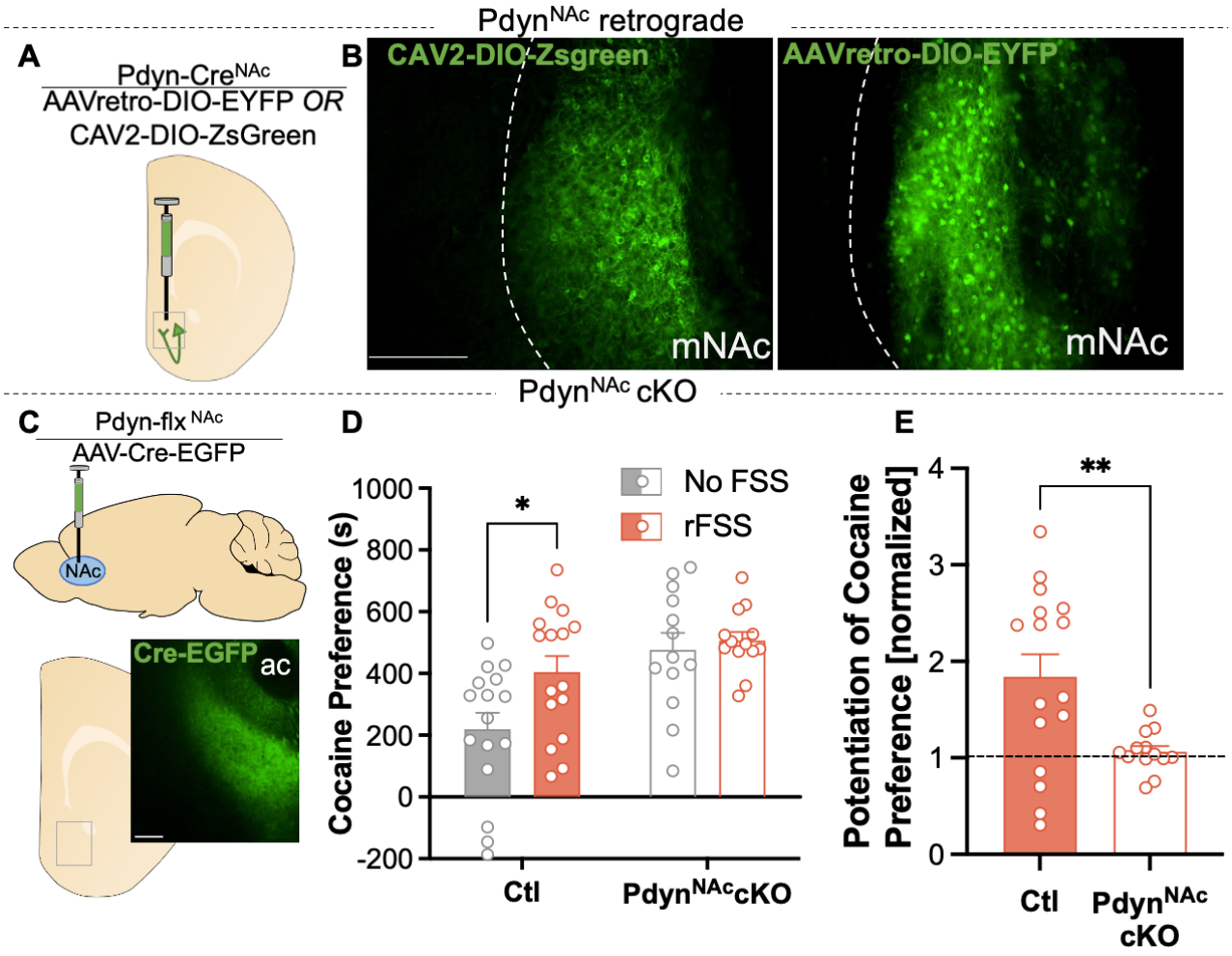

Figure 2.4. Nucleus accumbens dynorphin neurons provide the primary source of NAc dynorphin and are required for regulation of cocaine preference. (A) Cartoon showing injection of retrograde virus (CAV2-DIO-Zsgreen or AAVretro-DIO-EYFP) in the NAc of Pdyn-cre mouse. Rectangle shows imaging field. (B) Representative images showing native expression of retrogradely-delivered fluorophore (ZsGreen or EYFP) in the NAc of a Pdyn-Cre mouse. Scale bar=50μm. (C) Cartoon showing injection of virus delivering Cre-recombinase (AAV-Cre-EGFP) to the NAc of Pdyn-flx mice. Below: representative image showing native expression of AAV-Cre-EGFP in the NAc. (D) Cocaine preference scores of control and Pdyn^{NAc}cKO mice with and without prior swim stress (rFSS) (n=13-16). (E) Preference scores from panel D with each group normalized to its unstressed controls (n=13-16).

Figure 2.5

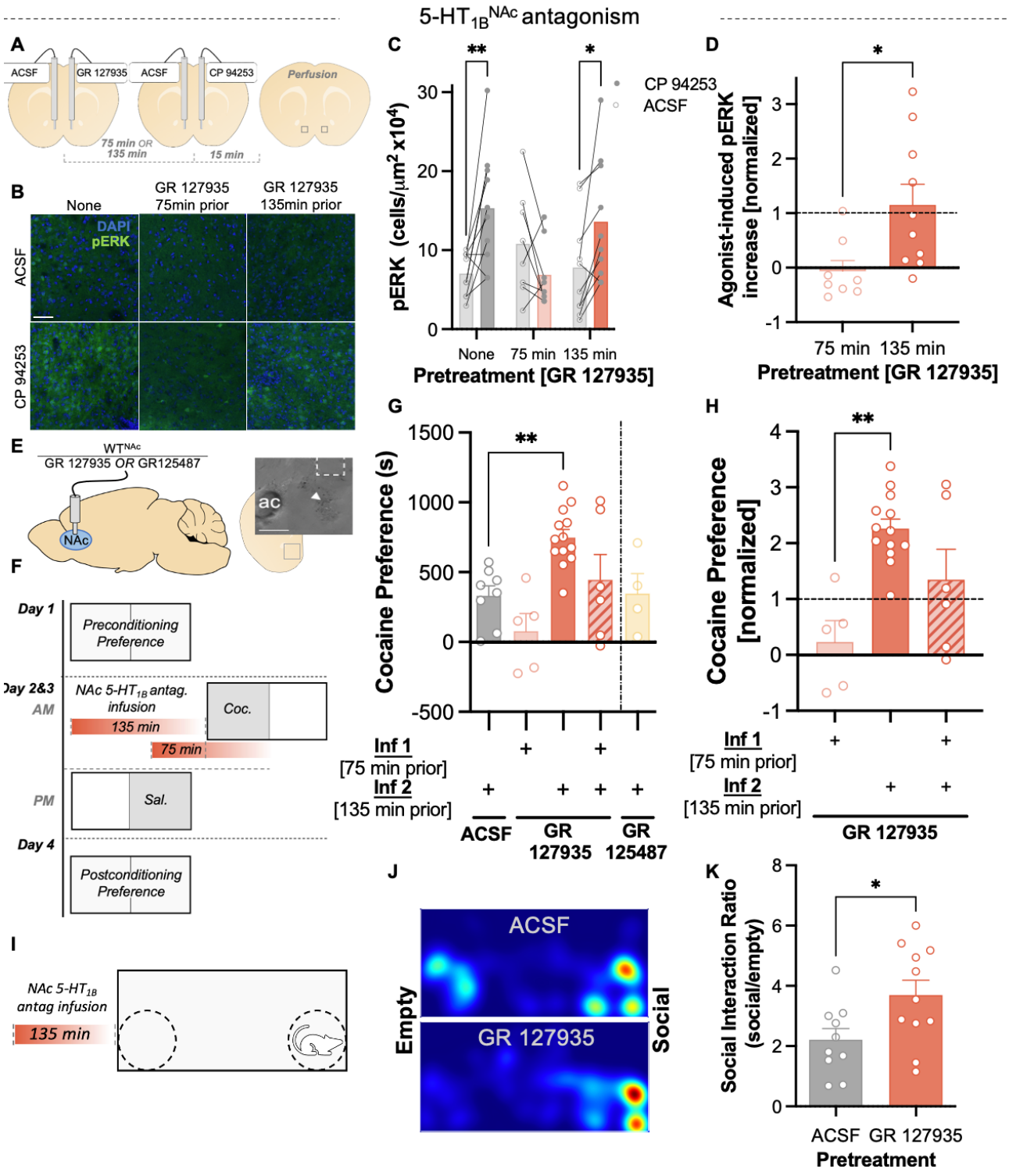

Figure 2.5. Prior 5-HT$_{1B}$ receptor antagonism in NAc potentiates subsequent cocaine and social preference. (A) Schematic of unilateral NAc infusion of 5-HT$_{1B}$ antagonist GR 127935, followed 75 or

135min later by infusion of 5-HT_{1B} agonist CP 94253 and perfusion. Rectangles show imaging field. (B) Representative images showing immunofluorescent lab phospho-ERK1/2 (pERK) in ACSF (control) hemisphere and hemisphere receiving 5-HT_{1B} ligand infusions. Scale bar= 50μm. (C) Quantification of pERK+ cells in control hemisphere and agonist-infused hemispheres following NAc infusion of GR 127935 prior to CP 94253 (n=8-10). (D) Quantification of pERK+ cells following GR 127935 pretreatment, expressed as percentage of agonist-induced increase in pERK compared to ACSF and normalized to agonist-induced increase without pretreatment (n=8-10). (E) Cartoon showing implantation of fluid cannula for infusion of 5-HTR antagonists into the NAc of WT mice. (inset) Image showing dye injection (arrow) and damage from cannula confirming placement in medial NAc. Scale bar=200μm. (F) Schematic of cocaine CPP procedure showing pretreatment with NAc infusions of 5-HT_{1B} antagonist (GR 127935) at time points 75min or 135min prior to cocaine conditioning. (G) Cocaine place preference scores (postconditioning-preconditioning preference) for mice pretreated with infusions of 5-HT_{1B} antagonist GR 127935 or ACSF in the NAc (n=4-13). (H) Preference scores of GR 127935 pretreatments from panel D normalized to ACSF pretreatment preference. (I) Schematic showing pretreatment NAc infusion of 5-HT_{1B} antagonist prior to three-chamber social interaction assay. (J) Representative heatmaps indicating the distribution of time spent in the social interaction apparatus after infusion of GR 127935 into the NAc 135min prior and ACSF control. (K) Social interaction ratio (time spent in social zone/time spent in empty zone) following pretreatment with GR 127935 (n=10-11).

Figure 2.6

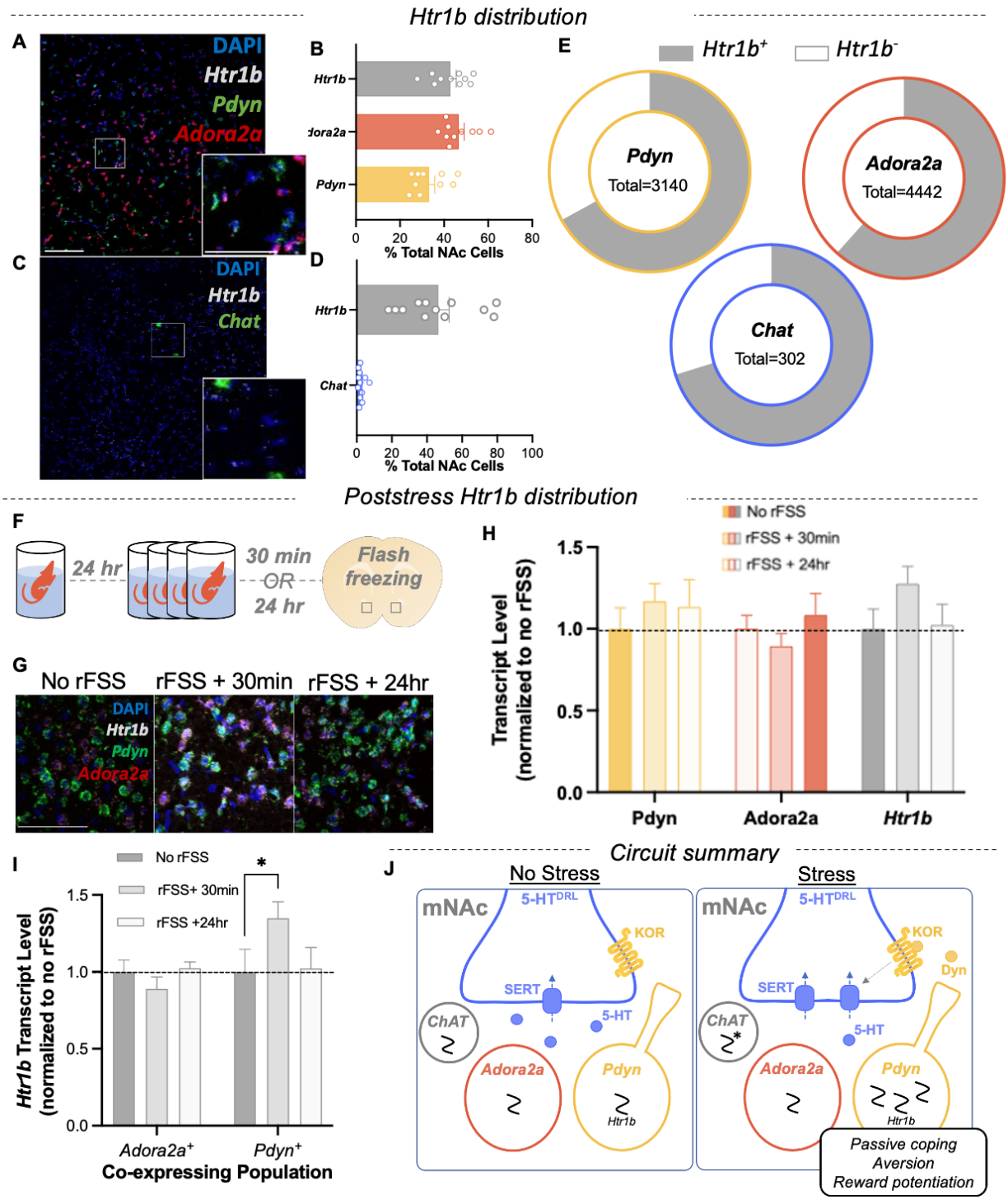

Figure 2.6. Stress induces a transient and cell-type specific increase in *Htr1b* expression in the NAc. (A) Representative image showing *in-situ* hybridization labeling *Pdyn, Adora2a, and Htr1b* transcripts in the medial NAc. Inset: higher magnification of rectangular region. Scale bar= 100μm, 25μm (inset). (B)

Quantification of percent of total cells in the medial NAc positive for *Htr1b*, *Pdyn*, and *Adora2a* (n=6). (C) Representative image showing *in-situ* hybridization labeling *and Htr1b* and *Chat* transcripts in the medial NAc. Inset: higher magnification of rectangular region. Scale bar=100μm, 25μm. (D) Quantification of percent of total cells in the medial NAc positive for *Htr1b* and *Chat* (n=6). (E) Proportion of each subpopulation that expresses *Htr1b.* (F) Schematic of rFSS procedure prior to flash freezing brains for *in-situ* hybridization (G) Representative images showing colocalization and intensity of labelling of *Pdyn, Adora2a, and Htr1b* transcripts in the medial NAc after stress. Scale bar=25μm. (H) Quantified total levels of *Pdyn, Adora2a, and Htr1b* following stress, normalized to no rFSS controls (n=6-8). (I) Quantified levels of *Htr1b* transcripts expressed in $Pdyn^{+}$ and $Adora2a^{+}$ cells following stress, normalized to no rFSS controls. (J) Summary schematic of NAc circuit mediating response to stressors, aversion, and reward potentiation. With exception of SERT, the schematic is simplified to include only entities measured or manipulated in this study. **Chat* transcript level was not assessed after stress

Chapter 3. Kappa opioid receptor activation in dopamine neurons disrupts behavioral inhibition

This chapter is adapted from the following manuscript:

Abraham AD, **Fontaine HM**, Song AJ, Andrews MM, Baird MA, Kieffer BL, Land BB, Chavkin C (2018) Kappa opioid receptor activation in dopamine neurons disrupts behavioral inhibition. *Neuropsychopharmacology* 43(2):362-372.

I conducted the marble burying assay, performed surgeries, assisted with operant behavioral assays, and contributed to the preparation of the manuscript. I contributed to the data or analysis of data that appears in the following figures.

Abstract:

The dynorphin/kappa opioid receptor (KOR) system has been previously implicated in the regulation of cognition, but the neural circuitry and molecular mechanisms underlying KOR-mediated cognitive disruption are unknown. Here, we used an operational test of cognition involving timing and behavioral inhibition and found that systemic KOR activation impairs performance of male and female C57BL/6 mice in the differential reinforcement of low response rates (DRL) task. Systemic KOR antagonism also blocked stress-induced disruptions of DRL performance. KOR activation increased 'bursts' of incorrect responses in the DRL task and increased marble burying, suggesting that the observed disruptions in DRL performance may be attributed to KOR-induced increases in compulsive behavior. Local inactivation of KOR by injection of the long-acting antagonist norBNI in the ventral tegmental area (VTA), but not the infralimbic prefrontal cortex (PFC) or dorsal raphe nucleus (DRN), prevented disruption of DRL performance caused by systemic KOR activation. Cre-dependent genetic excision of KOR from dopaminergic, but not serotonergic neurons, also blocked KOR-mediated disruption of DRL performance. At the molecular level, we found that these disruptive effects did not require arrestin-dependent signaling, because neither global deletion of G-Protein Receptor Kinase 3 (GRK3) nor cell-specific deletion of GRK3/arrestin-dependent p38α MAPK from dopamine neurons blocked KOR-mediated DRL disruptions. We then showed that nalfurafine, a clinically available G-biased KOR agonist, could also produce DRL disruptions. Together, these studies demonstrate that KOR activation in VTA dopamine neurons disrupts behavioral inhibition in a GRK3/arrestin-independent manner and suggests that KOR antagonists could be beneficial for decreasing stress-induced compulsive behaviors.

Introduction

Dynorphin, an endogenous opioid peptide released following stress, activates the kappa opioid receptor (KOR) to produce depression-like behaviors (Mague et al, 2003; Knoll and Carlezon, 2010) and aversion (Shippenberg and Herz, 1986). In humans, acute KOR activation with selective and highly efficacious KOR agonists or the natural products from the *Salvia divinorum* plant (Salvinorin A; a selective KOR agonist) produces potent psychotomimetic and hallucinogenic effects (Johnson et al 2010), suggesting that KOR activation may also contribute to cognitive disruptions following a behavioral stress experience.

Although it is challenging to measure psychotomimetic drug effects using animal models, KOR activation has been shown to alter multiple domains of cognition in rodents, including attention, memory, and impulsivity (Nemeth et al, 2010; Cole et al, 2013). KOR activation has no effect on impulse control (Paine et al, 2007) or impulsive choice, but does disrupt behavioral performance in a response inhibition task (Walker & Kissler, 2013). Degradation of response inhibition may be indicative of a loss of inhibitory control, a cognitive feature that is disrupted in psychiatric illnesses such as substance use disorder (Jentsch and Taylor, 1999) and compulsive disorders (Chamberlain et al., 2005). KOR activation following stress may exacerbate behavioral symptoms or contribute to the etiology of these diseases. Although KOR antagonists are in development for the therapeutic goal of decreasing stress-induced mood disorders and relapse of substance abuse (Carroll and Carlezon, 2013), it is unknown whether KOR antagonists may also be useful for decreasing stress-induced cognitive disruptions or compulsive behaviors.

Behavioral and cognitive disruptions in patients with obsessive-compulsive disorder (OCD) can worsen following stressful events (Fornaro et al., 2009). Many of the symptoms observed in OCD may be a consequence of loss of inhibitory control (Chamberlain et al., 2005) leading to obsessive or compulsive thoughts and behaviors. One method to investigate disruption of inhibitory control in rodents is through the use of the differential reinforcement of low response rates (DRL) task. The DRL task requires an animal to withhold responding for a set wait period prior to making a reinforced response (Sidman, 1955). Responses occurring before the end of the wait period (nonreinforced responses) reset the wait period.

This task has previously been used to measure temporal discrimination (Sidman, 1956), antidepressant efficacy (O'Donnell et al, 2005), and impulsive action (Selleck et al, 2015). Nonspecific opioid receptor activation in the prefrontal cortex disrupts performance in the DRL task (Selleck et al, 2015), demonstrating that opioid receptor activation decreases inhibitory control. Stress-induced release of dynorphin would be expected to disrupt cognitive performance, but the specific nature of these disruptions, the sites of action in brain, and the cellular mechanisms underlying these disruptions are not yet known. Here, we find that KOR activation in dopamine neurons of the ventral tegmental area (VTA) disrupts inhibitory control to increase compulsive responses. We also determine that KOR-mediated DRL disruptions are likely to use G-protein receptor kinase 3 (GRK3)/arrestin-independent intracellular signaling pathways, indicating that some G-biased KOR agonists may produce compulsive responses.

Materials and Methods

Subjects

Male and female C57BL/6 mice (n = 174) ranging from 3-10 months of age were used in these experiments.

Drugs

(±)U50,488H (U50,488) and norbinaltorphimine (norBNI) were provided by the National Institute of Drug Abuse Drug Supply Program (Bethesda, MD). U50,488 (5 mg/kg and 10 mg/kg) or norBNI (10 mg/kg) was dissolved in saline and administered intraperitoneally (i.p.) in a volume of 10 mL/kg. norBNI (2.5 ug/uL) dissolved in sterile artificial cerebrospinal fluid (ACSF) was intracranially microinjected.

Procedures

Differential Reinforcement of Low Rates of Responding (DRL)

Following training to discriminate between active and inactive nose poke holes, mice were trained in a 60-min differential reinforcement of low rates (DRL) procedure (Horwood et al, 2001). During a DRL session, a single nose poke led to food reward delivery. Following the first reinforced nose poke, subjects were required to withhold responding for a specified wait period. Nose pokes that occurred before the end of the wait period reset the wait period and were nonreinforced. Once trained for stable performance on the DRL task (below 40% error over at least 4 days; 15-25 training sessions), animals underwent sessions where they received saline treatment on a baseline DRL day, and a U50,488 treatment the following day

immediately prior to a DRL session. For microinjection studies, male mice were used that had previously been tested for the systemic effect of KOR activation in the DRL task. Male mice were trained in the DRL task, then received a single microinjection of norBNI (2.5 ug/uL; long acting kappa antagonism by JNK activation; Bruchas et al, 2007, Land et al, 2009) into the infralimbic PFC (n = 4), DRN (n = 4), or VTA (n = 10). Controls received an injection of ACSF into the VTA (n = 8). Mice recovered from surgery for 3 days prior to retraining for stable performance in the DRL task (3-5 days). Following retraining, mice received a saline baseline test day and U50,488 the following day. Conditional and global knockout mice and their littermate controls received two DRL sessions with 5 mg/kg U50,488 and one DRL session with 10 mg/kg U50,488. Each test was separated by at least 5-10 daily sessions of DRL training between the drug test days to ensure that there were no persistent effects of drug administration on DRL performance. Each drug test day was preceded by a saline baseline DRL test day.

Forced-Swim Stress

To induce stress, C57BL/6 male mice (n = 8) were exposed to a modified forced-swim test as previously described (McLaughlin et al, 2003). Briefly, the modified-Porsolt forced-swim paradigm used was a 2-day procedure in which mice swam in 30°C water for 15 min the first day following a baseline DRL session, then 6 min during each of four trials on the second day without the opportunity to escape. Mice were tested for DRL performance within 10 min following the second day of stress (Stress 1). One week later, mice received the 2-day FSS procedure (Stress 2) with norBNI (10 mg/kg; i.p.) or saline pretreatment (n = 4 per group) and DRL performance was tested the following day.

Marble Burying

C57BL/6 male mice were placed in a novel rectangular context (50.8 X 25.4 X 25.4 cm; corn cob bedding packed to 5cm depth) housed in a sound- and light-attenuating cabinet for a 30-min habituation period. Mice then received an injection of U50,488 (n = 10) or saline (n = 9) and were immediately returned to the context for another 30-min habituation period. Mice were removed from the context and 18 marbles (16 mm diameter) were placed on the bedding. Mice were immediately returned for a 30-min marble burying test. Total number of marbles buried (at least 2/3 covered) at the end of the 30-min session were counted by an experimenter blinded to drug treatment (Deacon, 2006).

Data Analysis

Error percentage was calculated by the number of responses that led to a reset of the wait time (nonreinforced responses) divided by total number of responses (reinforced + nonreinforced responses). Stable performance in the DRL task was defined as at least 4 days with <40% error (Sinden et al, 1986). After mice showed stable performance, they received baseline (saline) and U50,488 test days. Mice that did not show stable performance also received baseline and test days to match drug treatment between cage-mates. Thus, mice that showed greater than 40% error during the DRL baseline day were removed from analysis. Interresponse time (IRT) was the time between each nose poke on the active port. MATLAB software (version R2016a) was used to extract burst response data. Data were analyzed with t-tests or two-way ANOVAs as required by experimental designs for DRL, FR1, FR5, and marble burying experiments. Post hoc comparisons were conducted with Sidak's test. For all statistical tests, the α was set to 0.05.

Results

Systemic KOR activation disrupts DRL performance in male mice.

Following training for stable reinforcement under a DRL-15s schedule, we tested the effect of systemic KOR activation on DRL performance in male mice. The experimental paradigm is diagrammed (Figure 3.1a). Responses during a DRL session could be either reinforced or nonreinforced, and either response resets the 15-s wait period. A subset of nonreinforced responses occurred with interresponse time (IRT) intervals less than 1s. These 'burst responses' have been described as a consequence of reward omission and a disruption in positive feedback for reinforced responses (Kramer and Rilling, 1970). Mice (n = 13) were administered saline prior to a DRL session on a baseline day, and one day later received a KOR agonist (U50,488) immediately prior to a DRL session. A representative raster plot of responses in the DRL task is shown from a male mouse following saline then U50,488 pretreatment (Figure 3.1b).

Administration of U50,488 (5 mg/kg) significantly increased the number of nonreinforced responses (t_{12} = 2.78, p = 0.016) and significantly decreased the number of reinforced responses (t_{12} = 2.54, p = 0.026) during the DRL test session compared to the saline test session, but there was no significant difference between saline and U50,488 treatment in total response number (Figure 3.1c).

Treatment with U50,488 significantly increased percent error during the DRL session (t_{12} = 6.59, p < 0.0001) compared to the baseline saline day (Figure 3.1d). To investigate the temporal patterns of responding in the DRL task following KOR activation, we compared interresponse times (IRTs) during U50,488 and saline-treatment days (Figure 3.1e). There was a significant main effect on IRT (F (10,120) = 39.86, p < 0.0001), a main effect of Treatment (F (1,12) = 16.67, p = 0.002), and an interaction between IRT and Treatment (F (10,120) = 24.4, p < 0.0001). There was a significant difference between saline and U50,488 treatment in the 0-3s IRT bin (p < 0.0001), indicating a loss of inhibitory control of behavior (Selleck et al, 2015). The loss of inhibitory control caused by KOR activation was evident as a significant increase in burst responding (t_{12} = 2.85, p = 0.015) (Figure 3.1f).

Stress-induced release of dynorphin generated similar disruptions to those observed with systemic U50,488 treatment in a separate cohort of mice. Following a repeated forced swim stress procedure (Stress 1), mice (n = 8) showed a significant increase in % error (t_8 = 2.93, p = 0.022). These mice were then retrained and retested following a second repeated forced swim stress (Stress 2) with saline (n = 4) or norBNI (n = 4) pretreatment. Mice that received saline prior to Stress 2 showed a significant increase in % error (t_4 = 3.59, p = 0.037), but mice that received norBNI prior to Stress 2 did not show a significant increase in % error (Figure 3.1g). Together, these results demonstrate that pharmacological or stress-induced KOR activation impaired performance in the DRL task by disrupting behavioral inhibition and indicate that KOR activation increased compulsive responses to unexpected reward omissions.

<u>KOR activation does not affect FR responding and increases marble burying.</u>

In the DRL task, burst responses have been hypothesized to occur when the delivery of reward is ambiguous or omitted (Sidman, 1956). To determine whether the burst responding caused by KOR activation could be attributed to a nonspecific change in operant performance or compulsive increases in responding, we tested the effect of KOR activation in two fixed ratio tasks. Male mice (n = 7) were trained in a fixed ratio 1 (FR1) procedure, where one nose poke led to the delivery of one food pellet. Following stable performance in the FR1 task, mice were given saline on one day and U50,488 (5 mg/kg) the following day. There was no significant difference in the total number of reinforced responses following U50,488 treatment (Figure 3.2a), and unlike mice trained in the DRL task, all responses were separated

by at least 1s, showing that FR1 trained animals did not produce burst responses with saline or U50,488 treatment. We trained a separate cohort of male mice in an FR5 task (n = 6), where five nose pokes led to the delivery of one food pellet. KOR activation did not significantly alter the number of total responses during an FR5 session (Figure 3.2b) and produced no burst responses following a reinforced response. These experiments demonstrated that when reward delivery was predictable, KOR activation did not promote compulsive burst responses.

However, when tested in a task producing mild anxiety, such as in the marble burying test (Deacon, 2006), KOR activation with U50,488 significantly (t_{17} = 2.71, p = 0.015) increased the number of marbles buried (Figure 3.2c). These results suggest that KOR-mediated increases in burst responses may be specific to anxiogenic or ambiguous reward-contexts.

KORs in the VTA and in dopamine neurons are required for KOR-mediated DRL disruptions.

The infralimbic prefrontal cortex (PFC), dorsal raphe nucleus (DRN), and ventral tegmental area (VTA) contain KORs (Mansour et al, 1987) and have been implicated in response inhibition (Dalley et al, 2011). To identify the brain regions involved in KOR-mediated DRL disruptions, male mice were trained in the DRL task, then received either ACSF into the VTA (Control; n = 8) or a microinjection of norBNI (2.5 ug/uL) bilaterally into the PFC (n = 4), or VTA (n = 10) or unilaterally into the DRN (n = 4) (Figure 3.3a). For nonreinforced responses, a two-way ANOVA with Treatment (Saline; U50,488) and Brain Region (PFC; DRN; VTA; Control) as factors showed that there was a significant effect of Treatment (F (1,22) = 36.71, p < 0.0001), and a significant interaction between Treatment and Brain Region (F (3,22) = 10.16, p = 0.0002). Nonreinforced responses were different between saline and U50,488 treatment days in Control (p = 0.0003) and DRN (p = 0.0001) groups, but not in VTA or PFC (Figure 3.3b). There was a significant effect of U50,488 treatment (F (1,22) = 28.02, p < 0.0001) and Brain Region (F (3,22) = 4.22, p = 0.017) on percent error, and a significant interaction between Treatment and Brain Region (F (3,22) = 7.25, p = 0.002). U50,488 treatment significantly increased percent error in Control (p = 0.003), DRN (p = 0.024), and PFC (p = 0.004) groups, but not in the VTA group (Figure 3.3c). This suggests that KOR activation in the VTA was required for disruptions in DRL performance. For burst responses (Figure 3.3d), there was a significant effect of Treatment (F (1,23) = 5.34, p = 0.03). There was no significant effect of U50,488 on reinforced responses, total responses, or IRTs in VTA injected mice.

These results suggested that KOR activation in the VTA was required for the U50,488-mediated effects on DRL performance. Although the VTA is primarily comprised of dopaminergic neurons, it contains other cell types and is innervated by a broad variety of neurons (e.g. serotonergic or GABAergic) that would be affected by KOR inactivation (Polter and Kauer, 2014). Dopaminergic and serotonergic neuron activity is important for impulsivity (Dalley and Roiser, 2012) and KOR-mediated aversion (Ehrich et al., 2015), but cognitive disruptions may occur through distinct cellular mechanisms. We tested whether KOR-mediated disruptions in DRL performance occurred in male mice having either global KOR knockout (KOR KO; n = 13), KOR conditionally removed from ePet1-Cre expressing serotonergic neurons (KOR CKO^{PET}; n = 4), or KOR conditionally removed from DAT-Cre expressing dopaminergic neurons (KOR CKO^{DAT}; n = 11). Control mice (n = 18) were $KOR^{lox/lox}$ or $KOX^{lox/+}$ littermates from CKO colonies (Figure 3.3e).

For nonreinforced responses, a two-way ANOVA with Treatment (Saline; U50,488) and Genotype (Control; KOR CKO^{PET}; KOR CKO^{DAT}; KOR KO) as factors showed a main effect of Treatment ($F(1, 42) = 16.63$, $p < 0.001$) and a non-significant trend towards an interaction between Treatment and Genotype ($F(3, 42) = 2.59$, $p = 0.065$; Figure 3.3f). For percent error, there was a significant effect of Treatment ($F(1, 42) = 36.4$, $p < 0.001$), Genotype ($F(1,42) = 3.034$, $p = 0.04$), and an interaction between Treatment and Genotype ($F(3, 42) = 7.187$, $p < 0.001$). U50,488 treatment significantly increased percent error in Control ($p < 0.0001$) and KOR CKO^{PET} ($p = 0.0001$) groups, but not KOR CKO^{DAT} or KOR KO groups (Figure 3.3g). For burst responses, there was a significant effect of Treatment ($F(1,42) = 26.1$, $p < 0.0001$) and a significant interaction between Treatment and Genotype ($F(3,42) = 3.192$, $p = 0.033$). KOR activation significantly increased burst responses in Control ($p = 0.0007$) and KOR CKO^{PET} groups ($p = 0.0035$), but not KOR CKO^{DAT} or KOR KO groups (Figure 3.3h). Similar to pharmacological blockade of VTA KORs, genetic excision of KORs from dopaminergic neurons prevents KOR-mediated increases in percent error.

<u>KOR-mediated DRL disruptions are GRK3/arrestin-independent.</u>

The aversive effects of KOR activation have been attributed to GRK3/arrestin-dependent activation of the p38α mitogen-activated protein kinase (p38 MAPK) in dopamine neurons (Bruchas et al, 2007; Ehrich et al, 2015). We tested whether KOR-mediated cognitive disruptions had similar molecular requirements to KOR-mediated aversion by measuring KOR effects on DRL performance in mice with conditional knockout of p38 MAPK from dopaminergic neurons (p38αCKO^{DAT}). Male p38αCKO^{DAT} (n = 4) and p38α$^{lox/+}$ littermates (n = 5) showed a main effect of Treatment in percent error ($F(1, 7) = 28.5$, $p = 0.001$) but no effect of Genotype and no interaction between Genotype and Treatment (Figure 3.4a). Prior to p38 activation, GRK3 phosphorylates KOR and promotes arrestin binding to KOR to initiate MAPK signaling (Bruchas et al, 2006). To test whether the KOR-mediated DRL disruptions were arrestin-dependent, we used male G-Receptor Kinase 3 (GRK3) knockout mice (n = 14) and wild type littermates (n = 9). There was a significant effect of Treatment ($F(1,21) = 45.2$, $p < 0.001$), but no effect of Genotype and no interaction between Genotype and Treatment. We then tested whether a G-biased KOR agonist, nalfurafine (Schattauer et al., 2017), could produce deficits that were comparable to the unbiased KOR agonist U50,488. Nalfurafine (50 μg/kg) pretreatment produced a significant increase in percent error ($t12 = 9.125$, $p < 0.0001$). Together, these results demonstrate that KOR-mediated disruptions of DRL performance are GRK3/arrestin-independent.

Discussion

The present study specifies the cellular and molecular pathways underlying KOR-mediated increases in compulsive responses. First, we found that systemic KOR activation disrupted inhibitory control and decreased response efficiency in the DRL task by increasing nonreinforced responses and burst responses in both male and female mice. Systemic KOR antagonism blocked disruptions of DRL performance caused by stress-induced release of dynorphin in male mice. The burst responses induced by pharmacological KOR activation could not be attributed to a simple disruption of operant responding, as KOR activation did not produce burst responding in an FR1 or FR5 task. Instead, U50,488 administration promoted marble burying, suggesting that KOR activation increased compulsive behaviors in anxiogenic or uncertain environments. Second, we demonstrated that KOR-mediated disruptions in DRL performance were due to KOR activity in the ventral tegmental area and KOR activation on

dopamine neurons. Third, although arrestin-dependent signaling in dopaminergic neurons is required for KOR-mediated aversion (Ehrich et al, 2015), KOR-mediated cognitive disruptions can be generated via arrestin-independent intracellular signaling pathways. Together, these findings reveal a relationship between KOR activation and inhibitory control of behavior that may underlie interactions between stress and compulsivity.

Although KOR actions can have sex-dependent effects (Russell et al, 2014), we found that KOR agonism disrupted DRL performance in both male and female mice. Sex differences have been observed in stress circuitry (Goldstein et al, 2010) and impulsivity (Mitchell and Potenza, 2015), suggesting that there could be sex-specific effects of KOR activation on DRL performance. One issue in comparing male and female mouse behavior in the DRL task is that female rats acquire DRL more efficiently than male rats, and we observed the same sex difference in mice. This effect on DRL performance has been suggested to result from an effect of ovarian steroids (Beatty, 1973) or baseline differences in locomotor activity in males and females (van Hest et al, 1987). To account for baseline differences in DRL acquisition in the present study, female mice were trained on a DRL-25s protocol, rather than a DRL-15s protocol. Despite the differences in DRL procedure, KOR activation induced qualitatively similar deficits in males and females in the DRL task, demonstrating that the cognition disrupting effects of KOR activation are likely to be sex-independent.

Performance in the DRL task can be disrupted by several different factors, including dysregulation of temporal discrimination, general locomotor alteration, and the loss of inhibitory control (Kramer and Rilling, 1970). Alterations in temporal discrimination would shift the peak of observed IRTs (Cho and Jeantet, 2010), but in these experiments, there was no broad shift in IRTs observed following KOR activation. Instead, there were increases in responses occurring within the 0-3s IRT bin, indicating deficits in inhibitory control (Selleck et al, 2015). We assessed how inhibitory control may be affected in the DRL task by analyzing burst responses (IRTs <1s) and found that burst responses increased following U50,488 administration. If burst responses increased in operant tasks where reinforcement probability is consistently predictable (e.g. an FR1 schedule of reinforcement), it would suggest that KOR activation produced a broad increase in compulsive responding. However, we observed no increased burst responses and no differences in total responses in an FR1 or FR5 task following KOR activation.

Many studies have reported hypolocomotion induced by KOR activation (Paris et al., 2011), which suggests that the KOR-mediated increase in burst responses is unlikely to reflect a simple motoric effect. We found that compulsive responses could be observed following KOR activation in the marble burying task, and corroborated Rose et al. (2016), who demonstrated that KOR antagonism decreased drug-induced drug-marble burying. Compulsive responses have been hypothesized to arise as a reaction to environmental uncertainty to cope with ambiguous or threatening stimuli (Holaway et al, 2006). Novel objects in the marble burying task may provoke compulsive behavior, or uncertainty in the relationship between responses and outcomes could produce compulsive behavior in the DRL task. In contrast, during the FR tasks, there is a stable relationship between responses and outcomes, leading to a lack of burst responses following KOR activation. In addition to evidence suggesting that KOR activation can enhance dopamine D2 receptor-mediated compulsive responses (Perreault et al, 2007), our data indicates that KOR activity may have potent effects on compulsive behaviors via dopaminergic circuits.

Dopamine neurons in the ventral tegmental area encode discrepancies between expected and actual outcomes (Schultz et al, 1997), as well as convey information about whether outcomes are better or worse than expected (Hart et al, 2014). Alterations in reinforcement probability can modify dopamine neuron responsivity to aversive events (Matsumoto et al, 2016). KOR activation on dopamine neurons in the ventral tegmental area can produce aversion (Ehrich et al, 2015), and potentiate cocaine reward (Ehrich et al, 2014). We found that KOR activation in the ventral tegmental area, but not the dorsal raphe nucleus was necessary for KOR-mediated DRL disruptions. Mice with KOR blockade in the PFC increased percent error as a result of nonsignificant decreases in reinforced responses and increases in nonreinforced responses, rather than increases in burst responses. Local KOR activation in the PFC or in the VTA can decrease dopamine release in the PFC (Margolis et al, 2006; Tejeda et al, 2013) and dopamine depletion in the PFC has been shown to disrupt DRL performance by increasing burst responding (Sokolowski and Salamone, 1994). KOR blockade on dopamine neurons in the PFC may block burst responses, but this may not be sufficient to overcome the percent error increasing effects of KOR activation in the VTA. It is also possible that KOR activation in the VTA could dysregulate the dopamine signals received in the corticostriatal network to disrupt normal feedback mechanisms that control behavioral inhibition (Jentsch & Taylor, 1999).

Cre-driven excision of KOR can simultaneously remove KOR activity from somatic and terminal regions of particular neuronal populations. We compared the effect of conditional knockout of KOR from dopaminergic or serotonergic neurons against littermate controls and mice with global deletion of KOR. KOR activation in dopaminergic, but not serotonergic neurons, was necessary for disruptions in DRL performance. KOR activation on serotonergic neurons is important for affective processing (Bruchas et al, 2011) and cocaine reward potentiation (Schindler et al, 2012), but may not contribute to this particular feature of cognition. Conditional deletion of KOR from dopaminergic neurons prevented increases in burst responding, demonstrating that KOR activation on dopaminergic neurons may be important for generating responses to reward omissions. One challenge with a conditional deletion of KOR from dopamine neurons is that dopamine neurons in the substantia nigra (SN) also contain KORs (Tempel & Zukin, 1987). Although SN KORs do not contribute to aversion (Bals-Kubik et al, 1993), it is possible that KORs in the SN could produce the observed cognitive disruptions or compulsive behaviors. However, in combination with the microinjection experiments targeting the VTA, we show that KOR activation on dopamine neurons of the VTA disrupts inhibitory control.

Ehrich et al (2015) demonstrated that KOR-mediated aversion requires GRK3/arrestin dependent p38α MAPK activation in VTA dopamine neurons. However, we found no effect of global GRK3 deletion or p38α MAPK deletion from dopamine neurons on KOR-mediated DRL disruptions. These findings show that KOR effects on cognition are distinct from KOR-mediated effects on aversion and likely to be arrestin-independent. We also found that nalfurafine, a G-biased KOR agonist (Schattauer et al., 2017) could produce the observed DRL disruptions. An important implication of this finding is that a highly G-biased KOR agonist used in the treatment of pain or itch without producing dysphoria might still be complicated by unwanted cognitive side effects at high doses (Chavkin, 2011).

In summary, our studies demonstrate that KOR activation in dopamine neurons in the ventral tegmental area disrupts inhibitory control of behavior. Dopamine neurons have been shown to modulate compulsive behaviors in mice (Pascoli et al, 2015), and our results show that KOR activation in dopamine neurons may increase compulsive behaviors when reinforcement probability is ambiguous. In contrast, when reinforcement probability was well-predicted, KOR activation had no effect or promoted inhibition of behavior. Together, these studies suggest that KOR activation disrupts behavioral inhibition in a reward-

context-dependent manner. Our results demonstrate that KOR antagonism during periods of chronic stress could decrease cognitive disruptions and may be beneficial for treating stress-mediated increases in compulsive responses. In agreement with our preclinical findings, there are some case studies reporting that opioid antagonists, such as naltrexone, can decrease compulsive behaviors (Kim, 1998) and buprenorphine, a mu opioid receptor partial agonist and KOR antagonist, can decrease treatment resistant compulsive behaviors (Liddell et al, 2013). Future studies could identify the molecular mechanisms underlying KOR-mediated DRL disruptions in VTA dopamine neurons to generate novel therapeutic interventions for stress-induced cognitive disruptions.

Figure 3.1.

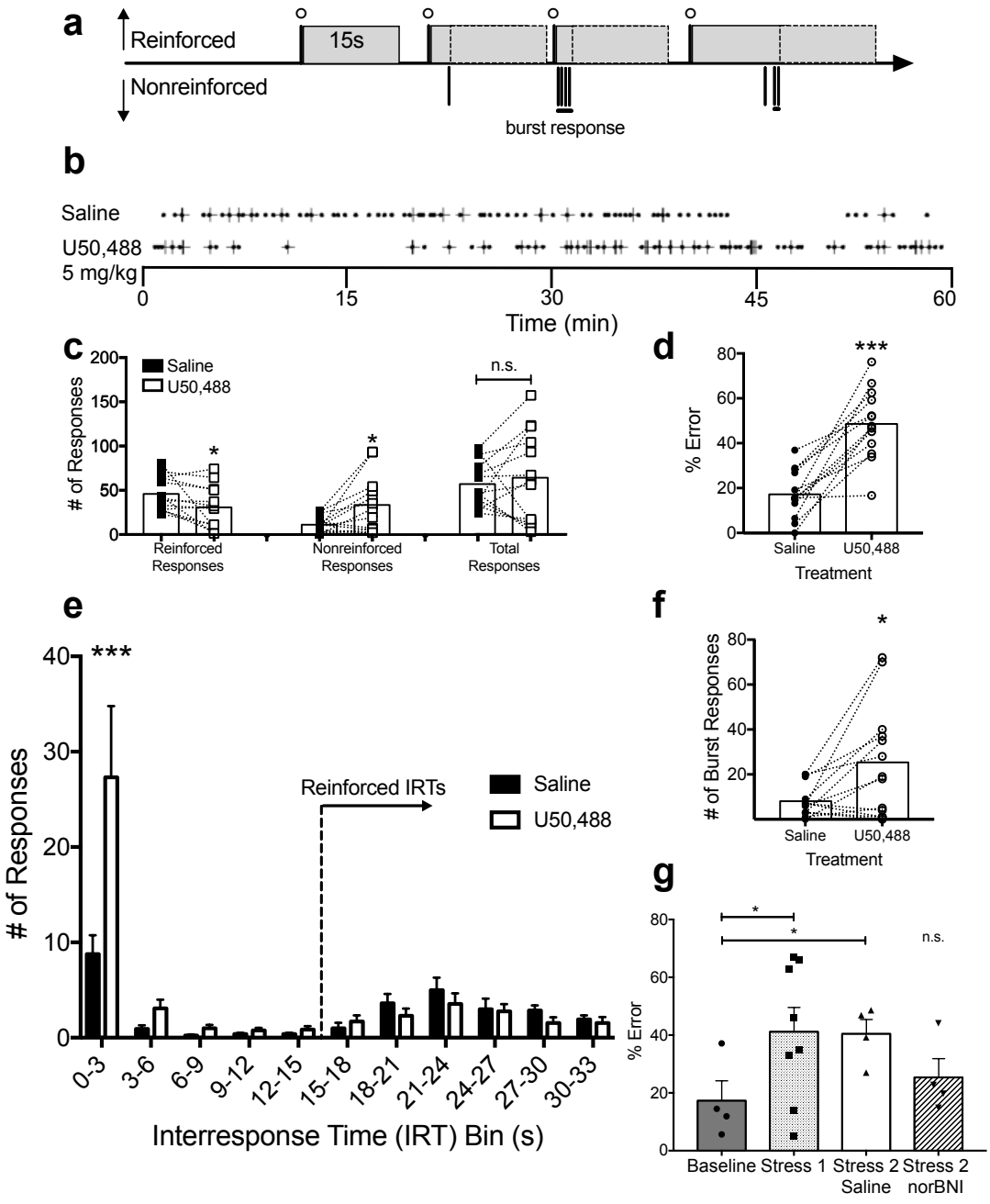

Figure 3.1. In male C57BL/6 mice, systemic KOR activation disrupted DRL performance. (a) Schematic of the DRL task. Reinforced responses (circle above vertical line) are shown as a positive deflection above the center time line and nonreinforced responses are shown as a vertical line below the center time line. Both reinforced and nonreinforced responses reset the 15s DRL wait period (gray box),

leading to longer wait periods (dashed box) if animals responded before the end of the wait period. Burst responses (horizontal line) were nonreinforced responses that occurred within 1s of the previous response. (b) To illustrate a typical data set, responses from a single male animal are shown during 60-min DRL sessions with both saline or U50,488 pretreatment. Closed circles represent reinforced responses and + symbols represent nonreinforced responses.(c) KOR activation by U50,488 decreased total number of reinforced responses and increased nonreinforced responses without altering total number of responses. The number of responses made by an individual mouse during a 60-min period following saline pretreatment (□) were compared to responses following U50,488 pretreatment (☐) by a paired t-test (*p<0.05) (d) KOR activation significantly increased percent error during the DRL session. (e) A histogram of interresponse times showing the number of responses per 3s bin. U50,488 increased responses occurring within 0-3s of the previous response. (f) KOR activation significantly increased the number of burst responses (an additional response <1s after the previous response). (g) Repeated forced swim stress increased percent error. Pretreatment with norBNI (KOR antagonist), but not saline, blocked increases in percent error following a second exposure to repeated forced swim stress compared to baseline. Error bars indicate S.E.M. *p < 0.05; ***p < 0.0001; n.s., not significant.

Figure 3.2.

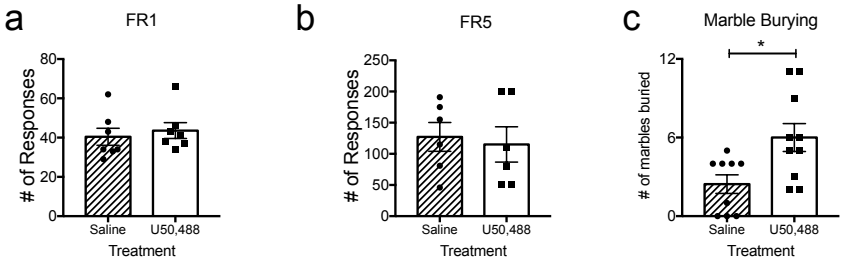

Figure 3.2. Systemic KOR activation increases marble burying. U50,488 pretreatment did not affect the total number of responses during an (a) FR1 or (b) FR5 session compared to saline pretreatment. (c) There was a significant increase in marble burying following U50,488 treatment. Error bars indicate S.E.M. *$p < 0.05$

Figure 3.3.

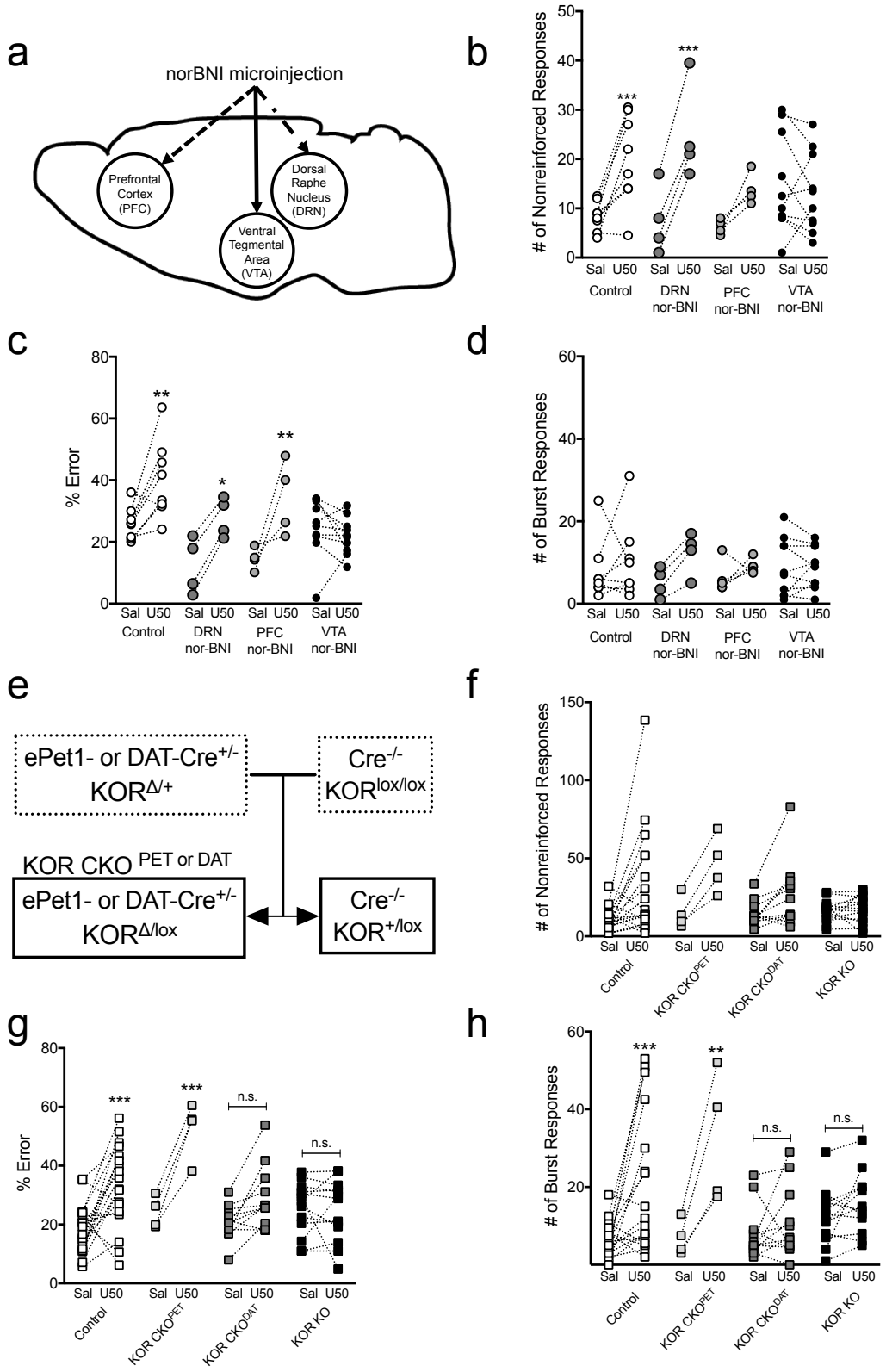
a
norBNI microinjection
Prefrontal Cortex (PFC)
Dorsal Raphe Nucleus (DRN)
Ventral Tegmental Area (VTA)
b
# of Nonreinforced Responses
Sal U50
Control
DRN nor-BNI
PFC nor-BNI
VTA nor-BNI
c
% Error
d
# of Burst Responses
e
ePet1- or DAT-Cre+/- KORΔ/+
Cre-/- KORlox/lox
KOR CKO PET or DAT
ePet1- or DAT-Cre+/- KORΔ/lox
Cre-/- KOR+/lox
f
# of Nonreinforced Responses
Control
KOR CKOPET
KOR CKODAT
KOR KO
g
% Error
n.s.
h
# of Burst Responses

Figure 3.3. KORs in the VTA and in dopamine neurons are required for KOR-mediated DRL disruptions. (a) Schematic shows microinjection sites for norBNI, a long lasting KOR antagonist. Mice received norBNI pretreatment five days prior to DRL test sessions. (b) There was a significant increase in the number of nonreinforced responses in Control and DRN/norBNI, but not VTA norBNI or PFC/norBNI following U50,488 treatment. (c) U50,488 caused a significant increase in percent error in Control, DRN/norBNI, and PFC/norBNI mice, but not VTA/norBNI injected mice. (d) There was a significant effect of U50,488 treatment on the number of burst responses, but no significant interaction between treatment and brain region. (e) Schematic shows breeding strategy for generating conditional knockout mice. Dashed boxes indicate parental mice. Parental mice were heterozygous for Cre-recombinase within serotonergic (ePet1) or dopaminergic (DAT) neurons and were heterozygous for a null KOR allele. Cre-recombinase negative mice with a floxed KOR gene were bred with heterozygous Cre-recombinase mice to produce conditional knockout mice with KOR specifically deleted from serotonergic or dopaminergic neurons or littermate controls. (f) U50,488 treatment significantly increased the number of nonreinforced responses but there was no significant interaction between treatment and genotype. KOR activation significantly increased percent error (g) and burst responses (h) in Control and KOR CKO^{PET} mice, but not KOR CKO^{DAT} or KOR KO mice. Error bars indicate S.E.M. *$p < 0.05$; **$p<0.01$, ***$p < 0.0001$.

Figure 3.4.

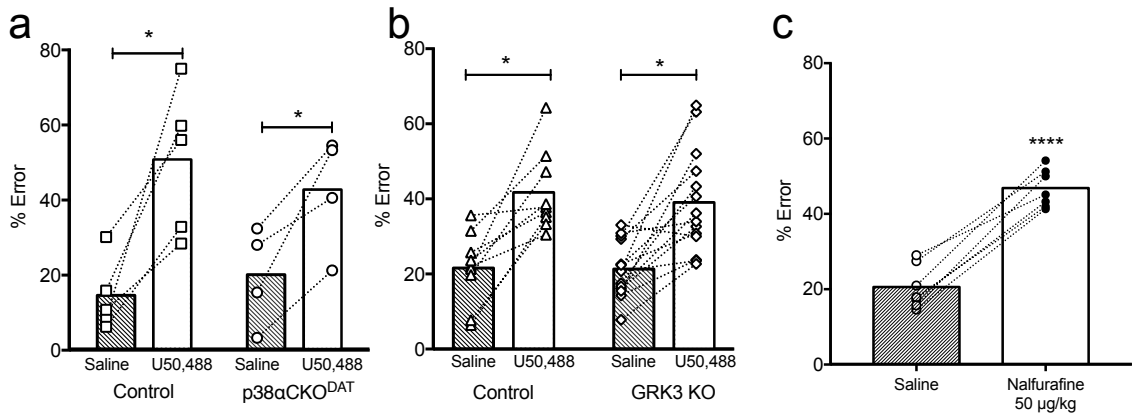

Figure 3.4. KOR-mediated DRL disruptions are arrestin-independent. (a) Conditional deletion of the p38α MAPK in dopamine neurons does not prevent KOR-mediated increases in percent error in the DRL task. (b) Global deletion of the G-Protein Receptor Kinase 3 (GRK3) does not prevent KOR-mediated increases in percent error in the DRL task. (c) The G-biased KOR agonist nalfurafine significantly increases percent error in the DRL task, indicating that DRL disruptions are arrestin-independent. Error bars indicate S.E.M. *$p < 0.05$.

Chapter 4. Estrogen regulation of GRK2 inactivates kappa opioid receptor signaling mediating analgesia, but not aversion

This section is adapted from the following manuscript for use in this dissertation:

Abraham, A.D., Schattauer, S.S., Reichard, K.L., Cohen, J.H., **Fontaine, H.M**., Song, A.J., Johnson, S.D., Land, B.B, Chavkin, C (2018) Estrogen regulation of GRK2 in female mice inactivates kappa opioid receptor signaling mediating analgesia, but not aversion. *Journal of Neuroscience*, 38:8031-8043.

I conducted the conditioned place aversion assays and assisted with the preparation of the manuscript. I contributed to the data and analysis that appears in the following figures

Abstract

Activation of kappa opioid receptors (KOR) produces analgesia and aversion via distinct intracellular signaling pathways, but whether G protein-biased KOR agonists can be designed to have clinical utility will depend on a better understanding of the signaling mechanisms involved. We found that KOR activation produced conditioned place aversion and potentiated conditioned place preference for cocaine in male and female C57BL/6N mice. Consistent with this, males and females both showed arrestin-mediated increases in phospho-p38 MAPK following KOR activation. Unlike in males however, KOR activation had inconsistent analgesic effects in females, and KOR increased GB/Γ -mediated ERK phosphorylation in males, but not females. KOR desensitization was not responsible for the lack of response in females because neither *Grk3* or *Pdyn* gene knockout enhanced analgesia. Instead, responsiveness was estrous cycle-dependent, as KOR analgesia was evident during low estrogen phases of the cycle and in ovariectomized (OVX) females. Estradiol treatment of OVX females suppressed KOR-mediated analgesia, demonstrating that estradiol was sufficient to blunt Gßγ -mediated KOR signals. G protein-coupled receptor kinase 2 (GRK2) is known to regulate ERK activation, and we found that the inhibitory, phosphorylated form of GRK2 was significantly higher in intact females. GRK2/3 inhibition by CMPD101 increased KOR stimulation of phospho-ERK in females, decreased sex differences in KOR-mediated inhibition of dopamine release and enhanced mu opioid receptor and KOR-mediated analgesia in females. In OVX females, estradiol increased the association between GRK2 and Gßγ. These studies suggest that estradiol, through increased phosphorylation of GRK2 and possible sequestration of Gßγ by GRK2, blunts G protein-mediated signals.

Introduction

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

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

Here, we report that KOR activation produced conditioned place aversion and potentiated cocaine conditioned place preference in both male and female C57BL/6N mice, but analgesic effects of KOR activation were attenuated in female mice. The analgesic effects of KOR activation were estradiol-sensitive in female mice. We then demonstrated that activation of the estradiol-sensitive G protein-coupled receptor kinase 2 (GRK2) in females increased sequestration of Gßγ signaling and reduced effects of KOR activation on intracellular signaling, inhibition of dopamine release, and agonist-mediated analgesia. Together, these studies identify an estradiol-mediated intracellular signaling mechanism leading to sexually dimorphic responses that may generalize to other G protein-coupled receptors (GPCRs) acting through Gi/o protein signaling.

Materials & Methods:

Subjects

Male and female C57BL/6N (B6) mice (n = 674) ranging from 8-16 weeks of age were used in these experiments. Breeding stocks were maintained in the UW Vivarium and some additional wildtype animals were obtained from Charles River. All experimental procedures were approved by the University of Washington Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) "Principles of Laboratory Animal Care" (NIH Publication No. 86-23, revised 1985). All testing was during the light phase of the 12-h light/dark cycle. Prodynorphin (Sharifi et al., 2001) or G-Protein Receptor Kinase 3 (Peppel et al., 1997) heterozygotes were bred to generate GRK3 (*Grk3*$^{-/-}$) or PDYN (*Pdyn*$^{-/-}$) knockout mice and wild type littermates. Mice were genotyped as described previously (Terman et al., 2004; McLaughlin et al., 2004). GRK3 or dynorphin knockout mice were phenotypically indistinguishable from wild type littermates in general locomotor behavior, weight, and lifespan. For estrous cycle determination, vaginal lavage was administered following behavioral testing and cells were placed on glass slides for cytology assessments (McLean et al., 2012).

Drugs

U50,488 (2.5 or 10 mg/kg), norbinaltorphimine (norBNI; 10 mg/kg), morphine (3 mg/kg), cocaine (7.5 or 15 mg/kg), and 5'GNTI (0.03 mg/kg) were provided by the National Institute of Drug Abuse Drug Supply

Program (Bethesda, MD) and were dissolved in saline for intraperitoneal (i.p.) administration in a volume of 10 mL/kg. U69,593 was purchased from Santa Cruz Biotechnology. Nalfurafine (50 ug/kg; NIDA Drug Supply) was dissolved in saline and administered subcutaneously (s.c.) in a volume of 10 mL/kg. GRK2/3 inhibitor CMPD101 (Tocris Bioscience) was dissolved in 10% ethanol/10% Cremaphor EL (Sigma-Aldrich)/80% saline (15 mg/kg) and administered i.p in a volume of 10 mL/kg. Estradiol (50 ug/kg; Cayman Chemical Co.) was dissolved in 0.1 % ethanol/0.1% Cremaphor EL/99% saline and administered i.p. in a volume of 10 mL/kg.

Procedures

Conditioned Place Aversion/Preference

Mice were tested for aversion to U50,488, a KOR selective agonist (Von Voigtlander and Lewis, 1982) in a two-chamber apparatus with distinct visual and tactile cues as previously described (Ehrich et al., 2015). All conditioning and testing sessions lasted 30-min and were recorded on video for analysis in Ethovision v3.0 (Noldus; Wageningen, Netherlands). On day 1 (pre-test), mice freely explored each side of the apparatus. Total time on each side was calculated, and mice were then conditioned with U50,488 (2.5 mg/kg) paired with their preferred side on subsequent days. On days 2 and 3 (conditioning), mice were confined to one side with saline treatment and >4 h later, confined to the other side following U50,488 administration. On day 4, mice were allowed to freely explore each side of the apparatus and time spent on the drug-paired floor during the test was measured. To test cocaine conditioned place preference, a three-chamber apparatus was used (McLaughlin et al., 2003). Mice were given a pre-test on day 1, then cocaine was paired with the less preferred side during conditioning on days 2 and 3. For repeated forced swim stress, mice were given a 15-min swim stress (30°C) following the pre-test session and four 6-min swims (separated by 6 min each) <10 min prior to the first cocaine conditioning session, as previously described (McLaughlin et al., 2003). During day 4 (post-test), mice were allowed to freely explore the apparatus. Preference score was determined by subtracting time on the drug-paired compartment during post-test from time on the drug-paired compartment during pre-test (Post-Pre).

Warm Water Tail Withdrawal Test

Mice were tested for latency (s) to withdraw tail (flick) from 52.5°C water to assess anti-nociceptive effects of KOR activation (Bruchas et al., 2007a). Mice were tested for basal latency to flick and treated with

either U50,488 (10 mg/kg), morphine (3 mg/kg), or saline and re-tested for latency to flick 30 min later. Doses for U50,488 analgesia were selected based on Schattauer et al. (2017). In males, 1.0-7.5 mg/kg U50,488 do not significantly change tail flick latency. Change in latency (s) to flick from the pre-test to the post-test was calculated. For experiments with the selective GRK2/3 inhibitor (CMPD101) (Thal et al., 2011), mice were tested for basal latency, treated with CMPD101 (1.5 mg/kg), tested 30 min later, then treated with U50,488 (10 mg/kg) or morphine (3 mg/kg) and re-tested 30 min later. Pregnant mice were tested with U50,488 prior to placement with a male, then re-tested with U50,488 at 6 d and 13 d following co-habitation. Investigators were blind to treatment groups during testing.

Pruritis

Antipruritic activity of nalfurafine was assayed as previously described (Schattauer et al., 2017). Mice were placed individually in observation boxes with a metal grid floor and were given 1 h to acclimate. Mice received either saline or nalfurafine (50 μg/kg; i.p.). Twenty min later, mice were injected subcutaneously on the midline of the back of the neck with saline or 5'-GNTI (0.03 mg/kg) and behavior was recorded on video for 15 min. The number of hind leg scratches directed to the back of the neck was counted. Data analyses were done by an investigator blind to sex and drug treatment.

Experimental Design and Statistical Analysis

Animal numbers are as follows: Conditioned place aversion (n= 13-16 per group); Conditioned place preference (n=7-19 per group); Tail flick (n = 3-6 per group for males; n = 7-19 per group for females); Time course for tail flick (n =6); Hot plate (n =6-9 per group); Pruritis (n = 10-15 per group); Dynorphin/GRK3 knockout tail flick (n = 5-6 per group);; CMPD101/U50,488 tail flick (n = 13); CMPD101/Grk3$^{-/-}$ (n = 9-14 per group); CMPD101/morphine tail flick (n = 8 per group). Data expressed as mean ± SEM were analyzed with Prism 7 (GraphPad; La Jolla, CA). For EC_{50}, 95% confidence intervals (CI) are reported. Group differences were determined using t-tests, ANOVA, or repeated measures ANOVA as described in the results. Post hoc comparisons were analyzed with Dunnett's or Sidak's test. For all statistical tests, α was set to 0.05.

Results:

KOR activation in females produces aversion, but not analgesia

In male B6 mice, KOR activation stimulates Gαi/Gßγ signaling to produce analgesia and decrease itch, whereas arrestin-dependent signaling produces aversion and increases drug-seeking behaviors (Schattauer et al., 2017; Ehrich et al., 2015). We first tested for sex-differences in arrestin-mediated behaviors following KOR activation. U50,488, an unbiased KOR agonist (Von Voigtlander and Lewis, 1982; Schattauer et al., 2012), produced conditioned place aversion in male and female B6 mice (2.5 mg/kg; Figure 4.1*A*). There was a main effect of drug ($F(1,52) = 12.32$, $p = 0.009$), and no main effect of sex or interaction between sex and drug. Planned comparisons between the saline and U50,488-treated mice showed a significant effect of U50,488 in males ($p = 0.027$) and females ($p = 0.02$). As previously reported, females were more sensitive to the conditioned place preference (CPP) effects of cocaine than males (Russo et al., 2003); significant cocaine CPP was evident in female mice at 7.5 mg/kg, whereas 15 mg/kg was required for males (Figure 4.1*B*). This was confirmed with a one-sample t-test, showing that 7.5 mg/kg cocaine in unstressed males was not significantly different from a preference score of 0, whereas unstressed females (7.5 or 15 mg/kg; $p<0.0001$) and unstressed males (15 mg/kg; $p = 0.009$) were significantly different from 0. Repeated forced swim stress (rFSS) has been shown to produce a KOR-dependent increase in cocaine-seeking behaviors (McLaughlin et al., 2003) and these effects are GRK3/arrestin-dependent (Schindler et al., 2012). Stress potentiated cocaine CPP in both males and females (Figure 4.1*B*). A 3 way ANOVA showed that there was no main effect of sex, but there was a main effect of cocaine dose ($F(1,115) = 79.9$, $p < 0.001$) and stress ($F(1,115) = 7.14$, $p = 0.009$). There was also a significant interaction between sex and dose ($F(1,115) = 33.9$, $p < 0.001$), with both males ($p < 0.001$) and females ($p = 0.011$) showing significant differences in preference scores between 15 mg/kg and 7.5 mg/kg of cocaine. These conditioned place aversion and stress-potentiated cocaine CPP studies indicated that arrestin-mediated behavioral effects of KOR activation were present in both males and females.

We then tested Gßγ-mediated behaviors and found that KOR activation produced significant antinociceptive responses in the tail flick assay in males, but not females. In the tail flick assay (Figure 4.1*C*), there was a significant interaction between sex and drug treatment ($F(1,43) = 4.71$, $p = 0.036$). Post hoc analyses showed that males treated with KOR agonist had a significant increase in latency to

flick compared to saline-treated males (p = 0.008) and were significantly different from females treated with KOR agonist (p = 0.049). Repeated testing of tail flick analgesia in females at 10 min intervals for 60 min following U50,488 administration did not reveal any significant effect of drug treatment (Figure 4.1*D*). Higher doses of U50,488 were not tested in females, as doses of up to 30 mg/kg do not produce significant analgesia in female C57BL/6 mice (Mogil et al., 2003). To interrogate alternative antinociceptive circuits, we next used the hot plate assay and confirmed that KOR-mediated analgesia was also absent in female mice in this assay of analgesia (Figure 4.1*E*). There was a significant interaction between drug treatment and sex ($F(1,26) = 12.2$, $p = 0.002$) on hot plate response latency. Males treated with U50,488 (10 mg/kg) had significantly different response latencies from saline-treated males ($p = 0.0002$) and females treated with U50,488 ($p = 0.001$) in the hot plate assay.

To determine whether females showed antipruritic (anti-itch) effects of KOR activation, another hypothesized G protein-mediated behavior (Schattauer et al., 2017), we tested the G-biased KOR agonist nalfurafine (50 ug/kg) in males and females following application of 5'-GNTI, a KOR antagonist that induces compulsive scratching behaviors (Inan et al., 2011). We found that 5'-GNTI produced equivalent baseline levels of scratching behaviors in males and females, and that significant antipruritic effects of nalfurafine were observed in both male and female mice (Figure 4.1*F*). There was a significant interaction between sex and nalfurafine treatment ($F(1,46) = 4.05$, $p = 0.05$). Post hoc analyses showed that nalfurafine significantly decreased scratching in males ($p < 0.0001$) and females ($p = 0.0005$) compared to vehicle controls. These studies demonstrate that KOR produces arrestin-dependent behavioral effects in male and female B6 mice, but some G protein-mediated effects are differentially regulated in males and females.

Pharmacological inhibition of GRK2/3 enhances opioid-mediated analgesia in females

Our biochemical and voltammetric data showed that GRK2/3 inhibition with CMPD101 enables Gßγ-mediated KOR intracellular and neural circuit level signals in females, suggesting that KOR-mediated analgesia may also be regulated by estrogen-mediated phosphorylation of GRK2. We tested whether

GRK2/3 blockade would unmask KOR analgesia in the warm water tail flick assay and found that CMPD101 pretreatment did not significantly alter baseline analgesic response. However, KOR activation in CMPD101 pretreated females produced a significant increase in tail flick latency ($F(1,12.5) = 31.7$, $p < 0.0001$; post-hoc: $p = 0.0003$). To assess the specificity of CMPD101, which inhibits both GRK2 and GRK3, we determined the necessity of GRK3 for the effects of CMPD101 on KOR-mediated analgesia. There was a significant difference between groups ($F(2,45) = 6.31$, $p = 0.004$). U50,488 in GRK3 knockouts and littermate controls did not produce a significant analgesic response, but U50,488 did increase tail withdrawal latency in female GRK3 knockout mice pretreated with CMPD101. The response in the presence of CMPD101 was significantly different from wildtype controls ($p = 0.013$) and GRK3 knockout mice ($p = 0.007$).

Based on our observations with KOR responses in females, we predicted that analgesic responses to mu opioid receptor (MOR) activation with morphine may also be attenuated by GRK2 activity in females. There was a significant interaction between drug treatment and sex ($F(1,27) = 5.21$, $p = 0.031$), and post-hoc analyses showed that females pretreated with saline showed a significantly different response to morphine compared to females pre-treated with CMPD101 ($p = 0.022$). Together, we observed that blockade of GRK2 enhances KOR- and MOR-mediated analgesic responses in females. These studies demonstrate that estradiol stimulates GRK2 phosphorylation to increase Gβ/γ sequestration and block G protein-mediated biochemical, circuit, and behavioral effects of opioid receptor activation (Schematic, Figure 4.2*D*).

Discussion

These studies demonstrate that females show blunted intracellular, neural circuit, and behavioral responses to the G protein-mediated actions of KOR agonists. In particular, elevated estradiol was found to underlie the reduction in KOR function in females. Furthermore, we specifically identified estradiol upregulation of GRK2 activity to mediate the observed sex differences in opioid analgesic responses. GRK2-dependent blunting of the analgesic effects of MOR agonist-induced analgesia was also observed in females, suggesting that estradiol-stimulated GRK2 may regulate other GPCR systems.

Sex differences in KOR function

Whereas the G protein-mediated analgesic effects were subject to estradiol regulation, dysphoric effects of KOR activation were observed in both male and female mice. Although there are cycle-independent effects of KOR activation on aspects of reward-seeking behaviors in females (Russell et al., 2014), we did not observe significant sex effects on conditioned place aversion. These aversive effects are mediated by activation of the p38 MAPK (Land et al., 2009; Ehrich et al., 2015, Schindler et al., 2012; Bruchas et al., 2007b; Bruchas et al., 2011) and we found that p38 MAPK phosphorylation was increased in both B6 males and females following KOR activation. In contrast, G protein-mediated molecular or cellular effects, such as ERK1/2 phosphorylation (Belcheva et al., 2005) or dopamine release inhibition (Ehrich et al., 2015), were blunted following KOR activation. The attenuation of G protein-mediated signals through estradiol/GRK2 regulation also decreased analgesic behavioral responses in B6 females following KOR activation. An alternate explanation for sex differences in the analgesic effects of KOR activation is that there are sex-dependent regional differences in neurocircuitry underlying analgesia, however this hypothesis cannot account for the CMPD101-sensitive differences in KOR-mediated ERK1/2 activation or inhibition of dopamine release.

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

agonists in females (Craft, 2003; Fillingim and Gear, 2004; Rasakham and Liu-Chen, 2011), but the mechanisms underlying this variability were previously unknown.

Estradiol blunts KOR-mediated analgesia

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

Estradiol can produce complex effects on mood and behavior through estrogen receptor activation or interactions with G protein-coupled receptor signaling (Martinez et al., 2016; Gillies and MacArthur, 2010). Based on the observation that KOR activation did not produce analgesia or ERK phosphorylation, we hypothesized that an estradiol-regulated signaling system that controls G protein-biased signals was likely to be a mechanistic target for sexually dimorphic responses. G protein-coupled receptor kinase 2 (GRK2) is phosphorylated following estradiol treatment, likely through estrogen receptor α (Dominguez et al., 2009), sequesters $G_{\beta/\gamma}$ subunits (Lodowski et al., 2003), and decreases ERK activation (Pitcher et al., 1999). GRK2 activity also desensitizes KOR activity in cardiac tissue (Chen et al., 2017), suggesting that estradiol modulation of GRK2 activity could underlie sex differences in KOR activity. Our results showed that in females, GRK2 phosphorylation is increased, and there was no change in GRK2 mRNA levels. We also observed increased association of GRK2 with $G_{\beta/\gamma}$ subunits following estradiol treatment in

ovariectomized females, indicating that estradiol increased GRK2 sequestration of $G_{\beta/\gamma}$ to blunt G protein-mediated signals.

GRK2 regulates opioid analgesia in females

We found that pharmacological inhibition of GRK2 with compound 101 (CMPD101) attenuated sex differences following KOR activation. CMPD101 is a selective inhibitor for members of the GRK2 subfamily (GRK2 and GRK3), with little activity at GRK1 or GRK5 (Thal et al., 2011). To ensure that the effects that we observed were selective to GRK2, we tested the effect of CMPD101 in female $Grk3^{-/-}$ mice and found that the CMPD101-mediated increase in KOR analgesia was not GRK3 dependent. Genetic deletion of GRK2 in mice causes death during embryonic development (Jaber et al., 1996), preventing an assessment of CMPD101 in GRK2 knockout mice. In addition to sequestration of $G_{\beta/\gamma}$, GRK2 can also interact with mitogen-activated protein kinase kinase (MEK) to inhibit phosphorylation of ERK, suggesting an additional level of regulation over GPCR signaling that may contribute to our observed GRK2-mediated effects. GRK2 has also been shown to desensitize melanocortin-1 receptor (Sánchez-Más et al., 2005), which may contribute to sex-dependent analgesic effects of KOR agonists (Mogil et al., 2003). Together, these studies showed that GRK2 inhibition is sufficient to decrease KOR-mediated sex differences at a biochemical, neural circuit, and behavioral level in mice.

Whereas G protein-mediated analgesic effects were decreased, anti-pruritic effects of KOR activation remained present in females. Although nalfurafine is a G-biased KOR compound (Schattauer et al., 2017), our results suggest that not all G protein-mediated effects may be subject to GRK2 regulation. For example, KOR activation can also disrupt cognition through G protein-mediated actions, but cognitive disruptions following KOR activation are not sexually dimorphic (Abraham et al., 2018). These findings suggest that there are some KOR/G protein-mediated behaviors that may not be regulated by GRK2 or may not require $G_{\beta/\gamma}$ activity. GRK2 interacts with $G_{\beta1}$ and $G_{\beta2,}$ but not $G_{\beta3}$ (Belcheva et al., 1998), indicating that there may be differential regulation of G_{β}-mediated behaviors depending on the G_{β} subunits or cell populations required for behavioral effects. Additionally, anti-pruritic and cognitive effects may be regulated by inhibitory interactions between $G\alpha_i$ and downstream effectors such as Ras, c-Jun N-

terminal kinase (JNK) or protein kinase A (Edamatsu et al., 1998; Dessauer et al., 2002; Pierre et al., 2009). Munanairi et al. (2018) reported that KOR-mediated decreases in itch are sex-independent, and may not require G-protein activation, instead using phospholipase C, supporting our observation of the anti-pruritic effects of KOR activation in males and females. The reported actions of KOR on diuresis (Leander 1983; Craft et al., 2000) and locomotor activity (Robles et al., 2014) are also not sex-dependent, suggesting that these behaviors are likely regulated by cellular and molecular mechanisms that are distinct from KOR-mediated analgesia. Additionally, KOR antagonists have also been reported to differ in efficacy between males and females (Laman-Maharg et al., 2018). To increase the efficacy and specificity of KOR agonists and antagonists, future studies could aim to dissociate the molecular signals or cellular populations producing analgesia from those producing diuretic, antipruritic, sedative, and cognitive effects of KOR activation.

Similar to the analgesic effects observed with KOR, blockade of GRK2 also promoted analgesic responses to morphine in females, a MOR-selective opiate (Goldstein and Naidu, 1989). GRK2 overexpression with MOR in cell culture increases activation of arrestin-dependent signals (Zhang et al., 1998), suggesting that GRK2 decreases MOR G protein-mediated signals, similar to GRK2-KOR interactions. Sex differences in morphine analgesia at low doses have been hypothesized to occur through regulation of G protein-coupled inwardly rectifying potassium channels (GIRKs; Mitrovic et al., 2003), which are also known to directly bind $G_{\beta/\gamma}$ for channel activation (Huang et al., 1995). Our studies demonstrated that sex differences in opioid receptor activity were the result of GRK2 activity and suggests that multiple inhibitory GPCRs could be subject to a similar sex-dependent regulation. Future studies could assess the role of GRK2 in decreasing Gαi protein-mediated behaviors in other GPCR systems.

Together, our studies demonstrate that KOR activation produces distinct analgesic effects in males and females in an estradiol- and GRK2-regulated manner. G protein-biased KOR analgesic compounds that are in clinical development may have sex-dependent effects, and careful consideration of estradiol interactions with opioid receptor actions is required for the implementation of biased KOR agonists in

clinical populations. Further understanding of the mechanisms underlying sexually dimorphic effects of opioid receptor activation could guide the development of highly efficacious non-addictive opioid drugs for analgesia in males and females.

Figure 4.1

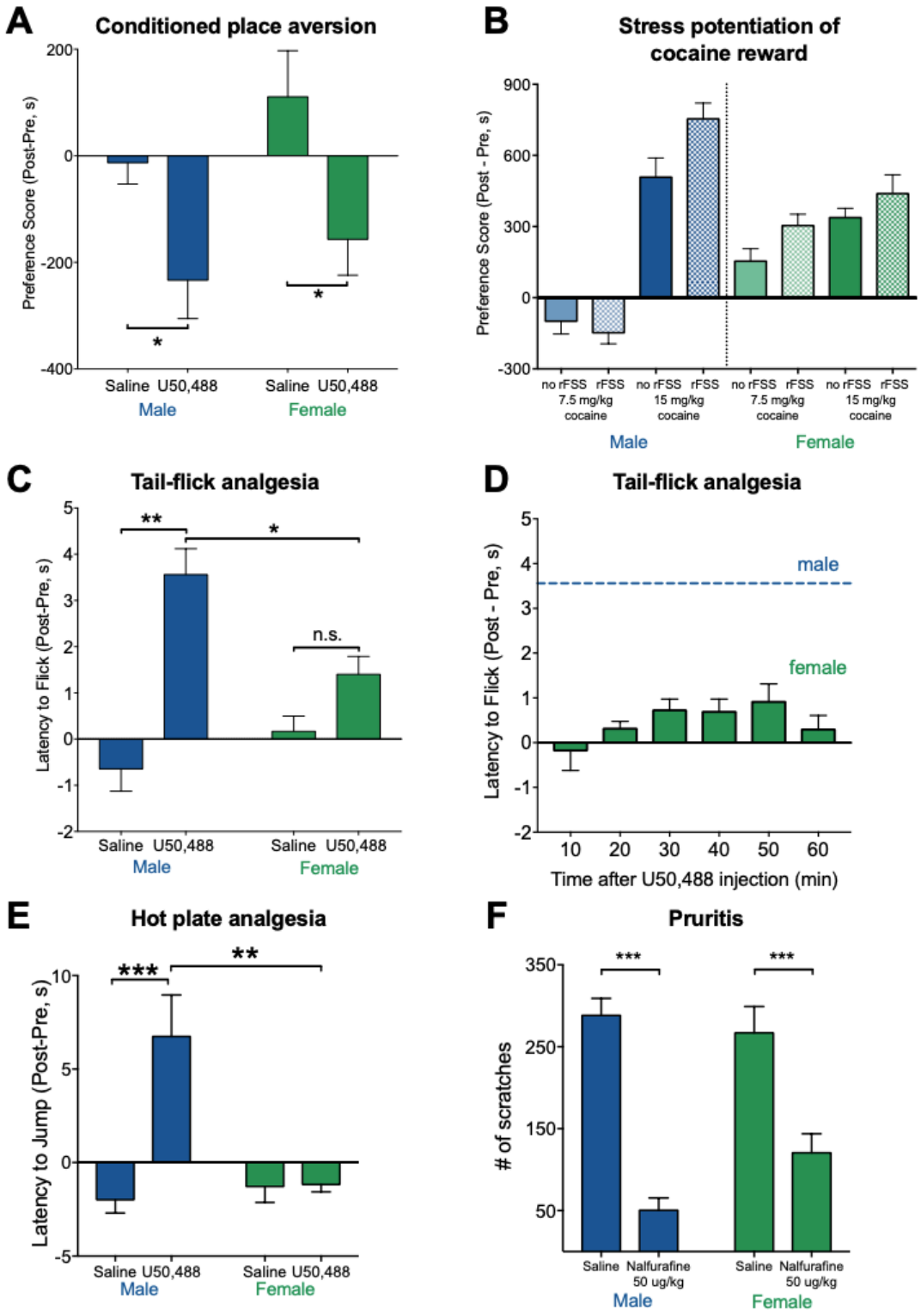
A
Conditioned place aversion
Preference Score (Post-Pre, s)
200
0
-200
-400
*
*
Saline U50,488
Male
Saline U50,488
Female
B
Stress potentiation of cocaine reward
Preference Score (Post - Pre, s)
900
600
300
0
-300
no rFSS
rFSS
7.5 mg/kg cocaine
no rFSS
rFSS
15 mg/kg cocaine
Male
Female
C
Tail-flick analgesia
Latency to Flick (Post-Pre, s)
**
*
n.s.
Saline U50,488
Male
Saline U50,488
Female
D
Tail-flick analgesia
Latency to Flick (Post - Pre, s)
male
female
10
20
30
40
50
60
Time after U50,488 injection (min)
E
Hot plate analgesia
Latency to Jump (Post-Pre, s)
***
**
Saline U50,488
Male
Saline U50,488
Female
F
Pruritis
# of scratches
350
250
150
50
***
***
Saline
Nalfurafine 50 ug/kg
Male
Saline
Nalfurafine 50 ug/kg
Female

Figure 4.1. KOR activation in females produces aversion, but not analgesia. (*A*) Conditioned place aversion to U50,488 (2.5 mg/kg) was observed in both males and females (n = 13-16 per group) compared to saline-treated mice (males $p<0.05$; females $p<0.05$). (*B*) Consistent with prior reports (Russo et al., 2003), female mice were more sensitive than males to cocaine conditioning. Repeated forced swim stress (rFSS; cross-hatched bars) increased cocaine preference (n = 7-19 per group) in males conditioned with 15 mg/kg cocaine and females with 7.5 mg/kg cocaine. There was no significant effect of sex on cocaine CPP potentiation, but there was a significant main effect of stress, and a significant interaction between sex and dose. (*C*) U50,488, but not saline, increased latency to tail withdrawal in males (n = 3 saline, n = 6 U50,488; $p<0.01$) in a warm water tail-flick assay, but U50,488 treatment of females produced a small increase latency that was not significantly different from saline treated mice (n = 11 saline, n = 27 U50,488). Latency to flick was significantly different between males and females treated with U50,488 ($p<0.05$). (*D*). To assess whether the lack of effect at 30 min post injection was due to a difference in the pharmacokinetics of U50,488, female mice were tested every 10 min and showed no significant effect at any time during the 60 min (n = 6). (*E*) Using the hot plate to interrogate different analgesic circuits, males treated with U50,488 (n = 6-9 per group) showed a significant ($p<0.001$) increase in latency to jump/lick in the hot plate assay compared to saline treatment and were significantly different ($p<0.001$) from U50,488 treated females. (*F*) Following application of 5'GNTI, a compound that induces compulsive scratching (Inan et al., 2011), males and females (n = 13; n = 12) treated with nalfurafine (50 µg/kg), a KOR agonist, had significantly decreased ($p<0.0001$; $p<0.001$) scratching behaviors compared to saline-treated males or females (n=15; n = 10). Error bars indicate SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$, n.s., not significant.

Figure 4.2

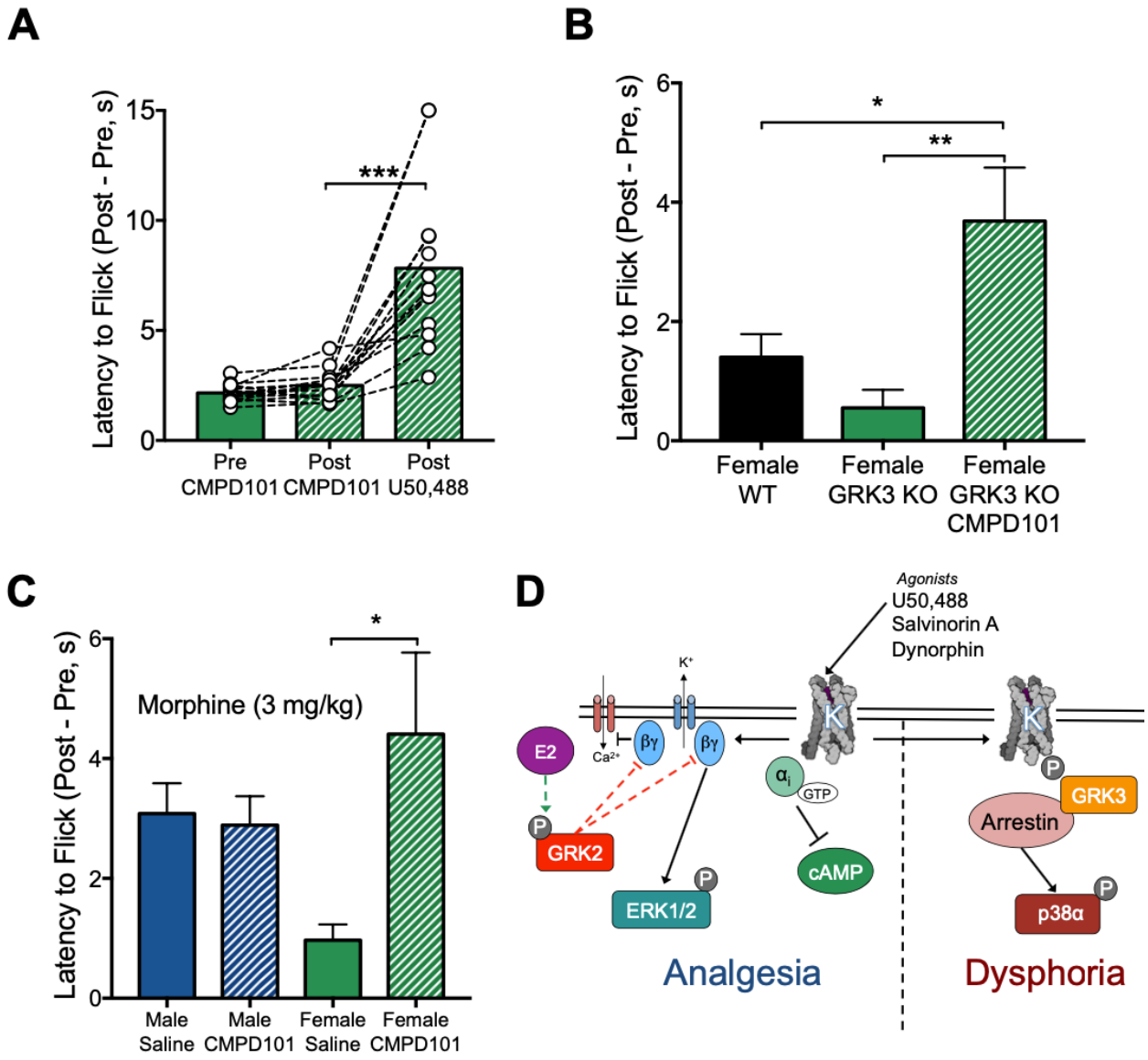

Figure 4.2. Pharmacological inhibition of GRK2/3 enhances opioid-mediated analgesia in females. (*A*) Pre-treatment with CMPD101 did not alter baseline tail flick latency in females (n = 13). CMPD101 pre-treatment significantly increased latency to flick with U50,488 treatment in females ($p<0.001$). (*B*) Female WT data is replotted from Figure 1C. Female $Grk3^{-/-}$ mice (n = 9-14 per group) pre-treated with CMPD101 showed a significant increase in latency to flick compared to WT females ($p<0.05$) or $Grk3^{-/-}$ ($p<0.01$) pretreated with saline. (*C*) A submaximal dose (3 mg/kg) of morphine produces analgesia in males (n = 7-8 per group), with no effect of CMPD101, but the analgesic effect of morphine is blunted in

females (n = 8 per group) compared to females pre-treated with CMPD101 ($p<0.05$). (*D*) In the proposed model, elevated estradiol (E2) in female mice results in GRK2 phosphorylation and enhanced sequestration of the Gβγ subunits. This results in reduced Gβγ signaling, including a reduction in analgesic responses. In contrast, GRK3/arrestin-mediated signaling is left largely intact, maintaining the aversive response. Error bars indicate SEM. *$p<0.05$; **$p<0.01$, ***$p<0.001$.

Chapter 5. Conclusion

Primary findings

There are several primary findings of the work reported in this dissertation. First, I detailed the serotonergic contribution to stress-induced potentiation subsequent cocaine preference. This study established a Dyn-KOR-5HT-5HT1B axis within the NAc that regulates response to rewarding and aversive stimuli. Further, I showed that decreases in NAc serotonin tone are critical to KOR-induced potentiation of cocaine preference, potentially by allostatic mechanisms involving postsynaptic 5-HT_{1B} receptors. Next, colleagues and I detailed effects of stress and KOR activation on cognitive disruptions, indicating that KOR activation of p38α -depdendent pathways in VTA dopamine neurons mediates this effect. Lastly, colleagues and I established sex differences in KOR signaling, wherein estrogen attenuates the G-protein dependent aspect of KOR signaling in females. As I was the primary contributor to work described in the first chapter, I will subsequently focus on the implications of that work below.

Implications for allostasis, Dyn/KOR system, and serotonin system

Nucleus accumbens dynorphin was necessary for regulation of basal cocaine preference and stress potentiation of that preference. A combination of genetic, optogenetic, and pharmacological manipulations converged to indicate that dynorphin release from this source induces a decrease in serotonin signaling at NAc 5-HT_{1B} receptors, which is sufficient to potentiate cocaine preference. This increase in cocaine preference was mediated by 5-HT_{1B} receptors, and *ISH* staining for 5-HT_{1B} transcript demonstrated a selective increase in this transcript within NAc^{Pdyn} cells. These findings suggest the presence of a reciprocal, feedforward mechanism within the NAc during stress (NAc^{Pdyn}-KOR-5HT-5HT_{1B}-NAc^{Pdyn}). The gain on this reciprocal circuit may be changed following stress if functional 5-HT_{1B} receptors are increased following stress. As 5-HT_{1B} is a G_i-coupled GPCR and likely decreases neurotransmitter release, an increase in this receptor would be expected to enhance serotonin-mediated inhibition of dynorphin. This may decrease cocaine-induced dynorphin release and increase cocaine preference. This prediction is consistent with the increase in basal preference observed in NAc^{PDyn} cKO mice. Recent studies have shown that stimulation NAc^{D1} (or NAc^{Pdyn}) neurons can have divergent, KOR-mediated effects on reward behavior. These effects depend on which NAc^{Pdyn} subpopulation is manipulated and specific stimulation pattern employed (Al-Hasani et al., 2015; Soares-Cunha et al., 2019). Such factors may be relevant to the cellular and behavioral

consequences of altered 5-HT_{1B} regulation of NAc^{Pdyn}. Lastly, 5-HT_{1B} activation may induce transcriptional changes NAc^{PDyn}. The SNRI desipramine blocks stress-induced activation of prodynorphin in the NAc, and the effect of desipramine in cell culture was replicated by SSRI treatment (Chartoff et al., 2009). This supports the presence of a reciprocal circuit and the potential for 5-HT_{1B} modulation of dynorphin expression. Future studies are needed to directly test these possibilities.

The strong aversion to inhibition of DRN serotonin neurons supports a 'mood' theory of serotonin function. We note that our optogenetic inhibition likely had a disproportionately large effect on neurons with high basal activity. Most serotonin neurons exhibit low intrinsic firing rates, but a small proportion fire more frequently (~4Hz) (Li et al., 2016). The molecular identity and projection targets of this population have not been established but may form a subsystem that is responsible for these behavioral effects. While we showed that sustained (30 min) inhibition of serotonin is aversive, it remains to be seen if more discrete periods of inhibition can also drive negative affect.

Stimulation of serotonin terminals in the NAc during KOR activation attenuated KOR-induced potentiation of cocaine preference. This is consistent with our hypothesis that decreases in NAc serotonin tone drive potentiation of subsequent cocaine preference, but we did not directly measure levels of NAc serotonin. The effects of stress on serotonin tone in the ventral striatum are controversial, with most studies finding an increase during FSS (Kirby et al., 1995, 1997; Lukkes et al., 2008; Price et al., 2002). However, many of these studies measured serotonin levels in the ventrolateral striatum, as opposed to the medial portion that we implicate. Intriguingly, measurements of serotonin tone in the lateral septum, a region adjacent to the mNAc, show decreases during forced swim stress (Kirby et al., 1995; Price et al., 2002). This indicates that more precise measurement of mNAc serotonin tone, such as with photometric techniques, may resolve these apparent discrepancies (Wan et al., 2020).

Implications for therapeutic application

Local infusion of 5-HT_{1B} antagonists into the NAc potentiated subsequent cocaine preference, and blockade of the receptors during cocaine conditioning prevented the expression of this enhanced preference. These data indicate that stress-induced reductions in 5-HT_{1B} signaling alter reward sensitivity. Alternatively, 5-HT_{1B} activation has been shown to affect stress-induced immobility. Although these findings are mixed, 5-HT_{1B} agonists appear to reduce immobility during the FST (Chenu et al., 2008; Ruf & Bhagwagar, 2009).

Additionally, 5-HT_{1B} receptor signaling is dysregulated following abstinence from prolonged cocaine exposure, further implicating stress dysregulation of this receptor system in altered cocaine preference (Pentkowski et al., 2009, 2012). In cell culture models, the surface expression of 5-HT_{1B} is dynamically regulated by extracellular levels of 5-HT and dependent on receptor activation (Carrel et al., 2011) Therefore, pharmacologically stabilizing 5-HT_{1B} receptor signaling levels may prevent stress potentiation of reward sensitivity and cocaine preference.

At the level of the receptor, there are several partial agonists currently in clinical use that may confer this effect, including vortioxetine and zolmitriptan (Fernández et al., 2017; Garcia et al., 2017). Consistent with this suggestion, zolmitriptan administration significantly attenuates cocaine self-administration in rats (Garcia et al., 2020). In a small clinical study, vortioxetine, which is an SSRI + 5-HT_{1A} agonist + 5-HT_{1B} partial agonist and is currently approved for depression in the United States, caused statistically significant reductions in drug taking and craving (Fernández et al., 2017). Future studies are needed to clarify the effects of 5-HT_{1B} partial agonists in the NAc on stress and sensitization to subsequent reward, which may guide therapeutic development.

Proteins which interact with 5-HT_{1B} may also be targeted to stabilize 5-HT_{1B} signaling levels. One such protein is the adapter protein p11. When in complex with annexin II, p11 increases the surface expression of 5-HT_{1B}, 5-HT_4, and other receptors (Chen et al., 2010; Deora et al., 2004). P11 expression is increased in the forebrain of stressed rats and decreased in the brains of depressed human subjects that have died by suicide (Oh et al., 2013; Svenningsson et al., 2006). Additionally, p11 KO mice exhibit depression like behaviors that are not responsive to SSRI treantment (Svenningsson et al., 2006). Lastly, p11 expression within D1-expressing NAc neurons controls expression of cocaine CPP (Arango-Lievano et al., 2014). These findings indicate that p11 may be involved in stress-induced dysregulation of 5-HT_{1B}, affect, and reward sensitivity. No therapeutics currently exist to modulate p11-induced 5-HT_{1B} trafficking. However, this trafficking may be dependent on phosphorylation of annexin II, which may be targeted for future therapeutic intervention (Deora et al., 2004).

Future Directions

Although our data suggest that 5-HT_{1B} expression within NAc^{Pdyn} neurons controls stress-induced potentiation of cocaine preference, further studies are needed to directly test this assertion. First, additional

RNAscope experiments should be conducted to determine if the increase in *Htr1b* is indeed specific to *Pdyn* neurons in the NAc. *Htr1b* expression in NAc^{ChAT} neurons as well as afferents from DRN serotonergic and cortical glutamatergic neurons should also be examined following stress to determine if stress-induced changes in afferents or other NAc populations may be involved involved. Next, RNAscope to *Htr1b* in these NAc subpopulations and afferents following optogenetic inhibition of DRN^{SERT} neurons may further test if these changes are driven by on decreases in 5-HT tone.

To test the cell-type specific necessity of $5\text{-}HT_{1B}$ actions in stress-potentiation of cocaine CPP, we may employ a recently developed, Cre-dependent virus that can knockout $5\text{-}HT_{1B}$ within Cre^{+} cells (Hunker et al., 2020). This virus, which employs clustered regularly interspaced short palindromic repeat (CRISPR) technology, can selectively knockout $5\text{-}HT_{1B}$ from the different cell populations within the NAc (*Pdyn, Adora2a,* and *Chat*) and $5\text{-}HT_{1B}$-expressing afferents. Subsequently, we may use these cKO mice to test whether selective excision of $5\text{-}HT_{1B}$ from NAc^{Pdyn} neurons prevents stress potentiation of cocaine preference.

References

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