Kappa Opioid Regulation of Dopamine in Dysphoria and Addiction

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Abstract

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Stress-induced release of dynorphin and subsequent activation of the kappa opioid receptor (KOR) has been hypothesized to mediate the dysphoric component of stress. Evidence suggests that this dysphoria can alternately block or potentiate the rewarding properties of addictive drugs, while also directly inducing aversion. These effects have historically been described as being mediated by the ability of KOR activation to inhibit dopamine release within the nucleus accumbens. However, a growing body of evidence suggests that this model is overly simplistic.

This dissertation focuses on improving our understanding of how KOR signaling pathways within dopamine neurons can mediate the aversive, anhedonic, and proaddictive properties of stress. We first attempt to understand if changes in dopamine signaling can model bivalent, KOR-induced changes in cocaine reward. These experiments show that a combination of KOR effects on the somatodendritic and terminal compartments of dopamine neurons induce similar shift in the activity of core-projecting dopamine neurons as are seen in the behavior of mice during a conditioned place preference experiment. We then proceed to demonstrate that activation of KOR within dopamine neurons of the VTA is both necessary and sufficient for mediating the aversive properties of KOR activation. Surprisingly, however, we find that it is phosphorylation of the receptor and subsequent activation of p38 MAPK, and not KOR-induced inhibition of dopamine release, that is the key mediator of this behavior.

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To my father, for his never-ending support.

To my mother, for teaching me to always ask questions and never trust the answers.

To the members of Team Hot Mess, you know who you are.

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And to Christine, always Christine.

"As for you, Morrel, this is the secret of my conduct towards you. There is neither happiness nor misery in the world; there is only the comparison of one state with another, nothing more. He who has felt the deepest grief is best able to experience supreme happiness. We must have felt what it is to die, Morrel, before we can appreciate the enjoyments of life.

"Live, then, and be happy, beloved children of my heart, and never forget, that until the day when God deigns to reveal the future to man, all human wisdom is contained in these two words: '*Wait and hope*.'"

Alexandre Dumas, *The Count of Monte Cristo*

Chapter 1

INTRODUCTION

Stress, drug addiction, and depression

Although stress exposure can have reinforcing or motivational aspects (Sinha, 2001), it can also induce negative effects. Many theories of drug addiction propose that in human drug users, exposure to stress can facilitate the transition from recreational drug use to habitual drug abuse (Piazza and Le Moal, 1998; Sinha, 2001; Volkow and Li, 2004). For example, overall stress levels have been shown to positively correlate with alcohol use (DeFrank *et al*, 1987) or escalation of nicotine and alcohol consumption (Wills *et al*, 1996). Acute exposure to stress also seems to facilitate drug use in addicted drug users, being associated with increased drinking behavior in alcoholics (Miller *et al*, 1974) and elevated smoking in smokers (Pomerleau and Pomerleau, 1987). It has been proposed that some percentage of drug users may be pursuing a maladaptive coping strategy which includes self-medicating for depression (Carroll *et al*, 1997; Volkow and Li, 2004) or inability to cope with external stressors (O'Doherty, 1991; Sinha, 2001). This is consistent with the observation that depressive disorders are over-represented in patients seeking treatment for cocaine abuse (Johanson and Fischman, 1989; Volkow and Li, 2004), and that the severity of depression is predictive of future cocaine use (Stulz *et al*, 2011). In abstinent cocaine users, stress exposure induces drug craving and the strength of that craving is predictive of the time to relapse (Sinha *et al*, 2006).

This is consistent with preclinical literature in animal models of drug addiction. Exposure to stressors such as electric footshock (Goeders and Guerin, 1994; Rácz *et al*, 2013), tail-pinch (Piazza *et al*, 1990), and social stress (Haney *et al*, 1995; Tidey and Miczek, 1997) facilitate the acquisition of cocaine or amphetamine self-administration, as well as increasing ethanol consumption and preference for ethanol over water. Further, the locomotor activity

(Piazza *et al*, 1989) and serum corticosterone concentration (Piazza *et al*, 1991) shown in response to exposure to a mild stressor (novel environment) are predictive of the ability to acquire amphetamine self-administration. Exposure to foot shock or repeated forced swim stress can also increase the maximum effort rats are willing to expend for an infusion of heroin or cocaine (Groblewski *et al*, 2014; Shaham and Stewart, 1994). After extinction of cocaine self-administration, forced swim stress can also reinstate drug-seeking behavior (Graziane *et al*, 2013; Polter *et al*, 2014). Stress also reinstates extinguished drug-seeking for cocaine (Lu *et al*, 2002; Redila and Chavkin, 2008; Sanchez *et al*, 2003), heroin (Shaham and Stewart, 1995), or morphine (Lu *et al*, 2000) in the conditioned place preference (CPP) paradigm. It potentiates the rewarding properties of morphine (Will *et al*, 1998) and cocaine (McLaughlin *et al*, 2003a; Schindler *et al*, 2010) in the CPP paradigm as well.

Stressful life events can also lead to depressive reactions (Hammen, 2005). Research from twin studies has shown that environmental stressors and genetic factors each explain a similar amount of variance in depression risk (Tennant, 2002). Two of the most widely used antidepressant-sensitive depression assays are based on stress exposure, the Porsolt forced swim test (Porsolt *et al*, 1977) and the social avoidance assay (Berton *et al*, 2006; Krishnan *et al*, 2007).

Despite the magnitude of research that has been conducted on these topics, the mechanism of interactions underlying the proaddictive and prodepressive properties of stress remains poorly understood. As such, the purpose of the research presented into this dissertation was to further illuminate the neural circuits and molecular signaling events underlying these interactions, with a focus on the role of dynorphin, the kappa opioid receptor (KOR), and their interactions with dopamine signaling. The remainder of this section will introduce the dynorphin / KOR signaling unit, its role in mediating the dysphoric component of stress, signal transduction pathways downstream of the KOR, how stress and aversive stimuli interact with dopamine signaling, manners in which KOR alters the rewarding properties of addictive drugs, and finally interactions between KOR and dopamine signaling.

The dynorphin / kappa opioid receptor signaling unit

The kappa opioid receptor (KOR) is a member of the seven transmembrane G protein-coupled receptor (GPCR) superfamily and the opioid receptor subfamily (Chen *et al*, 1993; Connor and Christie, 1999; Meng *et al*, 1993; Xie *et al*, 1994). In mice, it is encoded by the Oprk1 gene; (Chen *et al*, 1993; HGNC, 2014; Xie *et al*, 1994; Yasuda *et al*, 1993). The receptor was initially named and defined by the actions of the prototypic KOR agonist ketacyclazocine in the chronic spinal dog (Martin *et al*, 1976).

The existence of multiple KOR subtypes has been proposed based on the binding properties of different ligands (Rothman *et al*, 1989). However, only one example of the receptor has been cloned from any one species (Chen *et al*, 1993; HGNC, 2014; Xie *et al*, 1994; Yasuda *et al*, 1993). GPCRs can exist as homodimers when expressed singly, but it has been shown using heterologous receptor expression in cell culture models that KOR can form stable, functional heterodimers with the other opioid receptors (Jordan and Devi, 1999; Milligan, 2004). As would be expected, heterodimers show different ligand binding properties and different signaling properties when compared to homodimers of either opioid receptor type (Jordan & Devi 1999, Rozenfeld and Devi 2007). Therefore, it has been proposed that previously reported opioid receptor subtypes may be heterodimers (Jordan & Devi 1999). However, whether or not these heterodimers can be observed *in vivo* in the absence of heterologous co-expression is still a subject of controversy. As such, discussion of the function and expression of KORs forthwith will be restricted to the presumption that all effects are mediated by KOR-KOR homodimers, or the κ-1 subtype.

Like other opioid receptors, the KOR is coupled to pertussis toxin-sensitive G proteins of the G_i/G_o family (Attali *et al*, 1989a; Konkoy and Childers, 1989). As such, activation of the KOR is generally associated with inhibition of adenylate cyclase, cyclic AMP, and ultimately inhibition of cell excitability and a suppression of transmitter release (Chavkin, 2013; Grudt and

Williams, 1993; Konkoy and Childers, 1989; Meng *et al*, 1993). In human subjects, activation of the receptor by exogenous agonists is associated with diuresis, analgesia, psychotomimesis and dysphoria (Peters *et al*, 1987; Pfeiffer *et al*, 1986; Rimoy *et al*, 1994; Wadenberg, 2003).

The dynorphins are a class of neuropeptides arising from the precursor protein prodynorphin (Chavkin *et al*, 1983), and are the endogenous ligands of the KOR (Chavkin *et al*, 1982). When prodynorphin is cleaved, into the products dynorphin A, dynorphin B, big dynorphin, α-neo-endorphin, and β-neo-endorphin by proprotein convertase 2 (Day *et al*, 1998) in large dense-core vesicles (LDCV) (Yakovleva *et al*, 2006), and released via calcium-dependent exocytosis (Chavkin *et al*, 1983). These neuropeptides have the highest affinity for KOR, although they also show lower affinity for the delta opioid receptor, the mu opioid receptor, and the NMDA glutamate receptor (Chen *et al*, 1995; Young *et al*, 1983). Although far from universally the case, dynorphin-containing LDCVs are often found in dendritic spines and other post-synaptic compartments while KORs are often found on axonal terminals; as such, dynorphin release often seems to mediate retrograde inhibitory signaling (Drake *et al*, 1994).

KOR and the dysphoric component of stress

Varying stressors induce release of dynorphin, such as psychological stress (Takahashi *et al*, 1990), electric tail shock (Watkins *et al*, 1992), electric foot shock (Land *et al*, 2008; Menendez *et al*, 1993), forced swim stress (McLaughlin *et al*, 2003a), and social defeat stress (McLaughlin *et al*, 2006b). This in turn seems to mediate a number of the behavioral effects of stress. Stress-exposure paradigms induce analgesia sensitive to KOR antagonists, including psychological stress using a communication box (Takahashi *et al*, 1990), tail shock (Watkins *et al*, 1992), and foot shock (Menendez *et al*, 1993). Forced swim stress and social defeat stress both induce analgesia that is blocked by KOR antagonists and also dependent on expression of both dynorphin and KOR (Land *et al*, 2009; McLaughlin *et al*, 2003a, 2006b). Acute administration of KOR agonists is also analgesic in both humans (Gear *et al*, 1996) and animals

(McLaughlin *et al*, 2006a). Further, subanalgesic doses of KOR agonists can also prolong stress-induced analgesia (Starec *et al*, 1996).

Human subjects injected with KOR agonists report the experience as dysphoric (Pfeiffer *et al*, 1986; Wadenberg, 2003). KOR agonists also induce aversion in animal models via pairing with a number of modalities, including taste (Mucha and Herz, 1985), odor (Land *et al*, 2008), and place (Shippenberg and Herz, 1986). Stress is also aversive: animals will avoid an odorant or a context paired with exposure to stress. This avoidance behavior is sensitive to administration of the KOR antagonist nor-binaltorphimine (nor-BNI) and dynorphin-dependent; by contrast, cocaine-paired preference for an odorant is unaltered by pretreatment with norBNI (Land *et al.*, 2008). Aversion to a context paired with i.c.v. corticotrophin-releasing factor (CRF) is also norBNI-sensitive and dynorphin-dependent, as is aversion to the CRF R2 agonist urocortin III; however, the CRF R2 antagonist antisauvigine-30 fails to alter aversion to the KOR agonist U50,488. This is interpreted as suggesting that exposure to stress induces release of CRF, which in turn induces release of dynorphin via activation of CRF R2; it is this dynorphin release which then mediates the aversive properties of both stress and CRF (Land *et al.*, 2008).

KOR activation also has prodepressive effects in models of learned helplessness and behavioral despair. KOR agonists increase immobility in the forced swim test, an unconditioned response to stress (Katoh *et al*, 1990); such increases are interpreted as prodepressive symptoms of behavioral despair. Repeated exposure to forced swim stress also induces progressive increases in the time spent immobile; these changes are attenuated by KOR antagonists and dynorphin-dependent (Carr *et al*, 2010; Mague *et al*, 2003; McLaughlin *et al*, 2003a; Zhang *et al*, 2007). KOR antagonists can also increase the latency to immobility at doses that do not alter basal locomotor behavior (Pliakas *et al*, 2001).

Results in a number of other paradigms support a key role for KOR activation in stress and depression. Social defeat stress is a model of depression in which repeated exposure to attacks by an aggressor animal induce progressive increases in the amount of time spent in

defensive postures. These changes are also norBNI-sensitive and dynorphin-dependent (McLaughlin *et al*, 2006b). In the learned helplessness paradigm, exposure to uncontrollable shock stress induces a subsequent inhibition in the ability to escape a chamber in response to a cue predicting imminent foot shock, a deficit which is reversed by antidepressants (Takamori *et al*, 2001). Intracerebroventricular administration of norBNI induces an antidepressant-like decrease in both the number of escape failures and the latency to escape (Newton *et al*, 2002). In the intracranial self-stimulation (ICSS) paradigm, KOR agonists induce rightward shifts in the frequency-response curve and increases in the response threshold; such changes are interpreted as prodepressive increases in anhedonia (Todtenkopf *et al*, 2004).

Due to the necessity of dynorphin release and KOR activation for stress-induced aversion and other prodepressive effects of repeated stress exposure—which can also be replicated by agonist-induced KOR activation—it has been proposed that activation of the dynorphin/KOR system encodes the dysphoric component of stress (Land *et al*, 2008). However, the mechanism by which the KOR mediates this effect—and in which circuits KOR is acting to do so—remains unclear. One key aim of this dissertation was to determine the role of the mesolimbic dopamine circuit in mediating KOR-induced dysphoria and subsequent aversion. Another was to increase our understanding of the molecular signal transduction pathways acting downstream of the KOR receptor.

KOR signaling pathways and the role of p38 MAPK

Acute G protein signaling. The KOR is predominately coupled to pertussis toxin-sensitive G proteins of the G_i/G_o family (Attali *et al*, 1989b; Lai *et al*, 1995; Wiley *et al*, 1997). However, the receptor is also capable of coupling to pertussis-insensitive G proteins such as G_z or G_{16} when coexpressed in the same cells (Lai *et al*, 1995; Lee *et al*, 1998). There is some evidence that coupling to G_s is also possible (Shen and Crain, 1990). As a GPCR, the KOR is broadly capable of signaling in two modes: signaling mediated by acute activation of G proteins,

and signaling mediated by phosphorylation of the receptor by G protein receptor kinase 3 (GRK3) and subsequent activation of B arrestin 2 (Kendall and Luttrell, 2009). A number of the downstream effects of KOR activation are mediated by the former mechanism. KOR activation induces inhibition of adenylate cyclase (Attali *et al*, 1989b; Konkoy and Childers, 1989) and subsequent inhibition of cyclic adenosine monophosphate (cAMP) (Meng *et al*, 1993; Xie *et al*, 1994). This inhibition has been shown to be mediated by action of the G_{α} subunit (Hsia *et al*, 1984; Taussig *et al*, 1993). After dissociation of the G_{α} and $G_{\beta\gamma}$ subunits, the latter can activate K+ channels (Grudt and Williams, 1993). The best understood of these targets are G protein-gated inwardly rectifying K+ channels (GIRKs), particularly $K_{IR}3.1$ (Appleyard *et al*, 1999; Grudt and Williams, 1993; Henry *et al*, 1995). $G_{\beta\gamma}$ proteins activate GIRKs via direct interaction with the channel (Dascal, 1997; Sadja *et al*, 2003; Wickman and Clapham, 1995). Activation of these channels can directly inhibit neuron firing (Margolis *et al*, 2003) or inhibit terminal transmitter release (Lüscher and Slesinger, 2010). It has also been shown that KOR can couple to voltage-dependent Shaker-type K+ channels ($I_{K(A)}$) to inhibit glutamate release at the MF-CA3 synapse (Simmons and Chavkin, 1996). $G_{\beta\gamma}$ subunits also inhibit a variety of voltage-gated calcium channels (VGCCs), including L-type, N-type, and P/Q-type (Attali *et al*, 1989a; Rusin *et al*, 1997; Wiley *et al*, 1997; Xiang *et al*, 1990), which is mediated by direct interaction between the subunit and the channel. This can also inhibit transmitter release (Rawls *et al*, 1999; Rusin *et al*, 1997). $G_{\beta\gamma}$ subunits can also suppress transmitter release by reducing the quantal amplitude through direct $G_{\beta\gamma}$-mediated inhibition of synaptic vesicle fusion with the plasma membrane (Photowala *et al*, 2006). KOR activation can also increase internal calcium mobilization by activation of the inositol-tris-phosphate pathway (Spencer *et al*, 1997). Acute G protein signaling can also lead to activation of mitogen-activated protein kinase (MAPK) pathways, discussed in more detail below.

Desensitization and Arrestin-dependent signaling. Phosphorylation of the receptor subsequent to activation by agonist paired with clathrin-dependent endocytosis of a receptor-arrestin complex is a well-established mechanism of GCPR desensitization and is a key

mechanism of opioid tolerance (Kendall and Luttrell, 2009). In the KOR, this is mediated by phosphorylation of the receptor by GRK3 at the C-terminal amino acid serine 369 (S369) and subsequent activation of β Arrestin 2 (Appleyard *et al*, 1999). As such, desensitization is absent in models using heterologous expression of a mutated version of the receptor featuring a serine-to-alanine substitution at this site (KSA) (Appleyard *et al*, 1999). Similarly, analgesic tolerance induced by prior exposure to agonist is absent in animals lacking GRK3, as is agonist-induced increases in receptor phosphorylation (McLaughlin *et al*, 2004). However, arrestins also recruit other catalytically-active proteins to the receptor and form secondary signaling complexes (Kendall and Luttrell, 2009). Some of these proteins, such as Src, are critical for endocytosis and thus play dual roles similar to arrestins.

Extracellular Signal-Regulated 1/2 MAPK (ERK 1/2 MAPK) signaling. KOR-stimulated activation of ERK 1/2 MAPK follows two temporal phases, an early phase peaking after 5-30 minutes of receptor activation and a late phase peaking after 60-120 minutes of receptor activation (McLennan *et al*, 2008). The early phase of ERK 1/2 MAPK activation requires activation of Ras, protein kinase C ζ (PKCζ), phosphoinositide 3-kinase (PI3K), and elevations in calcium that seem to be mediated at least in part by L-type calcium channels (Belcheva *et al*, 1998, 2005; Bohn *et al*, 2000). In other GPCR models, arrestin-dependent activation of ERK 1/2 MAPK has been shown to be mediated by scaffolding with Raf1 and MEK1 (Luttrell *et al*, 2001), and it is presumed that activation downstream of the KOR is mediated by the same pathway. The functional differences in these two modes of ERK 1/2 MAPK signaling are still uncertain. However, it is known that KOR-stimulated activation of ERK 1/2 MAPK plays a role in differentiation of embryonic stem cells (Kim *et al*, 2006). Given that the treatment protocol in this paper utilized sustained activation of ERK 1/2 MAPK over 24 hours of incubation with a KOR agonist, this effect is presumably mediated by arrestin-dependent activation. Similarly, KOR activation increases proliferation in an ERK-dependent manner in both immortalized and primary astrocytes (McLennan *et al*, 2008). This effect is arrestin-dependent and is not observed in immortalized astrocytes after treatment with C(2)-methoxymethyl salvinorin B (MOM-Sal-B), a

biased agonist which only induces the early arrestin-independent phase of ERK activation in these cells. It is unclear in both of these cases whether the early arrestin-independent phase of ERK 1/2 MAPK activation might also be required. Activation of ERK 1/2 MAPK has been implicated in a wide variety of cellular processes, such as long-term potentiation and depression, membrane implantation of the dopamine transporter, and modulation of A-type K+ channels (Bolan *et al*, 2007; Bruchas and Chavkin, 2010; Morón *et al*, 2003; Thomas *et al*, 2008). Similarly, it is involved in a number of behaviors relevant to stress and drug reward, including reconsolidation, locomotor sensitization, and place preference (Thomas *et al*, 2008). It has also been shown that the level of ERK 1/2 MAPK expression in the VTA can correspond to both the rate of firing of VTA dopamine neurons and the level of stress (Iñiguez *et al*, 2010). However, it is currently unclear which of these may also be observed by KOR-stimulated activation of ERK.

c-Jun N-terminal kinase MAPK (JNK MAPK) signaling. KOR agonists are also capable of activating JNK MAPK. This is mediated via a pathway that is pertussis toxin-sensitive and requires contributions from $G_{\beta\gamma}$, Src, focal adhesion kinase (FAK), Rac, Cdc42, and Son-of-sevenless (Sos) (Bruchas *et al*, 2007b; Kam *et al*, 2004). The behavioral and cellular consequences of agonist-stimulated JNK activation are currently unknown. Intriguingly, a class of KOR antagonists also appear to function as collateral agonists, inducing long-lasting blockade of KOR signaling via activation of JNK (Bruchas *et al*, 2007b). This activation is insensitive to pertussis toxin, as well as pharmacological inhibition of Src and Raf, demonstrating that it is mediated by a separate pathway than KOR-stimulated activation of JNK by standard KOR agonists.

p38 MAPK signaling. Activation of p38 MAPK by KOR has been observed after heterologous expression in cultured cells, in primary cultures of both astrocytes and neurons, and in neurons *in vivo* (Bruchas *et al*, 2006, 2007a). In each case, it appears to be arrestin-dependent and is not observed in cells heterologously expressing KSA, the mutated version of the receptor lacking the GRK3 phosphorylation site (Bruchas *et al*, 2006), nor is it observed *in vivo* in mice lacking GRK3 (Bruchas *et al*, 2007a). Neuronal activation *in vivo* is observed both

after exogenous administration of agonist and after stress-induced dynorphin release (Bruchas *et al*, 2007a). KOR-stimulated activation of p38 MAPK plays a key role in mediating many cellular and behavioral effects of KOR activation. Pharmacological inhibition of p38 MAPK blocks prodepressive increases in immobility observed after repeated swim stress as well as conditioned place aversion (CPA) to a KOR agonist. This appears to be an effect specific to behavioral responses to KOR activation, as it fails to alter CPP to cocaine and conditioned taste aversion (CTA) to lithium chloride. As such, it has been proposed that p38 activation mediates the dysphoric aspects of KOR activation (Bruchas *et al*, 2007a).

The actions of p38 MAPK within serotonergic neurons of the dorsal raphe nucleus (DRN) appear to play a key role in KOR-mediated dysphoria. Inactivation of KOR within the DRN by local administration of the KOR antagonist nor-BNI blocks CPA to the KOR agonist U50,488 (KOR-CPA). Further, although KOR-CPA is not observed in KOR knockout mice, it is rescued by lentiviral restoration of KOR signaling in the DRN. Similar results are seen with stress-induced reinstatement of an extinguished place preference for cocaine. Intriguingly, this rescue of CPA and stress-induced reinstatement requires phosphorylation of the KOR, as it is not observed after lentiviral expression of KSA (Land *et al*, 2009). Further research has demonstrated that conditional knockout (CKO) of p38α MAPK from serotonergic neurons using Cre-recombinases expressed under control of either the serotonin transporter (SERT) (Zhuang *et al*, 2005) or the serotonin cell type-specific transcription factor ePet1 (Scott *et al*, 2005) blocks KOR-CPA (Bruchas *et al*, 2011). A similar block is observed after virally Cre-induced knockout of p38α MAPK within the DRN (Bruchas *et al*, 2011). CKO of p38α MAPK from serotonergic neurons also blocks stress-induced potentiation of cocaine CPP expression, stress-induced reinstatement of cocaine CPP, and social avoidance after exposure to an uncontrolled social defeat stress. These effects appear to be mediated by p38α MAPK-mediated regulation of surface expression of SERT (Bruchas *et al*, 2011; Schindler *et al*, 2012; Zhu *et al*, 2005). Administration of a KOR agonist or stress-induced release of dynorphin induce increases in paroxetine-sensitive serotonin uptake and selective increases in SERT surface expression. Intriguingly, this effect

appears to be driven selectively by terminals of serotonergic neurons projecting to the ventral striatum (Schindler *et al*, 2012), suggesting that either p38α MAPK or a downstream mediator is absent in the axon terminals of other serotonergic neurons. KOR-CPA is not observed in global SERT knockouts, confirming a key role for p38α MAPK-SERT interactions in the aversive properties of the KOR (Schindler *et al*, 2012). p38α MAPK is also capable of inducing heterologous desensitization of the GIRK channel $K_{IR}3.1$ in both cultured cells and in serotonergic neurons of the DRN via Src phosphorylation of tyrosine-12 (Clayton *et al*, 2009; Lemos *et al*, 2012a). The behavioral relevance of this phosphorylation event is currently unclear.

It is unknown what effects KOR-induced activation of p38 MAPK may mediate in other cell types, particularly neurons which release other monoamines such as norepinephrine and dopamine. The ability of p38 MAPK to enhance SERT surface expression was first observed in CHO cells after heterologous expression of the transporter (Zhu *et al*, 2005). In this system, activation of p38 MAPK was induced by incubation with anisomycin and effects were confirmed to be mediated by p38 MAPK by sensitivity to a selective p38 inhibitor, SB203,580. Under similarly heterologous expression conditions, the norepinephrine transporter (NET) and the dopamine transporter (DAT) were shown to be similarly regulated by p38 MAPK activation, with the former showing a SB203,580-sensitive increase in uptake and the latter showing a SB203,580-sensitive decrease in uptake. Confirming this, inhibition of p38 alters uptake in a number of DAT point mutants heterologously expressed in CHO cells (Lin *et al*, 2003). p38 MAPK is capable of phosphorylating DAT on the N-terminal tail threonine 53, a site which has been shown to be phosphorylated *in vivo*. Further, phosphorylation at this site is expected to induce functionally-relevant conformational changes of the transporter (Gorentla *et al*, 2009). There are many other manners in which p38 MAPK could potentially alter dopamine signaling. For example, cells undergoing nerve growth factor withdrawal-induced apoptosis undergo an increase in monoamine oxidase (MAO) expression, an effect which is blocked by inhibition of p38 MAPK (De Zutter and Davis, 2001). However, inhibition of p38 MAPK or expression of a dominant-negative p38 MAPK leads to increases in MAO-A activity. Conversely, expression of a

constitutively active version of p38 MAPK results in an inhibitory phosphorylation of MAO-A (Cao *et al*, 2009). These effects are consistent across a number of cell lines, including primary culture of cortical neurons. Given that MAO-A plays a key role in the metabolism of dopamine (Youdim and Riederer, 1993), this is another mechanism by which p38 MAPK activation could regulate dopamine signaling. p38 MAPK has been shown to be capable of regulating neuronal signaling in a wide variety of other respects, including effects on synaptic plasticity (Boudreau *et al*, 2007; Rumbaugh *et al*, 2006), regulatory phosphorylation of sodium channels (Hudmon *et al*, 2008), and KOR-induced inhibition of transmitter release (Capik *et al*, 2013). As such, another key aim of this dissertation was to examine the behavioral and functional consequences of KOR-stimulated activation of p38α MAPK in dopamine neurons.

Dopamine, aversion, and stress

The anhedonia hypothesis. Aversive stimuli have long played a key role in attempts to understand the signal or signals mediated by dopamine signaling. The first well-known attempt to grapple with this mechanistically was the anhedonia hypothesis (Wise, 1982). This hypothesis took its root from the basic observation that drugs that activated or increased dopamine signaling were reinforcing in a manner that was sensitive to dopaminergic lesion by 6-OHDA, as well as to dopaminergic antagonism by neuroleptics. Specifically, neuroleptic administration was proposed to block the reinforcing properties of food or drugs of abuse such that animals reacted as though they were in an extinction session. Given the further knowledge that neuroleptics block the euphoric aspects of amphetamine in human subjects, this theory proposed that dopamine signaling was necessary to signal hedonic and euphoric properties of reward. When dopamine signaling is antagonized, it would therefore block those hedonic aspects. As a corollary, this would further suggest that direct inhibition of dopamine signaling would be dysphoric and aversive (Wise, 1982; Wise *et al*, 1978). This hypothesis was bolstered by the observation that increases in dopamine signaling within the nucleus accumbens (NAc)

seemed to be a general mechanism of addictive drugs, and that some aversive drugs (such as the KOR agonist U50,488) inhibited dopamine release in the same region (Di Chiara and Imperato, 1988). Further, it is consistent with the observation that withdrawal from repeated drug use induces a depressive-like state of dysphoria and long-lasting inhibitions in accumbal dopamine (Barr *et al*, 2002; Imperato *et al*, 1992b).

Terminal changes in dopamine efflux in response to aversive stimuli. However, there are a number of observations that conflict with this hypothesis. First and foremost is that hedonic responses seem to be separable from dopamine signaling. For instance, selective lesion of the midbrain dopamine nuclei does not alter facial responses to either ingestive or nonpalatable tastes (Berridge *et al*, 1989), implying that hedonic and aversive sensation is intact. For another, a robust body of literature shows that aversive, stressful, and fear-inducing stimuli such as foot shock, restraint, social defeat stress, foot shock, tail pinch, forced locomotion, bitter taste, or an anxiogenic drug can induce increases in dopamine and metabolite concentrations in terminal regions including the NAc, medial prefrontal cortex (mPFC), and dorsal striatum (Abercrombie *et al*, 1989; Bassareo *et al*, 2002; Bertolucci-D'Angio *et al*, 1990; D'Angio *et al*, 1987; Imperato *et al*, 1991; Robinson *et al*, 1987; Di Scala and Sandner, 1989; Thierry *et al*, 1976; Tidey and Miczek, 1996; Yadid *et al*, 2001). This may be due to a general activation of motivational circuits, as striatal levels of glutamate, aspartate, and GABA are also increased (Keefe *et al*, 1993). Further, data suggest that the increase in dopamine is mediated by increases in the firing of dopamine neurons, as local infusion of either the AMPAR antagonist CNQX or the NMDAR antagonist APV into the NAc via microdialysis fail to alter tail-shock-induced increases in dopamine, while no increases are seen after blockade of action potentials via infusion of TTX into the medial forebrain bundle (Keefe *et al*, 1993). In fact, it has also been reported that local administration of APV into the NAc core actually potentiates stress-induced increases in dopamine (Doherty and Gratton, 1997).

One problem with interpreting this data is that relief from an aversive stimulus should be interpreted as rewarding. So experiments which show an increase in dopamine within an

active avoidance paradigm (McCullough *et al*, 1993) or after the termination of an aversive stimulus (Bassareo *et al*, 2002; Di Chiara *et al*, 1999; Imperato *et al*, 1992a) could be showing increases in dopamine that mediate a reward signal. Further complicating this concern is the fact that many of these experiments have been conducted using microdialysis, a technique with a limited temporal resolution. As such, many of these experiments are performed in a context in which both the onset and offset of a shorter aversive stimulus occurs during a single sample period (Bertolucci-D'Angio *et al*, 1990), and thus potentially obscuring an acute inhibition in dopamine signaling during the stimulus prior to a subsequent rise in dopamine signaling after the relief from the stimulus. Other experiments are performed in paradigms in which mice undergo prolonged exposure to stress over extended periods of time in which secondary adaptations in dopamine signaling (or in the animals' perception of the stimulus) may be occurring (Imperato *et al*, 1991). Some experiments show an increase in terminal dopamine release in response to presentation of a cue or context paired with an aversive stimulus, which could alternately be interpreted as communicating information about the aversive nature of the stimulus or as communicating a prediction error response when the predicted aversive stimulus is not delivered (Pezze *et al*, 2001; Tidey and Miczek, 1996; Wilkinson *et al*, 1998; Young *et al*, 1993). There is also some evidence that the response may be related to the magnitude of the aversive stimulus (Horvitz, 2000; Sorg and Kalivas, 1991). Contrary to the majority of findings, inhibitions of dopamine release within the NAc have been reported in response to exposure to olfactory or taste cues previously paired with an aversive stimulus (Jeanblanc *et al*, 2002; Mark *et al*, 1991).

Changes in dopamine neuron firing in response to aversive stimuli. Single-unit recording of the firing of dopamine neurons in the substantia nigra pars compacta (SNc) and the VTA has told a similarly complicated story. Early reports suggested there might be two populations of dopamine neurons: one activated by an aversive stimulus such as a tail pinch or an air puff, and another inhibited by the same stimuli (Chiodo *et al*, 1979, 1980). However, further research consistently reported that most dopamine neurons were either inhibited or showed no

response to aversive stimuli, with only a minimal population showing excitation (Mirenowicz and Schultz, 1996; Ungless *et al*, 2004). Much of this information is fit into a prediction error model. In these reports, the majority of dopamine neurons show an increased excitation in response to an unpredicted reward or a reward-predicting cue, but are more likely to show a pause in firing in response to an unpredicted air puff or an air puff-predicting cue (Mirenowicz and Schultz, 1996), or an intense pinch (Schultz and Romo, 1987). Similarly, an inhibition of dopamine neuron firing is observed if a predicted reward is delayed or of a reduced magnitude than expected (Hollerman and Schultz, 1998; Tobler *et al*, 2005). However, even within this literature reports persisted of a subset of neurons that are excited by aversive stimuli, predominately in neurons which also fired in response to primary rewards or reward-predicting cues (Mirenowicz and Schultz, 1996; Schultz and Romo, 1987).

A new model of value and salience encoding by dopamine neurons. Recent research suggests that these discrepancies may be accounted for by a combination of recording sites and how a dopamine neuron is defined based on electrophysiological parameters. When recording from dopamine neurons from the VTA or the SNc—either for single-unit or for whole-cell patch-clamp studies—it is common to determine the molecular identity of a neuron *a priori* based on one or a few electrophysiological traits, such as firing rate, afterhyperpolarization, input resistance, sensitivity to autoreceptor activation by quinpirole, action potential width, or the presence of the I_h cation current mediated by hyperpolarization-activated nucleotide-gated (HCN) channels (Chu and Zhen, 2010; Lammel *et al*, 2008; Margolis *et al*, 2006b, 2008, 2010). These criteria were originally based on their reliability as a predictor of dopaminergic content in the SNc (Aghajanian and Bunney, 1977; Grace and Bunney, 1980). However, recent studies which have focused on the properties of labeled, tyrosine hydroxylase-immunoreactive (TH-ir) neurons, have called into question the usefulness of those criteria for sorting VTA neurons. This evidence suggests that these criteria may segregate neurons as much by projection site as by transmitter content. For example, although quinpirole sensitivity does positively correlate with action potential width when recording from VTA neurons, amygdala-projecting dopamine

neurons seem to lack the former while having short action potential durations (Margolis *et al*, 2008). Based on these studies, it seems to be the case that classical dopamine neuron criteria primarily identify dopamine neurons in the medial SNc which project to the dorsolateral striatum and dopamine neurons throughout the parabrachial pigmental nucleus of the VTA (PBP) which project to the lateral shell of the NAc. By contrast, a seemingly distinct population of dopamine neurons is found in the more ventromedial portions of the VTA (including ventromedial parts of the PBP) which projects to mPFC, basolateral amygdala, NAc core, and medial NAc shell. This latter group seems to be comprised of neurons which are smaller, show reduced DAT expression, show a higher firing rate, show a higher basal AMPAR/NMDAR ratio, and lack an afterhyperpolarization and I_h current (Lammel *et al*, 2008, 2011). However, further complicating these reports is the possibility that there are species differences in the composition of these groups: for instance, it has been reported in rat that mPFC-projecting dopamine neurons show inhibition due to activation of the D2R dopamine receptor subtype as well as KOR activation in a GIRK-dependent manner (Margolis *et al*, 2003, 2006a, 2008). By contrast, it has been reported in mouse that mPFC-projecting dopamine neurons not only lack autoreceptor-mediated inhibition of cell firing but further do not seem to express appreciable levels of D2 receptors or GIRK2 channels (Lammel *et al*, 2008). Even within a single species, dopamine neurons do not seem to universally segregate into the groups described above: lateral shell and medial shell-projecting dopamine neurons both show a cocaine-induced increase in AMPAR/NMDAR ratios (interpreted as suggesting an induction of glutamatergic long-term potentiation), while mesocortical and nigrostriatal neurons do not. Similarly, mesocortical and lateral shell-projecting dopamine neurons show a stress-induced increase in AMPAR/NMDAR ratios, while nigrostriatal and medial shell-projecting dopamine neurons do not (Lammel *et al*, 2011).

This new grouping strategy also seems to be supported by experiments sorting neurons by their response to aversive stimuli. Experiments supporting the prediction error hypothesis which predominately show an inhibition of dopamine neuron firing in response to aversive

events are generally recorded from the SNc (Hollerman and Schultz, 1998; Mirenowicz and Schultz, 1996; Schultz and Romo, 1987; Tobler *et al*, 2005). Similarly, it has been reported that VTA recordings which show an inhibitory response to aversive stimuli predominately sample from the more dorsolateral extension of the PBP, where recordings from lateral shell-projecting dopamine neurons would dominate (Brischoux *et al*, 2009). But a recent experiment in which dopamine neurons were recorded and juxtacellularly labeled from throughout the VTA found that although TH-ir neurons from the more dorsal PBP were found to consistently show an inhibition in response to a tail pinch, more ventromedial TH-ir neurons were excited by the same stimulus (Brischoux *et al*, 2009). Another experiment defining dopamine neurons by electrophysiological firing properties found a similar result when comparing the responses to liquid rewards and airpuffs (Matsumoto and Hikosaka, 2009). Further, the lateral habenula—a glutamatergic nucleus which seems to be the source of aversive and negative reward signals in VTA dopamine neurons (Matsumoto and Hikosaka, 2007)—seems to be positively coupled to mesocortical dopamine neurons via direct monosynaptic projections and negatively coupled to other dopamine neurons via an indirect pathway including the GABAergic rostromedial tegmental nucleus (Bourdy *et al*, 2014; Jhou *et al*, 2009; Lammel *et al*, 2012).

An attempt to integrate this data has led to a recent hypothesis attempting to define the signals being mediated by these separate groups of dopamine neurons (Bromberg-Martin *et al*, 2010). In this model, there are three broad signals mediated by dopamine neurons. The first signal is motivational value, used to direct reward seeking and process the evaluation and valence of outcomes. This is encoded in dopamine neurons of the medial SNc and lateral PBP, which correspond to the "classic" dopamine neurons which Lammel *et al.* (2008) found to predominately project to the dorsolateral striatum and NAc lateral shell. The second signal is motivational salience, used to support orienting of attention, arousal and cognitive processing. This is encoded in dopamine neurons of the ventromedial VTA, which correspond to the dopamine neurons reported to have nonstandard electrophysiological responses that project to mPFC, basolateral amygdala, NAc core, and NAc medial shell. This second signal is also found

in a separate population of dopamine neurons in the dorsolateral SNc (Bromberg-Martin *et al*, 2010; Mirenowicz and Schultz, 1996). Intriguingly, it has been observed that dorsal SNc dopamine neurons predominately project to striatal matrix, whereas ventral SNc dopamine neurons predominately project to striatal patches. As such, there may also be differences in the role of these dorsal striatal compartments in mediating motivational value versus salience responses. The third signal is a general alerting signal which can be found in both populations.

Attempts to validate this hypothesis are complicated by the specificity of how sampling and interventions at these recording sites are detailed in the literature. Many studies simply define their target as "nucleus accumbens" or "VTA," with no attempt given to elucidate whether a given subregion may have been preferentially sampled or manipulated. There is also reason to be concerned that injections of drug or virus may often not be sufficiently specific to uniquely hit a single subregion. Further, the data from Lammel *et al.* (2008) now suggests that the core/shell dichotomy may be insufficient, but rather that we need to segregate the shell by the medial-lateral axis. Nevertheless, there is some evidence to support this hypothesis. For example, it had previously been observed that stress-induced increases in dopamine signaling were strongest and most consistently reported in the PFC, followed by the NAc, and with the dorsal striatum showing the weakest response if any (Horvitz, 2000). Further, there are more reports from NAc and dorsal striatum than PFC showing rebound increases in dopamine release after relief from an aversive stimulus (Abercrombie *et al*, 1989; Di Chiara *et al*, 1999; Kalivas and Duffy, 1995). More recently, voltammetric recordings from the core of the NAc have shown an increase in the frequency of phasic dopamine release events during social defeat stress (Anstrom *et al*, 2009). A similar increase in the frequency of burst firing events of putative VTA dopamine neurons was also observed afterwards, which seemed to be limited to those neurons which were predisposed to higher burst levels. This is consistent with the prior observation that dopamine neurons with positive responses to aversive stimuli were more predisposed to burst firing than those with negative responses (Brischoux *et al*, 2009); as well as with the observation that mesocortical, mesoamygdaloid, NAc core-projecting, and NAc medial shell-projecting

dopamine neurons have a greater excitatory response to current injections and a higher maximum firing rate than nigrostriatal and NAc lateral shell-projecting dopamine neurons (Lammel *et al*, 2008). Further evidence that burst firing is critical for mediating the excitatory response to aversive stimuli in dopamine neurons was provided by the observation that it is severely attenuated in mice in which an essential subunit of the NMDAR has been conditionally knocked out of dopamine neurons (Zweifel *et al*, 2011). Burst firing is also severely attenuated in these conditional knockouts (Zweifel *et al*, 2009). Intriguingly, these mice show an inability to associate fear conditioning with either a paired cue or context, instead showing a generalized fear and anxiety response. This suggests that the excitatory response is not critical for evaluating the valence of an outcome, but rather with determining how to orient it towards a contextually appropriate response, consistent with the salience hypothesis.

A pair of recent, seemingly contradictory optogenetic studies may also make more sense in light of this hypothesis. In one study, light-stimulated activation of burst firing (but not tonic firing) in VTA dopamine neurons via channelrhodopsin induced a state of stress-susceptibility in the social avoidance assay. Conversely, light-stimulated inhibition of VTA dopamine neurons via halorhodopsin induced a state of stress-resilience. This suggests a key role of dopamine neuron burst firing in mediating the saliency of the social defeat stress to generate the avoidance response (Chaudhury *et al*, 2013). In the other study, light-stimulated inhibition of VTA dopamine neurons induced a depression-like inhibition of struggling in the tail-suspension test along with increased anhedonia in a sucrose preference test; whereas light-stimulated induction of burst firing in dopamine neurons decreased immobility in the Porsolt forced swim test. This suggests a key role of dopamine burst firing in mediating antidepressant responses (Tye *et al*, 2013). It is possible that whereas burst firing in ventromedial salience-mediating dopamine neurons promotes localization of the cue and improves the ability to elicit aversive cue-dependent avoidance responses, burst firing in dorsolateral value-mediating decreases the dysphoric encoding of a stimulus. Consistent with this interpretation, the latter paper reports VTA injection coordinates that include a more dorsal portion of the VTA than the former paper

(Chaudhury *et al*, 2013; Tye *et al*, 2013). Further, it has also been shown that direct inhibition of dopamine neurons via optogenetic activation of halorhodopsin in either the VTA or the SNc is sufficient to support both real-time and conditioned place aversions (Ilango *et al*, 2014; Tan *et al*, 2012). This could be mediated via effects in ventromedial SNc / dorsolateral VTA motivational value-encoding neurons. However, further experiments will clearly be required to determine if these effects are truly mediated by distinct subsets of dopamine neurons.

Long-term stress-induced alterations in dopamine signaling. Another related question is how dopamine signaling is altered on longer time scales after exposure to stress. Experiments using microdialysis show that after a chronic stress paradigm involving repeated exposure to uncontrollable foot shock over 7 days to 3 weeks, an inhibition of basal dopamine signaling is observed that lasts at least 14 days after the final stress exposure (Gambarana *et al*, 1999; Mangiavacchi *et al*, 2001; Scheggi *et al*, 2002). Unfortunately, this question seems to be under-studied using other chronic stress paradigms. By contrast, evidence strongly suggests that dopamine neuron excitability is increased after stress exposure. As such, a single cold water swim test increases the AMPAR/NMDAR ratio in VTA dopamine neurons (Saal *et al*, 2003) as well as blocking long-term potentiation at GABAergic synapse via a KOR-dependent mechanism (Graziane *et al*, 2013; Polter *et al*, 2014). Both of these mechanisms would suggest an increase in the excitability of VTA dopamine neurons. Further research has shown that this AMPAR/NMDAR ratio increase is observed in both mesocortical and NAc lateral shell-projecting dopamine neurons, but not mesolimbic medial-shell projecting or nigrostriatal dopamine neurons (Lammel *et al*, 2011). This suggests that these stress-induced increases in excitability could potentially play key roles in both value and salience signaling. Consistent with these results, a number of studies have reported that repeated exposure to the social defeat assay induces an increase in dopamine neuron firing (Iñiguez *et al*, 2010; Krishnan *et al*, 2007) which seems to be primarily mediated by a selective increase in burst firing (Anstrom *et al*, 2009; Cao *et al*, 2010). The stress-mediated increase in the AMPAR/NMDAR ratio is blocked by the glucocorticoid receptor antagonist RU486, and the increase in burst firing requires

glucocorticoid receptor expression in neurons expressing the D1 receptor (Barik *et al*, 2013; Saal *et al*, 2003). Given that D1-expressing medium spiny neurons predominately coexpress dynorphin (Steiner and Gerfen, 1998), it is possible that KOR signaling may also play a role in this effect; (however, not via changes in the AMPAR/NMDAR ratio (Graziane *et al*, 2013)). The increase in dopamine neuron firing frequency is also required for repeated social defeat to induce social avoidance behaviors in mice (Barik *et al*, 2013; Krishnan *et al*, 2007).

How is it that repeated stress exposure can potentiate dopamine neuron firing while inhibiting dopamine release? Repeated immobilization stress or exposure to the learned helplessness paradigm can induce an increase in dynorphin immunoreactivity in both the core and shell of the NAc (Shirayama *et al*, 2004). This could be mediated by repeated stress-induced increases in dopamine release, which can lead to increases in dynorphin expression via repeated activation of D1 receptors in D1R / dynorphin-expressing medium spiny neurons (Steiner and Gerfen, 1998). As such, it may be the case that heightened dopamine release during repeated stress exposure leads to a dysphoric state mediated in part by a compensatory increase in tonic dynorphin signaling at dopamine terminal sites, while the responsivity of dopamine neurons themselves is increased. Consistent with this, it was reported that after a 4 week exposure to chronic mild stress, the ability of a tail pinch to induce increases in dopamine signaling was increased in both the NAc shell and the PFC (Di Chiara *et al*, 1999). This same study also showed that dopamine release in response to an unpredicted reward was inhibited after stress exposure, suggesting that dopamine neuron responsivity is altered in a circuit-specific fashion.

The current body of evidence suggests that both stress-induced increases and decreases in dopamine signaling play a key role in mediating aversive responses and dysphoria. As such, two key aims of my dissertation research were to improve our understanding of the role played by the inhibition of dopamine release in mediating the aversive properties of KOR activation, and also in mediating interplays between stress-induced dysphoria and stress-induced increases in cocaine reward.

Interactions between KOR activation and the rewarding properties of addictive drugs

Reward-inhibiting properties of KOR activation. Administration of KOR agonists alone is aversive (Mucha and Herz, 1985; Shippenberg and Herz, 1986). Early research proposed that this aversion was mediated by the ability of KOR agonists to suppress dopamine signaling, as discussed in more detail below (Di Chiara and Imperato, 1988; Shippenberg *et al*, 1993). Given the knowledge that dopamine seems to play a key role in the rewarding properties of drugs of abuse, and that human cocaine addicts show alterations in dynorphin expression in the ventral and dorsal striatum (Hurd and Herkenham, 1993), research has focused on potential interactions between KOR signaling and the addictive properties of drugs of abuse. Early research focused on the possibility that KOR agonists may have antiaddictive properties, particularly focusing on the models of locomotor sensitization and reward sensitization, with the latter focused on changes in response in the CPP paradigm.

It was initially reported that chronic pretreatment with a KOR agonist shortly before cocaine administration over three days blocked the expression of locomotor sensitization to cocaine on a fifth day (Heidbreder *et al*, 1993, 1995). Evidence suggests that this is due to a block of the development of sensitization itself, as similar locomotor responses to a cocaine challenge are observed in cocaine- and U50,488- or saline-pretreated animals from 2-21 days after the last day of a sensitization pretreatment paradigm (Shippenberg *et al*, 2001). The ability of repeated treatment by KOR agonists alone to alter the locomotor response to a subsequent psychostimulant challenge is unclear, as varying publications have reported either a decrease, increase, or no effect at all (Collins *et al*, 2001; Crawford *et al*, 1995; Fuentealba *et al*, 2007; Heidbreder *et al*, 1995). Although these experiments did vary by agonist, dose, and pretreatment time, there is no clear trend in the reported data. Potentially clarifying matters, Chartoff *et al* (2008) reported that effects of the KOR agonist Salvinorin A on acute cocaine-

induced locomotor activity are context-sensitive. If repeatedly administered in home cages, it fails to alter the locomotor response to cocaine; whereas if it is repeatedly administered in the same locomotor activity chambers as the cocaine challenge, they found that it increases the locomotor response.

Similar responses were seen in research focusing on the reward-sensitizing properties of cocaine. Animals with five days of repeated cocaine experience will show a CPP for cocaine at lower doses than drug-naïve animals. This reward-sensitizing effect is blocked if a KOR agonist is administered 15 minutes prior to cocaine during the sensitization phase, even in the absence of treatment with a KOR drug during the CPP procedure itself (Shippenberg *et al*, 1996). Both of these effects are reasonably long-lasting, given that they can be observed if CPP training is initiated from 3-10 days after cessation of the sensitization phase. Block of reward sensitization was also seen if animals only received KOR agonist pretreatment on days 3-5 of a five day cocaine pretreatment paradigm (Shippenberg and Rea, 1997). However, no effect was seen on morphine-induced reward sensitization or on cross-sensitization to morphine in cocaine-pretreated animals (Shippenberg *et al*, 1996). Cocaine reward is also blocked acutely in drug-naïve animals if a KOR agonist is administered 15 minutes prior to cocaine training in the CPP paradigm (Crawford *et al*, 1995; McLaughlin *et al*, 2006a).

Schenk *et al* (1999) found that the pretreatment with the KOR agonist U69,593 in extinguished animals blocks cocaine-primed but not amphetamine-primed reinstatement for self-administration of cocaine. Cocaine-primed reinstatement was also blocked by the KOR agonists enadoline and spiradoline in squirrel monkeys (Rüedi-Bettschen *et al*, 2010). U69,593 also failed to alter reinstatement to cocaine self-administration induced by the dopamine uptake inhibitor GBR 12909 or the cocaine analog WIN 35,428, although it did block reinstatement by the cocaine analog RTI-55 (Schenk *et al*, 2000). A later publication did report that pretreatment with U69,593 may be able to inhibit amphetamine-primed reinstatement for self-administration of amphetamine, but closer examination of this data set suggests that the effect is driven by the failure of amphetamine to induce reinstatement in all of the groups save

at one combination of dose and time bin for the vehicle-treated controls (Schenk and Partridge, 2001). In light of the models of stress-induced reinstatement that are discussed below, it is important to note at this time that none of these experiments examining the influence of KOR agonists on drug-primed reinstatement examined the effects of KOR agonists alone on animals that have extinguished drug-taking.

As mentioned above, ICSS is a model of anhedonia and mania in which animals press a level for an electric stimulation of the medial forebrain bundle. Treatment with cocaine dose-dependently decreases the threshold stimulation frequency, a result which is alternately interpreted as an increase in reward/hedonia or an increase in mania; this effect is blocked by acute pretreatment with the KOR agonist U69,593 (Tomasiewicz *et al*, 2008). Repeated pretreatment over eight days with the KOR agonist Salvinorin A (SalA) similarly inhibits the ability of cocaine to decrease the threshold stimulation frequency (Potter *et al*, 2011). However, in this case the response to cocaine was tested twenty-four hours after the last SalA administration. Although they replicated previous findings that SalA acutely elevated the threshold, they also observed that twenty-four hours after SalA the threshold was decreased, a finding they attributed to an opponent-like process. As such, if the data was compared to a baseline threshold measured prior to SalA administration, the response to cocaine was consistent regardless of SalA treatment. Further, this argues that the rewarding properties of ICSS were elevated 24 hours after Salvinorin A administration.

Concerning the putative antiaddictive effects of KOR activation, it is intriguing to note that the effects seem to be at least somewhat selective for cocaine. This observation is based on the lack of an effect on nicotine psychomotor sensitization (Heidbreder *et al*, 1995), reward- or cross-sensitization for morphine CPP (Shippenberg *et al*, 1996), and self-administration reinstatement induced by a number of non-cocaine modulators of dopamine signaling (Schenk and Partridge, 2001; Schenk *et al*, 1999, 2000). It has, however, been found that acute (10 min.) pretreatment with the KOR agonists U50,488 and E-2078 10 minutes before morphine administration block morphine CPP, while failing to alter apomorphine CPP at either a 10

minute or 45 minute pretreatment time point (Funada *et al*, 1993). Thus, it might be the case that direct interactions between the aversive properties of KOR activation and the rewarding properties of addictive drugs discussed below are broadly applicable, whereas effects of KOR on blocking reward or locomotor sensitization are limited to cocaine.

It is also important to observe that the vast majority of literature on this topic is focused on tightly acute pretreatment time points, with generally 15 or fewer minutes elapsing between pretreatment with a KOR agonist and administration of cocaine. At these close time points, KOR activation is aversive in a CPP paradigm (McLaughlin *et al*, 2006a), so it is possible that the dysphoric properties of KOR activation simply cancel out the rewarding properties of cocaine activation when they co-occur.

Reward-potentiating properties of KOR activation. More recently, experiments have focused on proaddictive effects of KOR activation and the role of KOR in mediating stress-induced effects on drug reward. If mice are subjected to either forced swim stress or social defeat stress prior to training with cocaine in a CPP paradigm, they show a potentiated place preference, interpreted as an increase in drug reward. This potentiation is blocked by pretreatment with the KOR antagonist norBNI and is dependent on expression of prodynorphin and the KOR (McLaughlin *et al*, 2003a, 2006a, 2006b). Intriguingly, it has also been demonstrated that although pretreatment with the KOR agonist U50,488 15 minutes prior to cocaine blocks its rewarding properties, replicating the experiments described above, the reward-potentiating effects of stress can be replicated by pretreating with U50,488 60 minutes prior to cocaine (McLaughlin *et al*, 2006a). Thus, KOR agonists can either block or potentiate the rewarding properties of cocaine, based on the pretreatment time point. Stress can similarly potentiate the expression of a previously learned place preference for cocaine or nicotine, an effect that is norBNI-sensitive and replicable by U50,488 administration 60 minutes prior to the CPP post-test (Schindler *et al*, 2010; Smith *et al*, 2012). However, U50,488 5 minutes prior to the CPP post-test fails to alter the expressed CPP.

After undergoing training in CPP, repeated exposure to the contextual cues in the absence of drug induces extinction. However, exposure to stress can reinstate the place preference. Stress-induced reinstatement to cocaine and nicotine CPP requires KOR activation by dynorphin and is replicated by the KOR agonist U50,488 (Jackson *et al*, 2013; Redila and Chavkin, 2008).

Stress exposure also reinstates self-administration of cocaine in a manner dependent on KOR activation (Beardsley *et al*, 2005) in the VTA (Graziane *et al*, 2013). This effect has been replicated with the KOR agonists spiradoline and enadoline, and has been observed in various species including rats and squirrel monkeys (Valdez *et al*, 2007). Acute administration of the KOR agonist U50,488 can also induce reinstatement of alcohol and nicotine administration (Funk *et al*, 2014; Grella *et al*, 2014). Repeated swim stress also increases the maximum effort Wistar rats are willing to expend for a unit dose of cocaine, an effect which is blocked by treatment with norBNI (Groblewski *et al*, 2014). Further, the ability of foot shock stress to induce increases in ethanol consumption and ethanol preference over drinking water requires expression of dynorphin (Rácz *et al*, 2013).

Evidence also suggests that withdrawal-induced dynorphin release may play a key role in the administration of some addictive drugs, as evidenced by antagonist experiments. Rats which have been trained to self-administer ethanol vapor and were subsequently rendered ethanol-dependent via extended ethanol vapor exposure show a potentiation in their self-administration. This effect is attenuated by treatment with the KOR antagonist norBNI (Walker and Koob, 2008; Walker *et al*, 2011). Note that nor-BNI failed to alter ethanol self-administration in rats which did not undergo the ethanol dependence induction paradigm, suggesting a lack of dynorphin signaling in the basal (non-ethanol dependent) state. Similarly, rats allowed extended access (12 hours) to heroin self-administration also show a norBNI-dependent escalation over repeated trials that is not observed in animals with shorter access (1 hour) (Schlosburg *et al*, 2013). Prodynorphin knockout also induces a reduction in the breakpoint for nicotine at high

doses, although it is unclear if this is due to effects on the rewarding or aversive properties of nicotine (Galeote *et al*, 2009).

There is a long and complicated history of the interactions between KOR activation and the rewarding properties of addictive drugs that, at first glance, is seemingly contradictory. On the one hand, a large body of research argues that KOR agonists are antiaddictive and block the rewarding and reinforcing properties of drugs of abuse. On the other hand, another large body of research argues that the KOR mediates dynorphin-dependent, stress-induced increases in drug reward, an effect which can be mimicked by administration of exogenous KOR agonists. One question is, what is the mechanism of these disparate effects on drug reward? The idea that the dysphoric effects of KOR activation could block the rewarding properties of drugs of abuse when coadministered is intuitive, but how could stress-induced dysphoria increase drug reward? A model has been proposed to answer this question based on subjective changes in reward state (Bruchas *et al*, 2010). In this model, stress-induced KOR activation mediates a state of dysphoria. This is perceived by the animal as a downward shift in the basal hedonic state. Subsequent administration of a drug of abuse (such as cocaine) elevates the reward state of an animal; but it could be the case that this is elevated reward state is potentiated if it is also perceived by the animal as rescuing it from the dysphoric state. This model proposes that the KOR-induced potentiation of cocaine CPP is mediated by a summation of these two effects—the rescue from dysphoria, and the further induction of euphoria. However, this model is dependent on the idea that this effect is time-dependent: at close coadministration time points (approximately fifteen minutes or less), cocaine can rescue an animal from dysphoria but cannot elevate the internal reward state further. This balance is expressed in the CPP paradigm as a lack of place preference. At more distant pretreatment time points (if cocaine is administered sixty minutes after a KOR agonist), then cocaine not only rescues the animal from dysphoria but also increases the internal reward state to a level similar to that seen when cocaine is administered alone. This hypothesis has not been directly tested. Given the importance of dopamine in the rewarding properties of cocaine as discussed above, and its

potential importance in mediating the dysphoric properties of KOR activation as discussed below, another key aim of my dissertation research was to determine if coadministration of a KOR agonist (U50,488) and cocaine could modulate dopamine signaling in a manner similar to the proposed shifts in the reward state.

Interactions between KOR and dopamine signaling

Terminal effects of KOR on released dopamine. KOR mRNA is highly expressed throughout a number of regions that play key roles in dopamine signaling (Mansour *et al*, 1994). It is found in both the VTA and the SNc as well as the dopaminergic-serotonergic caudal linear raphe. It is also found in terminal regions such as the NAc, the dorsal striatum, amygdala, mPFC, the bed nucleus of the stria terminalis, the ventral pallidum, and the globus pallidus. Prodynorphin mRNA and dynorphin protein is robustly expressed throughout the NAc and striatum, primarily in medium spiny neurons of the direct pathway which also express substance P and the D1R dopamine receptor subtype (Anderson and Reiner, 1990; Steiner and Gerfen, 1998); local release by these neurons is probably the primary source of dynorphin signaling on striatal dopamine terminals (Svingos *et al*, 1999). Within the NAc, most KOR expression is in axonal structures, and the majority of that is associated with synaptic vesicles and plasma membranes, generally in axon terminals (Meshul and McGinty, 2000; Svingos *et al*, 1999). Further, a majority of the KOR immunoreactivity is in profiles that either also contain DAT or are apposed to terminals containing DAT (Svingos *et al*, 2001). Intriguingly, the majority of KOR-immunoreactive axons within the NAc are not in direct contact with dynorphin-immunoreactive profiles, implying that dynorphin in this region may operate by bulk signaling, diffusing to the receptor from distal sites (Svingos *et al*, 1999). Dynorphin protein is also abundant in fibers and terminals throughout the SNc and VTA; originating from sites including NAc, amygdala, and the hypothalamus (Fallon *et al*, 1985), and potentially also the dorsal raphe nucleus (Fu *et al*, 2010; Lemos *et al*, 2012a).

As such, it is not surprising that KOR activation is capable of robustly regulating dopamine signaling. Systemic or i.c.v. injections of KOR agonists lead to a strong, often long-lasting (on the order of hours), suppression of terminal dopamine signaling in the NAc, dorsal striatum, and mPFC (Di Chiara and Imperato, 1988; Donzanti *et al*, 1992; Ebner *et al*, 2010; Gray *et al*, 1999; Spanagel *et al*, 1990; Zhang *et al*, 2005). Evidence suggests that this suppression may be predominately induced by local inhibition at the site of dopamine terminals, as it is observed using microdialysis after direct injection into any of those dopamine terminal sites in awake animals (Donzanti *et al*, 1992; Spanagel *et al*, 1992; Tejeda *et al*, 2013; You *et al*, 1999; Zhang *et al*, 2004a, 2004b), and is also seen in slices in response to application of KOR agonists (Gauchy *et al*, 1991; Heijna *et al*, 1992; Lemos *et al*, 2012b; Schlösser *et al*, 1995; Werling *et al*, 1988). This inhibition is also observed in synaptosomes and in cultured dopamine neurons, confirming that the inhibition is mediated by effects within the dopamine terminal (Ronken *et al*, 1993a, 1993b). Inhibition of dopamine release also depends on expression of KOR in dopamine neurons, as it is absent in conditional knockout mice (Chefer *et al*, 2013a; Tejeda *et al*, 2013). Surprisingly, the precise mechanism of KOR-mediated inhibition of dopamine release is not understood and has not been investigated in detail. It is generally presumed to be mediated by direct $G_{\beta\gamma}$ interactions discussed above, but this has not been directly tested. Given that it is known that KOR can inhibit transmitter release by different mechanisms in different cell types (Ford *et al*, 2007; Hjelmstad and Fields, 2003; Simmons *et al*, 1994), this is to some extent an open question.

Somatodendritic effects of KOR on dopamine neuron firing. SNc and VTA dopamine neurons also express KOR somatodendritically, but it is less clear what functional role these receptors serve. Although intra-VTA injection of a KOR agonist is aversive, it fails to alter dopamine release within the NAc (Bals-Kubik *et al*, 1993a; Devine *et al*, 1993; Spanagel *et al*, 1992). This is consistent with the observation that although KOR mRNA is highly expressed in the SNc and VTA, [^{3}H]U69,593 binding is lower in these regions compared to the adjacent SNr (Mansour *et al*, 1994). Further, although it has been shown that KOR activation can inhibit the

firing of SNc dopamine neurons, the effect is much weaker by dose than the effects seen on inhibition of dopamine release in terminal regions, with an 8 mg/kg i.v. dose of U50,488 being required to induce a 40% inhibition of cell firing, while a 40% or greater inhibition of terminal dopamine release is frequently reported after a 4-5 mg/kg i.p. dose of the same drug (Walker *et al*, 1987). Nevertheless, KOR agonists can directly hyperpolarize dopamine neurons and inhibit their firing through postsynaptic activation of GIRK channels (Margolis *et al*, 2003). Indeed, evidence suggests that all neurons in the VTA that show direct hyperpolarization in response to KOR agonists are dopaminergic (Ford *et al*, 2006; Margolis *et al*, 2003).

Another possibility is that circuit differences in different populations of neurons mediate the inability of VTA KOR activation to inhibit NAc dopamine. Injections of U50,488 into the SNc reduce dopamine in the dorsal striatum (You *et al*, 1999). Further, one study has found that in rat VTA, dopaminergic cells labeled by retrograde tracer injections into the mPFC were hyperpolarized by U69,593 whereas dopaminergic cells labeled by tracer injection into the NAc were unaffected. Consistent with this, they also found that direct injection of U69,593 into the VTA inhibited dopamine efflux in the mPFC while failing to alter dopamine efflux in the NAc (Margolis *et al*, 2006a). This was interpreted as suggesting that NAc-projecting dopamine neurons are only regulated by KOR at the level of the terminal. However, contradictory data suggest that this may be a species difference. This study, along with previous studies showing an inability of intra-VTA KOR agonists to alter NAc dopamine, were performed in rats. Another study performed in mice has shown that KOR activation in dopaminergic cells labeled by tracer injection into the NAc induces an inhibitory outward current which is not observed in dopaminergic cells labeled by tracer injection into the BLA (Ford *et al*, 2006). Other experiments performed in mice have reported that mesocortical dopamine neurons have substantially lower levels of GIRK2 mRNA and protein than mesostriatal neurons, suggesting a reduced sensitivity to hyperpolarization by GIRK channels (Lammel *et al*, 2008). Further, the same study found that mesocortical dopamine neurons did not show I_h currents, whereas Margolis *et al* (2006) reported increased I_h currents in mesocortical dopamine neurons relative to mesolimbic

dopamine neurons. However, the ability of somatodendritic KOR activation to alter NAc dopamine signaling has never been directly tested in mice. As such, it is still not clear if this is a species difference or if the data can be parsimoniously explained in another fashion, perhaps involving differences in sampling sites or populations of NAc-projecting dopamine neurons.

KOR activation can also induce inhibition of dopaminergic IPSCs in dopamine neurons, a phenomenon which has been attributed to direct inhibition of dendritic dopamine release (Ford *et al*, 2006, 2007). The precise mechanism is unknown, but it does not involve voltage-dependent calcium channels, Shaker-type K+ channels, or protein kinase A, as it is unaltered by inhibitors for any of these proteins (Ford *et al*, 2007).

KOR effects on dopamine uptake. It is possible that KOR activation could also alter dopamine signaling through interactions with DAT. It has been shown that a single administration of U69,593 can induce increases in dopamine uptake in NAc synaptosomes using rotating disk voltammetry (Thompson *et al*, 2000). However, these experiments were performed in the absence of inhibitors for non-DAT methods of dopamine uptake, such as other monoamine transporters or low-affinity high-capacity transporters. Given that these other transporters can promiscuously take up dopamine, they may have mediated this increase in clearance (Daws, 2009; Hagan *et al*, 2011; Zhou *et al*, 2005). Other studies have not replicated these results: recordings of electrically stimulated dopamine release from the NAc core and shell do not show any changes in $t_{1/2}$ 15, 60, or 180 minutes after administration of Salvinorin A (Ebner *et al*, 2010). Similarly, whole brain synaptosomes do not show any change in the DAT-specific component of uptake after repeated forced swim stress using a protocol known to evoke dynorphin release in the NAc (Schindler *et al*, 2012).

KOR effects on other transmitters. KOR activation can also possibly effect dopamine transmission by altering signaling of other neurotransmitters. KOR activation can also inhibit glutamate, GABA, and serotonin within the NAc (Hjelmstad and Fields, 2001, 2003; Tao and Auerbach, 2002). Glutamate and GABA release are both inhibited by presynaptic mechanisms

(Hjelmstad and Fields, 2003); the mechanism of KOR-induced suppression of serotonin signaling is not entirely clear, but involves at least in part a p38 MAPK-dependent increase in SERT implantation into the plasma membrane (Bruchas *et al*, 2011; Schindler *et al*, 2012). Within the VTA, KOR activation can also inhibit glutamate release by a presynaptic mechanism and GABA by an unknown mechanism (Ford *et al*, 2006; Margolis *et al*, 2005a). KOR-mediated effect on VTA GABA are also circuit-specific: $GABA_A$ IPSCs are greatly inhibited in BLA-projecting but minimally affected in NAc-projecting VTA dopamine neurons; whereas $GABA_B$ IPSCs are more strongly inhibited in NAc-projecting than BLA-projecting VTA dopamine neurons. This is interpreted as suggesting that separate GABA inputs are being regulated by KOR activation in cells projecting to those sites. KOR activation can also block the induction of long-term potentiation at GABAergic synapses (LTP_{GABA}) onto dopamine neurons (Graziane *et al*, 2013; Polter *et al*, 2014).

Long-term effects of KOR activation on dopamine signaling. Repeated KOR activation can also induce long-term alterations of dopamine signaling. Repeated pretreatment with the KOR agonist U69,593 leads to a potentiation of cocaine- or amphetamine-stimulated increases in NAc dopamine 24 hours after the last treatment despite a lack of change in basal dopamine levels (Fuentealba *et al*, 2007; Heidbreder *et al*, 1998). The mechanism of this effect is unknown, but could involve KOR-induced changes in expression of the D2R. 2 days but not 10 days after a similar pretreatment paradigm, D2R binding by [^{3}H]sulpiride was significantly reduced in dorsal striatum. A similar trend was seen in the NAc at 1 and 2 days after pretreatment ended, but it was not statistically significant (Izenwasser *et al*, 1998). A later study demonstrated that D2R-stimulated inhibition of dopamine release in the dorsal striatum by the agonist quinpirole was similarly inhibited at the 2 day time point, which would suggest that the changes in binding are mediated by changes in expression within dopamine neurons (Acri *et al*, 2001). A similar loss of response of NAc dopamine to quinpirole one day after repeated U69,593 pretreatment has been observed (Fuentealba *et al*, 2006). No changes in D1 receptor binding by [^{125}I]iodosulpiride were observed after repeated U69,593, but in the NAc and dorsal striatum increases in TH

immunoreactivity and decreases in DAT binding by [^{125}I]RTI-121 were observed 3 days after repeated U69,593 pretreatment (Collins *et al*, 2001). The latter is consistent with the observation that 3 days after repeated U69,593 pretreatment, dopamine uptake is inhibited as measured in NAc synaptosomes using rotating disk voltammetry (Thompson *et al*, 2000). Inhibition of D2R expression and increases in TH expression are also consistent with the observation that after repeated U69,593, the ability of local 40 mM K+ to stimulate dopamine release is increased in both mesencephalic cultures and the NAc (Dalman and O'Malley, 1999; Fuentealba *et al*, 2006). Changes in dopamine neurons at the somatodendritic level after repeated KOR activation appear to not have been studied in detail at this time.

The extent to which KOR signaling changes after prior activation in dopamine neurons is not clear at this time. Although KOR can undergo arrestin-dependent desensitization in the manner described above, it was recently observed that exposure to repeated swim stress failed to alter U69,593-induced inhibition of electrically evoked dopamine release in the NAc core (Lemos *et al*, 2012b). However, it was previously shown in NAc slices that shortly after treatment with 100 nM U50,488 (a dose ineffective at inhibiting dopamine release), the response to 1 μM U50,488 was weakened or entirely blocked (Schlösser *et al*, 1995). Another study using microdialysis in anesthetized rats found that although U69,593-induced inhibition of K+-induced dopamine release was unaltered after repeated pretreatment, basal dopamine concentrations were now inhibited by a previously ineffective low dose of U69,593 (Fuentealba *et al*, 2006). From a behavioral perspective, CPA to U69,593 is blocked if training is started shortly after a pretreatment paradigm (Shippenberg *et al*, 1988). However, it is not clear if this effect is mediated by tolerance to the drug or by a latent inhibition towards its dysphoric effects. It is also worthy of note that dynorphin expression is sensitive to changes in dopamine signaling. Dynorphin expression is reduced after knockout of D1R or neurotoxic lesion of dopamine neurons with the selective neurotoxin 6-OHDA; in either case, it is normalized by treatment with D1R agonists (Steiner and Gerfen, 1998). Further, cocaine and amphetamine administration both increase dynorphin mRNA and peptide levels (Daunais *et al*, 1993; Steiner

and Gerfen, 1998). Given that medium spiny neurons that express dynorphin also predominately express D1R, it is possible that the ability of the dynorphin-KOR system to regulate dopamine release is normally controlled via changes in dynorphin signaling rather than KOR signaling.

One key aim of my dissertation was to determine if p38 MAPK signaling was involved in inhibition of dopamine release. Another was to determine if in mice, KOR activation in the somatodendritic compartment of NAc-projecting dopamine neurons could contribute to alterations in NAc dopamine release.

Dopamine and the aversive properties of KOR

It has been widely hypothesized that the aversive properties of KOR activation are mediated by terminal inhibition of dopamine release in the NAc (Carroll and Carlezon, 2013; Di Chiara and Imperato, 1988; Xi and Stein, 2002), but this hypothesis is primarily correlative and has not really been directly tested. Injection of the dopaminergic neurotoxin 6-OHDA into the NAc—thus selectively lesioning NAc-projecting dopamine neurons—blocks CPA to the KOR agonist U69,593 (Shippenberg *et al*, 1993). CPA is also not observed following chronic treatment with the D1 antagonist SCH 23390 during training if it is administered either systemically or directly injected into the NAc (Shippenberg and Herz, 1987, 1988; Shippenberg *et al*, 1993). Given that SCH 23390 is itself aversive (and therefore is administered prior to both vehicle and U69,593 in these CPP experiments), it has been proposed that the aversive properties of KOR activation are occluded by the aversive properties of D1R antagonism in these experiments. It is also interesting to note that SCH 23390 blocks the acquisition of cocaine CPP when administered 30 minutes before cocaine training, but potentiates the expressed place preference when administered before a post-test (Cervo and Samanin, 1995). If inhibition of dopamine release is the mechanism for KOR's aversive, reward-blocking, and reward-potentiating effects; it is plausible that they could be occluded or duplicated by administration

of an antagonist that also blocks dopamine signaling at a key site of action. However, one major caveat to this hypothesis, as mentioned above, is that alterations of D1 activity also drive changes in D1R expression (Steiner and Gerfen, 1998). As such, it is possible that alterations in the aversive response to U69,593 are mediated by D1R-induced fluctuations in dynorphin signaling during the course of the experiment. Further, CPA to the KOR agonist U50,488 is still observed in dopamine-deficient mice, suggesting that KOR-mediated effects on dopamine signaling may not be necessary for its aversive properties (Land *et al*, 2009).

The KOR agonist U50,488 induces CPA if it is directly injected into the NAc, VTA, mPFC, or the lateral hypothalamus (Shippenberg *et al*, 1993). The latter is the source of a dense glutamatergic projection to the VTA (Kempadoo *et al*, 2013); as such, injection of a KOR agonist at any of these points could in theory lead to inhibition of dopamine at target sites including the NAc. However, as discussed above, injection of KOR agonist into the VTA fails to alter dopamine signaling in the NAc (at least in rat), instead inhibiting dopamine release within the mPFC (Margolis *et al*, 2006a). CPA to KOR agonists is also blocked after local norBNI injection into the NAc or mPFC (Land *et al*, 2009; Tejeda *et al*, 2013). But recent experiments discussed above have suggested that the key role of KOR activation within the NAc may be to modulate serotonergic signaling rather than dopaminergic signaling. In this model, KOR activation in NAc-projecting serotonin terminals activated p38 MAPK, which in turn leads to an increase in SERT membrane implantation in terminals.

Finally, CPA to the KOR agonist U69,593 is blocked after conditional knockout of KOR from dopamine neurons, as is inhibition of dopamine release within the NAc (Chefer *et al*, 2013a; Tejeda *et al*, 2013). Further, global restoration of KOR to all neurons in the VTA restores both the aversion and the inhibition of NAc dopamine. However, although this demonstrates a key role of KOR activation on dopamine neurons, it does not demonstrate a key role of KOR effects on dopamine signaling *per se*. As such, as stated above, a key aim of my dissertation research was to attempt to better determine the role of KOR-mediated inhibition of dopamine release and KOR activation in dopamine neurons on mediating its aversive properties.

Chapter 2

Kappa opioid receptor activation potentiates the cocaine-induced increase in evoked dopamine release recorded in vivo in the mouse nucleus accumbens

This chapter was formatted for this thesis from the following article previously published: "Kappa opioid receptor activation potentiates the cocaine-induced increase in evoked dopamine release recorded in vivo in the mouse nucleus accumbens." Ehrich JM, Phillips PE, Chavkin C. (2014) *Neuropsychopharmacology.* In press.

J.M.E. designed and performed all of the experiments in this chapter, and wrote this chapter. P.E.M.P. also participated in the writing of experiments. C.C. also participated in designing of experiments and the writing of this chapter.

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ABSTRACT

Behavioral stressors increase addiction risk in humans and increase the rewarding valence of drugs of abuse including cocaine, nicotine and ethanol in animal models. Prior studies have established that this potentiation of drug reward was mediated by stress-induced release of the endogenous dynorphin opioids and subsequent kappa opioid receptor (KOR) activation. In this study, we used *in vivo* fast scan cyclic voltammetry to test the hypothesis that KOR activation prior to cocaine administration might potentiate the evoked release of dopamine from ventral tegmental (VTA) synaptic inputs to the nucleus accumbens (NAc) and thereby increase the rewarding valence of cocaine. The KOR agonist U50,488 inhibited dopamine release evoked by either medial forebrain bundle (MFB) or pedunculopontine tegmental nucleus (PPTg) activation of VTA inputs to the shell or core of the mouse NAc. Cocaine administration increased the dopamine response recorded in either the shell or core evoked by either MFB or PPTg

stimulation. Administration of U50,488 fifteen min prior to cocaine blocked the conditioned place preference (CPP) to cocaine, but only significantly reduced the effect of cocaine on the dopamine response evoked by PPTg stimulation to NAc core. In contrast, administration of U50,488 sixty min prior to cocaine significantly potentiated cocaine CPP and significantly increased the effects of cocaine on the dopamine response evoked by either MFB or PPTg stimulation, recorded in either NAc shell or core. Results of this study support the concept that stress-induced activation of KOR by endogenous dynorphin opioids may enhance the rewarding valence of drugs of abuse by potentiating the evoked dopamine response.

INTRODUCTION

In humans, uncontrolled stress exposure has been shown to increase vulnerability to drug abuse, with stress levels showing positive associations with alcohol consumption (DeFrank *et al*, 1987) escalation of nicotine use, and increased alcohol consumption (Miller *et al*, 1974; Pomerleau and Pomerleau, 1987). In animal models, acute stress exposure also leads to elevated self-administration of amphetamine (Piazza *et al*, 1990) and cocaine (Goeders and Guerin, 1994). Neuroendocrine mediators of the stress response including corticotropin-releasing factor (CRF) and glucocorticoids have been implicated in stress-induced reinstatement (Mantsch *et al*, 2014; Wise and Morales, 2010). In addition, a specific role of the dynorphin/kappa opioid receptor (KOR) system in stress-induced dysphoria and anxiety responses underlying the pro-addictive effects of stress has been suggested by antagonism or genetic disruption of the dynorphin/KOR system (Bruijnzeel, 2009; Knoll and Carlezon, 2010; Tejeda *et al*, 2012). For example, the ability of stress exposure to potentiate both cocaine reward and nicotine reward in the conditioned place preference (CPP) paradigm was found to be blocked by the KOR antagonist norbinaltorphimine (norBNI) and required the expression of both dynorphin and KOR (Bruchas *et al*, 2010; McLaughlin *et al*, 2003a; Schindler *et al*, 2010; Smith *et al*, 2012). Initially, the observation that KOR activation could potentiate the rewarding properties of drugs of abuse was surprising because co-administration of KOR agonists block the reward-sensitizing properties of cocaine (Shippenberg *et al*, 1996) and cocaine-induced reduction of ICSS thresholds (Tomasiewicz *et al*, 2008). Furthermore, KOR agonists inhibit dopamine release and produce aversive behavioral responses (Di Chiara and Imperato, 1988; Shippenberg and Herz, 1988).

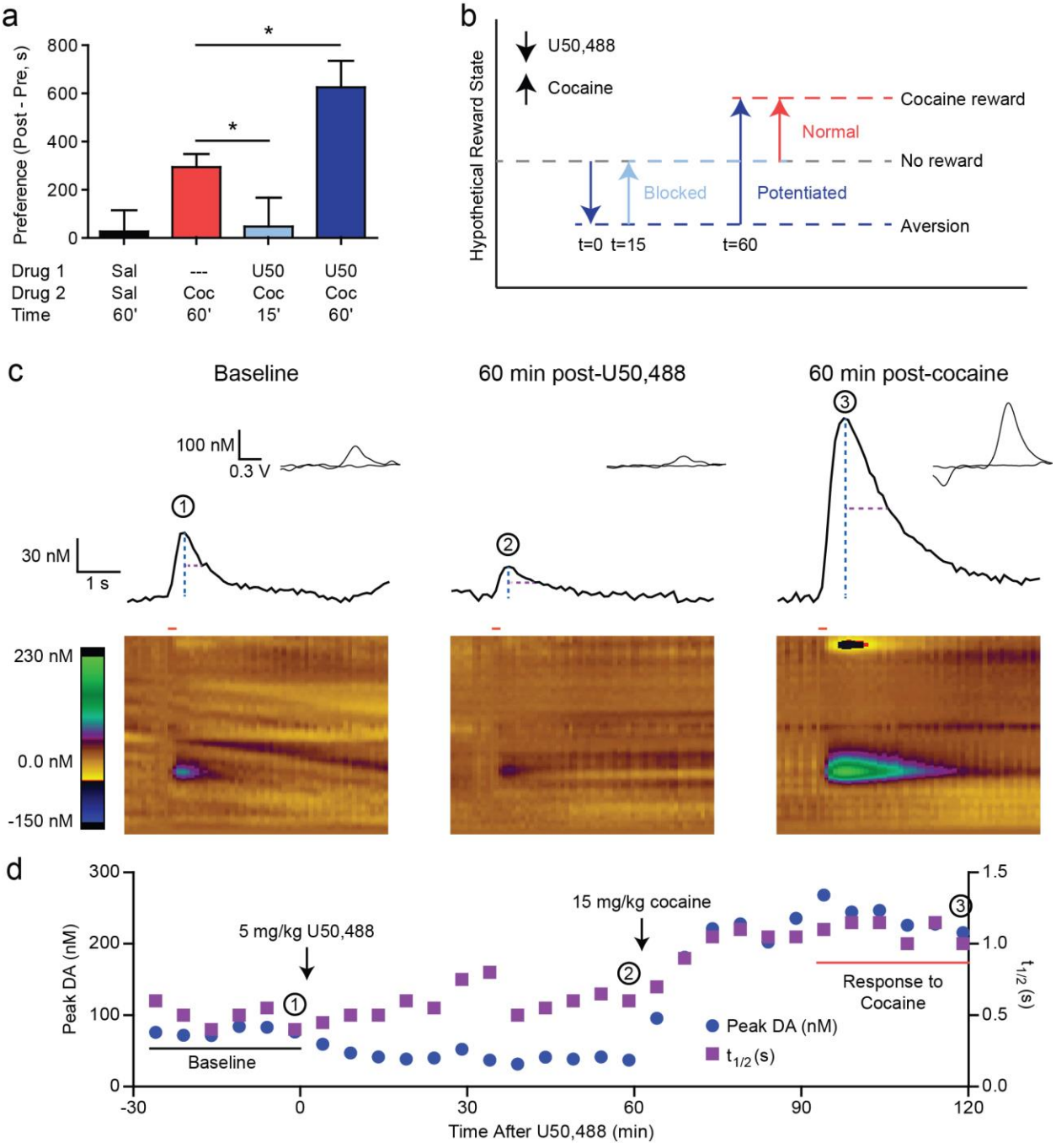
This apparent contradiction was rationalized by proposing that the dysphoric effects of KOR activation could produce aversion and block reward when paired directly with administration of a euphorigenic drug of abuse, but the magnitude of drug reward would be potentiated if it were experienced while the subject was in a dynorphin/KOR-induced dysphoric

state (Bruchas *et al*, 2010). In support of this hypothesis, pretreatment with the KOR agonist U50,488 sixty min prior to cocaine potentiated its rewarding properties in a manner similar to that seen after repeated stress exposure; whereas pretreatment with U50,488 fifteen min prior to cocaine blocked cocaine reward (Figure 2.1A) (McLaughlin *et al*, 2006a).

The prevailing view is that the dysphoric effects of aversive drugs result from inhibition of dopamine release from the VTA inputs to the NAc (Carroll and Carlezon, 2013; Shippenberg *et al*, 2001), via activation of KOR on dopaminergic nerve terminals (Tejeda *et al*, 2012). The ability of KOR agonists to induce aversion has been shown to require activation of D1 receptors in the NAc, and it is blocked by selective lesioning of mesolimbic but not nigrostriatal dopamine neurons (Shippenberg *et al*, 1993). Intriguingly, although both intra-NAc and intra-VTA microinjection of U50,488 in rat result in conditioned place aversion (Bals-Kubik *et al*, 1993a), intra-NAc but not intra-VTA injection of KOR agonists induce a reduction in NAc dopamine (Devine *et al*, 1993; Margolis *et al*, 2006a; Spanagel *et al*, 1992). This failure of intra-VTA KOR agonist administration to alter NAc dopamine levels has been attributed to the observation that in rat, medial prefrontal cortex (mPFC)-projecting but not NAc-projecting dopaminergic neurons show direct inhibition after administration of the KOR agonist U69,593 (Margolis *et al*, 2006a). Based on this result, it has been proposed that NAc-projecting neurons express functional KOR exclusively on terminals whereas mPFC-projecting neurons express KOR in both terminal and somatodendritic compartments (Margolis *et al*, 2006a; Tejeda *et al*, 2013). However, in mouse it has been shown that U69,593 can directly inhibit NAc-projecting dopaminergic neurons (Ford *et al*, 2006). The discrepant results of these experiments may be due to a species difference or some other experimental factor. As such, it is not clear if KOR activation in the terminal and somatodendritic compartments can separately regulate NAc dopamine signaling in mice.

If the dysphoric actions of KOR are mediated by presynaptic inhibition of dopamine release, then the potentiation of cocaine reward resulting in enhanced CPP might be evident in the interactions controlling accumbal dopamine levels. In this model (Figure 2.1B), KOR agonist

pretreatment at short pretreatment times (15 min or less) would be predicted to block cocaine reward by cancelling cocaine-induced effects on dopamine signaling. At longer pretreatment times, reduced dopamine release effectively resets the baseline, allowing an increased behavioral response to cocaine caused by the increased elevation of dopamine levels relative to this modified baseline. In the present study, we tested this model using FSCV to measure evoked dopamine release in the core or shell of the NAc following medial forebrain bundle (MFB) or pedunculopontine tegmental nucleus (PPTg) stimulation, which stimulate dopaminergic axons directly or excitatory afferents to the VTA (Floresco *et al*, 2003; Zweifel *et al*, 2009).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Baseline
60 min post-U50,488
60 min post-cocaine
100 nM
0.3 V
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230 nM
0.0 nM
-150 nM
d
Peak DA (nM)
5 mg/kg U50,488
15 mg/kg cocaine
Response to Cocaine
Baseline
Peak DA (nM)
t1/2 (s)
Time After U50,488 (min)

Figure 2.1. Kappa opioid receptor activation affects cocaine reward and dopamine responses in a time-dependent manner.

(a) Preference scores for animals receiving cocaine after no pretreatment or after pretreatment with U50,488 either 15 or 60 min prior shows the ability of KOR activation to either block or potentiate cocaine reward depending on time of pretreatment. Modified from McLaughlin 2003. Error bars represent SEM. *(b)* A conceptual model to explain the behavioral results depicted in (a). If cocaine and KOR agonist are co-administered at close time points (20 min or less), then KOR agonist-induced decreases in reward state ("Aversion") and cocaine-induced increases in reward state simultaneously co-occur and thus lead to a mutual block of preference and aversion ("Blocked"/"No reward"). At longer pretreatment time points, KOR agonist-induced dysphoria leads to a re-setting of the animal's internal reward state. When cocaine is then administered ("Cocaine reward"), it leads to a subjectively greater experience of reward ("Potentiated," relative to saline-pretreated controls, "Normal") even if the absolute magnitude of the reward state experienced after cocaine is unchanged. *(c)* Representative images showing analysis of FSCV data at baseline, after administration of U50,488, and after subsequent administration of cocaine. Bottom: color plots showing current generated by oxidation and reduction at the site of the working electrode during application of the triangle wave form. Red line shows time of MFB stimulation (6 pulses at 30 Hz, 300 uA). Middle: current by time plot shows evoked dopamine release (peak amplitude marked in blue) and uptake (half-life marked in purple) . Scale bars mark 30 nM dopamine and 1 s. Top: the cyclic voltammagram from the time of peak current confirms that the recorded current is induced by oxidation and reduction of dopamine. Scale bars mark 100 nM dopamine and 0.3 V from applied waveform. *(d)* Representative data from the entire recording of the animal shown in (c). Blue circles show change in the peak amplitude of the evoked dopamine current over time after administration of U50,488 (after t = 0 as marked) and cocaine (after t = 60 as marked). Purple squares show the half-life of evoked dopamine for each recording. Data shown in (c) is taken from time points 1, 2, and 3 as labeled.

METHODS

Conditioned Place Preference. Male C57Bl/6 wild-type mice were used in place-conditioning studies, as previously described (McLaughlin *et al*, 2003a). Mice were placed in a three-chamber box prior to any treatment and allowed to freely explore. After two intervening days of treatment with saline, U50,488 (5 mg/kg, *i.p.*), and/or cocaine (15 mg/kg, *s.c.*) in one chamber and saline in the other, on the fourth day mice were again allowed to freely explore the chamber. The difference in time spent in the drug-paired chamber on the pre- and post-training test days was recorded as the place preference.

In vivo FSCV. Male C57Bl/6 wild-type mice were anesthetized with 1.5 g/kg urethane. Sixty min later, anesthesia was confirmed and mice were mounted on a Model 900 stereotaxic alignment system (David Kopf Instruments). Carbon fiber (grade 34-700; Goodfellow Corporation) was pulled through a glass pipette using a Sutter P-97 puller to form a microelectrode. The fiber was then cut to approximately 120-140 μm past the glass seal. To detect dopamine, the potential at the working electrode was held at -0.4V versus an Ag/AgCl reference electrode, then ramped to +1.3V and then back to -0.4V at 400V/s. This waveform was applied at a rate of 10Hz. Carbon fiber microelectrodes were implanted in the NAc (A/P = +1.52mm, M/L = +/- 1.15mm, D/V = -3.9-4.1 (core) or -4.2-4.4 (shell) from bregma) and the reference electrode was implanted in contralateral cortex. Bipolar parallel stimulation electrodes were implanted either in the medial forebrain bundle (MFB) (A/P = -2.40mm, M/L = +/-1.10mm, D/V = -4.5-5.20mm from bregma) or the PPTg (A/P = -0.68mm from lambda, M/L = +/- 0.70mm, D/V = -2.50-3.20mm from bregma).

Electrode calibrations. Electrodes were calibrated by recording the in vitro response to an injection of 1 μM dopamine in aCSF. For each electrode, this was performed three times and the result was averaged. The average of all of these calibrations (61.4 nA current per 1 μM dopamine, data not shown) was used to convert current to dopamine concentration.

Histology and confirmation of recording sites. At the end of each experiment, urethane (0.5 ml of 1.5 g/kg) was injected into the mouse prior to inducing a lesion at the site of the working

electrode and, in a subset of animals, the lateral pole of the stimulating electrode. The mouse was then intracardially perfused with ice-cold PBS 4% paraformaldehyde and the brain was removed. After cryoprotection with 30% sucrose, brains were sliced at 40 μm and then stained with cresyl violet to identify the recording site (Figure 2.2) and the stimulation site (Figure 2.3). Recording position was also verified by measuring the change in evoked response as the electrode tip was lowered through the anterior commissure (Figure 2.4). Data were excluded from the subsequent analysis if a lesion was not clearly present in the core or shell and the anterior commissure was not found during positioning of the working electrode. Similarly, data were excluded from subsequent analysis if stimulation lesions or stimulation electrode tracks were found not to be in the vicinity of the PPTg.

Stimulus sweeps. For all stimulation sweeps, one stimulation parameter (e.g. current intensity, duration, or frequency) was varied while the other two were fixed. For stimulation sweep data paired with U50,488 or cocaine administration, two sweeps were collected at each stimulus parameter performed in first in an ascending then descending intensity for each parameter. The results of these two sweeps for each set of stimulus parameters were averaged together for a single replicate. These pairs of sweeps were performed before and starting 30 minutes after administration of U50,488 (5 mg/kg *i.p.*) or cocaine (15 mg/kg *s.c.*). Pilot data demonstrated that U50,488 administration induced a consistent inhibition of evoked dopamine release for at least the period from 30-90 minutes after injection whereas cocaine administration plateaued for a period from 30-60 minutes after injection. As such, sweeps were performed of all 3 stimulus parameters for U50,488 while only sweeps of duration and frequency were performed for cocaine. For stimulation sweep data comparing basal levels of MFB- and PPTg-evoked dopamine from NAc shell and core, only one sweep was performed. This was either collected from the first sweep performed in animals receiving U50,488, or a single sweep which was performed prior to taking recordings from animals in experiments studying interactions between U50,488 and cocaine.

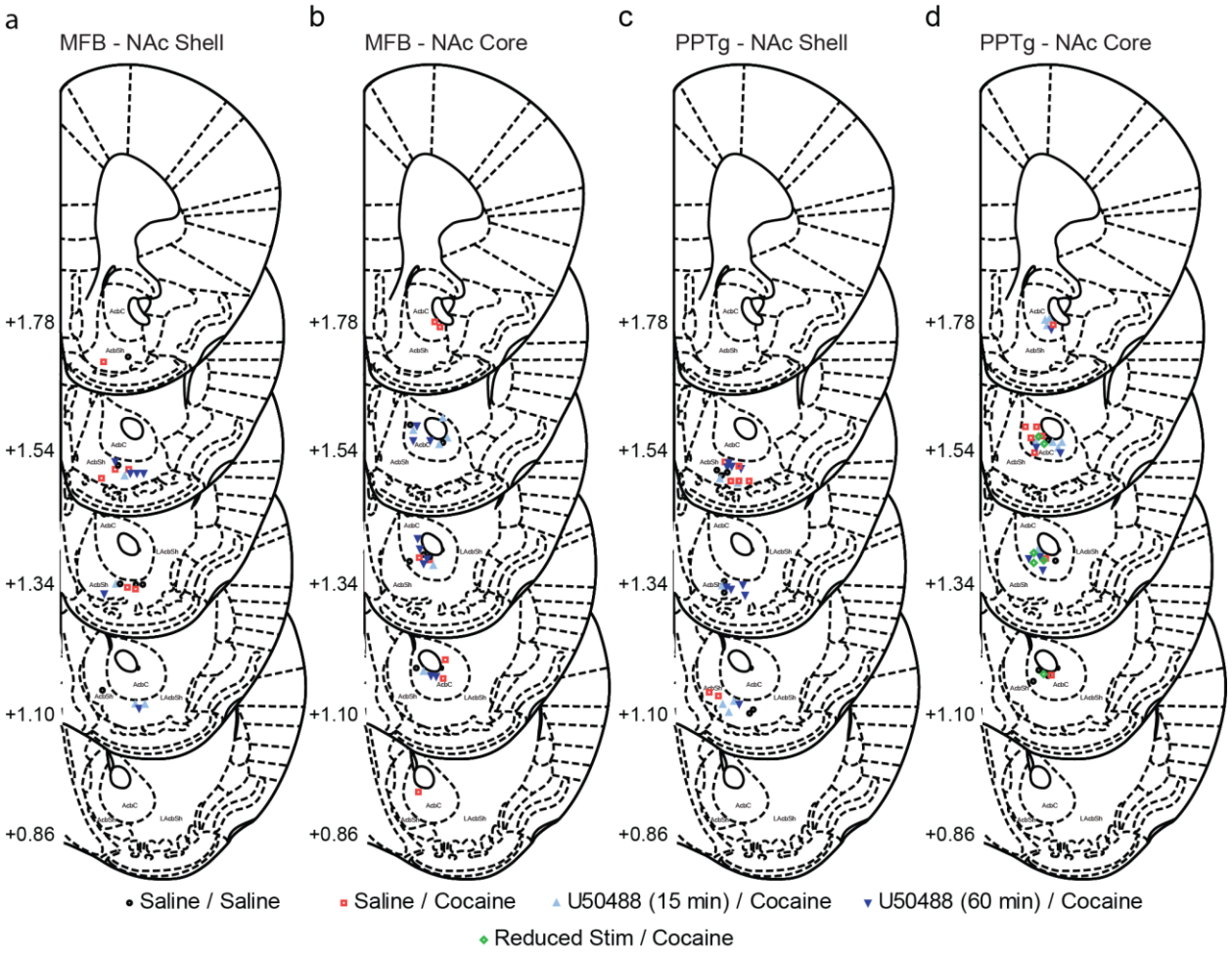

Figure 2.2. Recording sites for experiments analyzing interactions between KOR activation and cocaine.

(a) Histologically confirmed recording sites for animals in which MFB-evoked dopamine release was recorded from the shell of the nucleus accumbens. For one animal in the U50,488 (60 min) / Cocaine group, inclusion was based on electrode lowering data after we were unable to histologically confirm the recording site. *(b)* Histologically confirmed recording sites for animals in which MFB-evoked dopamine release was recorded from the core of the nucleus accumbens. *(c)* Histologically confirmed recording sites for animals in which PPTg-evoked dopamine release was recorded from the shell of the nucleus accumbens. For two animals in the Saline / Cocaine group, inclusion was based on electrode lowering data after we were unable to histologically confirm the recording site. *(d)* Histologically confirmed recording sites for animals in which PPTg-evoked dopamine release was recorded from the core of the nucleus accumbens.

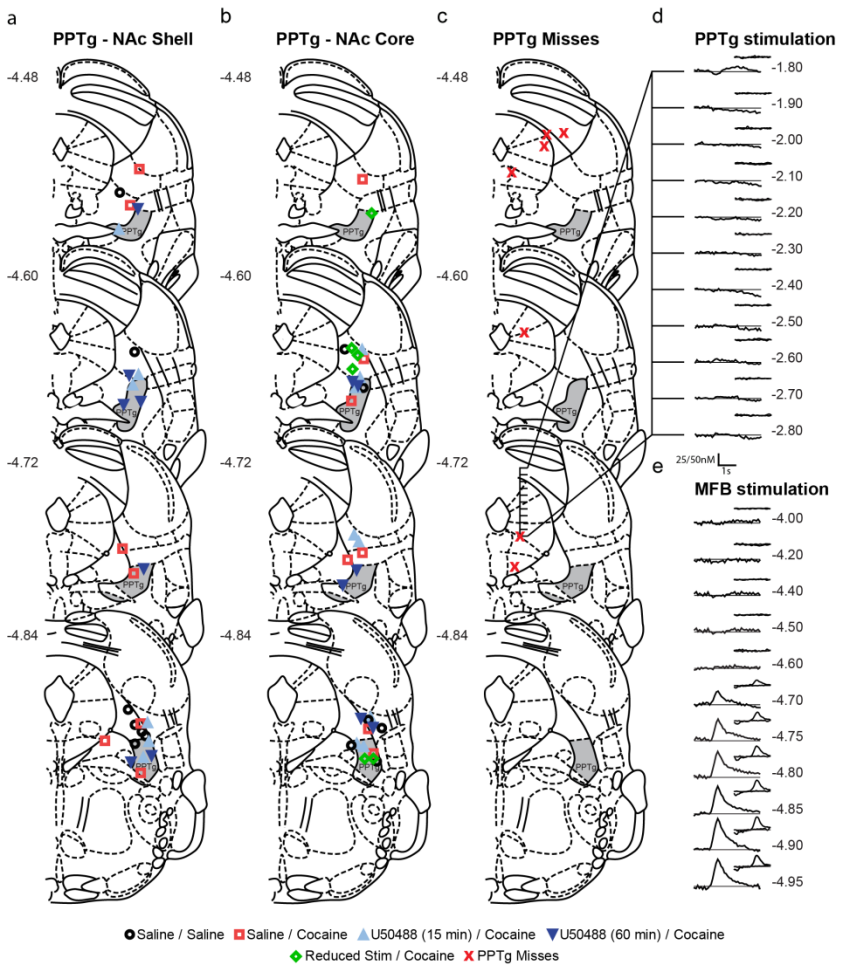

Figure 2.3. Histological analysis of stimulation sites for experiments analyzing interactions between KOR activation and cocaine confirms that stimulation electrodes must be near PPTg to evoke terminal dopamine release.

(a) Histologically confirmed stimulation sites show the location of the lateral stimulating electrode for animals in which PPTg-evoked dopamine release was recorded from the shell of the nucleus accumbens. *(b)* Histologically confirmed stimulation sites show the location of the lateral stimulating electrode for animals in which PPTg-evoked dopamine release was recorded from the core of the nucleus accumbens. *(c)* Histologically confirmed stimulation sites show the location of the lateral electrode for animals in which midbrain electrical stimulation failed to evoke dopamine release from the nucleus accumbens, but medial forebrain bundle stimulation successfully evoked dopamine release. *(d)* Raw current over time plots show a lack of dopamine release resulting from electrical stimulation (60 pulses at 60 Hz, 200 μA) while lowering the stimulation electrode to the site marked in (c). Insets accompanying each stimulation show the respective cyclic voltammagram displaying an absence of the stereotyped dopamine wave form. *(e)* Raw current over time plots show dopamine release evoked from electrical stimulation (24 pulses at 60 Hz, 50 μA) while lowering the stimulation electrode into the medial forebrain bundle with the recording electrode in the same position as the currents recorded in (d). Insets accompanying each stimulation show the respective cyclic voltammagram displaying the stereotyped dopamine wave form. Scale bar shows 25nM/50nM respectively for current by time plots and cyclic voltammagrams, and 1s for current by time plots.

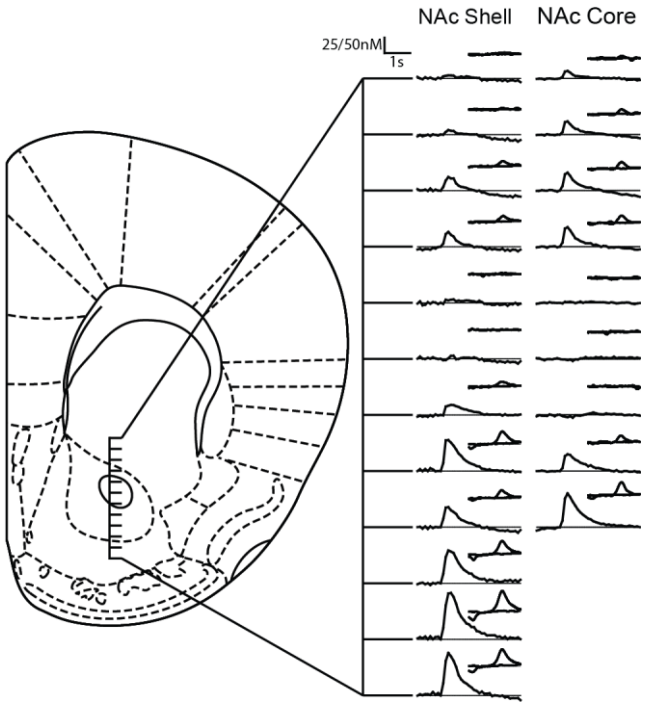

Figure 2.4. Electrode lowering data confirm that PPTg stimulation electrically evokes dopamine release in the nucleus accumbens and that evoked dopamine release can determine position of anterior commissure.

Left, cartoon adapted from Franklin & Paxinos 2007 shows intended recording site in nucleus accumbens core & shell. *Right,* raw current over time plots show representative examples of dopamine release resulting from electrical stimulation (60 pulses at 60 Hz, 150 μA) of the PPTg at different depths of the working electrode, starting at 1.2 mm from the final recording site for the shell of the nucleus accumbens and 0.9 mm from the final recording site for the core. Dopamine currents were not seen while the electrode was lowered through the anterior commissure, providing additional confirmation of the recording site location. Insets accompanying each stimulation show the respective cyclic voltammagram displaying the stereotyped dopamine wave form. Scale bar shows 50nM/100nM respectively for current by time plots and cyclic voltammagrams, and 1s for current by time plots.

Stimulus parameters. A series of pilot experiments were performed utilizing sweeps of varying stimulus intensities to optimize the recording parameters in both shell and core (Figures 1.5-1.8). A pulse width of 4.0 ms was used for all stimuli performed throughout all experiments. Varying the current intensity, the number of applied pulses, and the stimulation frequency applied to the MFB affected the amplitude of the evoked responses recorded in shell and core, and there was no significant difference in sensitivity between the two recording sites (Figure 2.5A-C). Varying the stimulation parameters applied to the PPTg also affected the amplitude of the evoked dopamine responses recorded in shell and core, however, the amplitude of the evoked response was significantly smaller in core than shell (Figure 2.5D-F). Furthermore, the stimulus required to evoke half-maximal response was significantly smaller following PPTg stimulation than MFB (Figure 2.5G-I). U50,488 (5mg/kg, i.p.) significantly reduced the dopamine response evoked by MFB stimulation at each of the current intensities 100-400 μA, applied pulse numbers >=20 and stimulus frequencies >=40 Hz (Figure 2.6A-C). Although a main inhibitory effect of U50,488 was observed in each case, a significant interaction was also observed for each stimulus parameter, indicating that for MFB-evoked dopamine the effect of KOR activation varied as stimulus parameters were varied (Figures 1.6A, 1.6C, and 1.6D). A subsequent analysis revealed that the stimulus current required to evoke the half-maximal response was also significantly increased after U50,488 administration (Figure 2.6B). As a result, for MFB experiments stimulus parameters of 6 pulses at 30 Hz and 300 uA were chosen to reflect the physiological burst-firing pattern of a dopaminergic neuron (Hyland *et al*, 2002). Parallel analysis of U50,488 effects on PPTg stimulated responses also showed a main inhibitory effect of U50,488 in each case; however, a significant interaction was only observed for stimulus duration (Figure 2.7A-C). Follow-up analysis revealed that U50,488 administration did not significantly affect the stimulus required to evoke the half-maximal response to PPTg stimulation (Figure 2.7D-F). We next analyzed the effects of cocaine administration on PPTg-stimulated dopamine across different stimulus parameters (Figure 2.8). The ability of cocaine to increase terminal dopamine concentrations was demonstrated by a main effect of cocaine

administration for varying stimulus durations and frequencies; however, an interaction was again only observed for stimulus duration (Figure 2.8A-B). Follow-up analysis revealed that cocaine administration did not significantly affect the stimulus required to evoke the half-maximal response to PPTg stimulation (Figure 2.8C-D). Based on this analysis, we chose a set of PPTg stimulation parameters able to produce stable and significant responses to U50,488 and cocaine (30 pulses at 60 Hz and 150 uA, applied every 300 sec). These PPTg stimuli evoked similar levels of dopamine release to that evoked by the MFB stimulation parameters (Figure 2.9).

Drugs. (+/-) U50,488H and cocaine hydrochloride were provided by the National Institute of Drug Abuse Drug Supply Program. Urethane was obtained from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in sterile saline for use *in vivo*.

FSCV Data collection. Experiments begin with 60 min of stimulation every 600s. The recordings from the last six of these stimuli were averaged together to form a baseline. As illustrated in Figure 2.1D, after a stable baseline of dopamine responses was established, mice were injected with either saline or U50,488 (5 mg/kg, *i.p.*). Then 15 min later, one group of mice received an injection of cocaine (15 mg/kg, *s.c.*; the U50,488 fifteen min pretreatment group). The other three groups of mice received injections of saline or cocaine 60 min after the first injection. For experiments analyzing interactions between U50,488 and cocaine, the last six stimuli prior to the first injection were averaged together to form the first baseline. The response to cocaine was determined by averaging across the six stimuli comprising the period from 35-60 min after cocaine injection or the second saline injection. The response to U50,488 (data collected only from animals receiving U50,488 sixty min prior to cocaine) was determined by averaging across the six stimuli comprising the period from 35-60 min after U50,488 injection. The acute response to cocaine or saline (%2nd baseline dopamine) was calculated using an alternate baseline comprising the six stimuli collected prior to the last injection. For MFB experiments, dopamine clearance rate was estimated as t1/2, which is the time for the dopamine current to recover to half of peak.

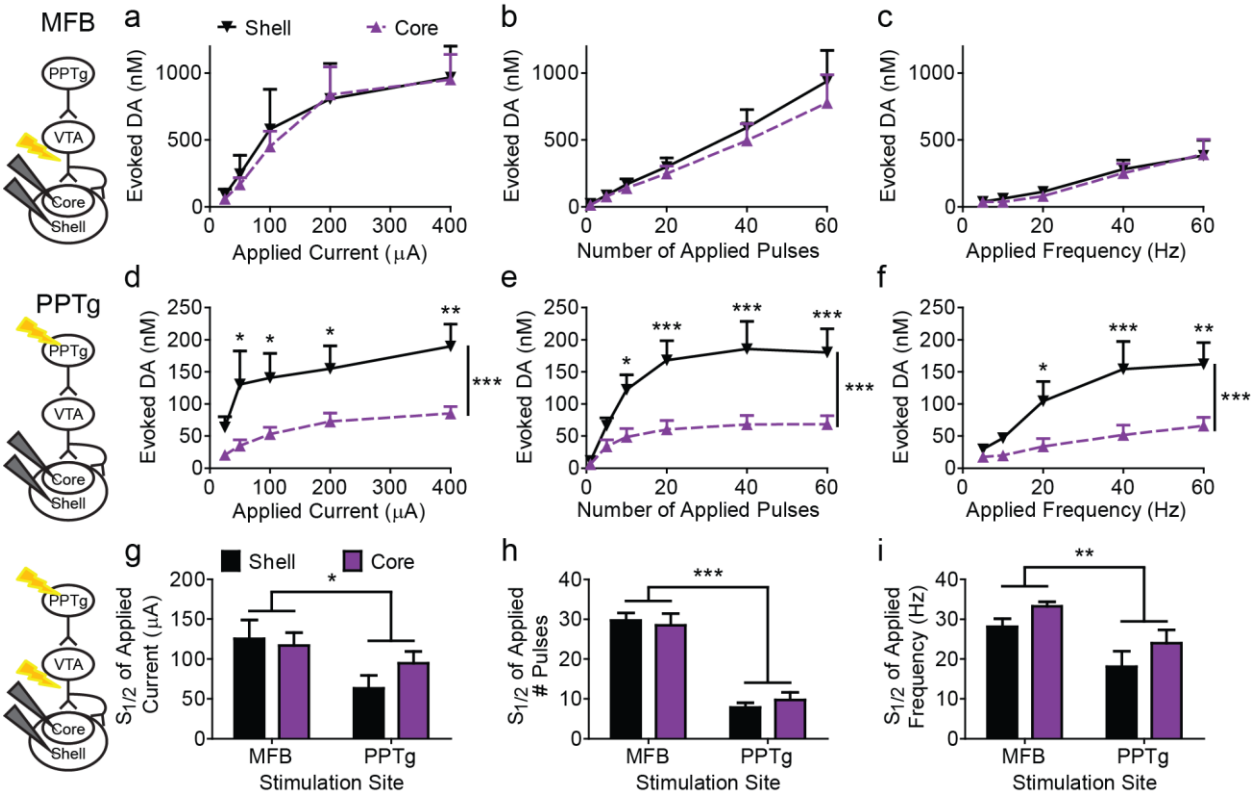

Figure 2.5. PPTg stimulation preferentially activates shell-projecting dopamine neurons, and PPTg stimulation saturates at weaker stimulus parameters than MFB stimulation.

To better understand the separate ability of electrical stimulation of the MFB or the PPTg to evoke dopamine release in the core and shell of the nucleus accumbens, a sweep of stimulus parameters (current, duration, and frequency) was performed for each stimulation and recording site. Stimulus intensities consistently increased recorded levels of evoked dopamine regardless of stimulus parameter, stimulation site, and recording site. For MFB stimulations, evoked dopamine responses were consistent across recording site. By contrast, levels of PPTg-evoked dopamine were greater in the NAc shell than the NAc core, suggesting that shell-projecting dopamine neurons receive stronger inputs from the PPTg than core-projecting dopamine neurons. Finally, the half-peak strength of stimulus intensity was consistently greater for responses to MFB stimulation than PPTg.

(a) Results are shown of experiments in which sweeps of MFB stimulus current intensities were conducted with a fixed duration (60 pulses) and frequency (60 Hz) while recording from the NAc core or shell. Repeated measures two-way ANOVA shows a main effect of applied current ($F_{(4, 60)}$=8.814, $p < 0.0001$) but no difference between recording sites ($F_{(1, 60)}$=0.1524, $p > 0.05$) and no interaction ($F_{(4,60)}$=0.05722, $p > 0.05$) was observed. *(b)* Results are shown of experiments in which various durations of MFB stimuli were applied at a fixed intensity (150 μA) and frequency (60 Hz) while recording from the NAc core or shell. Repeated measures two-way ANOVA shows a main effect of stimulus duration ($F_{(5, 72)}$=17.43, $p < 0.0001$) but no difference between recording sites ($F_{(1, 72)}$=0.9001, $p > 0.05$) and no interaction ($F_{(5, 72)}$=0.1544, $p > 0.05$) was observed. *(c)* Results are shown of experiments in which various stimulus frequencies were applied to MFB with a fixed intensity (150 μA) and duration (30 pulses) while recording from the NAc core or shell. Repeated measures two-way ANOVA shows a main effect of stimulus frequency ($F_{(4, 60)}$=12.92, $p < 0.0001$) but no difference between recording sites ($F_{(1, 60)}$=0.1690, $p > 0.05$) and no interaction ($F_{(4, 60)}$=0.04380, $p > 0.05$) was observed. *(d)* Results are shown of experiments in which various current intensities were applied to PPTg while duration (60 pulses) and frequency (60 Hz) were fixed and recording from the NAc core or shell. Repeated measures two-way ANOVA shows a main effect of applied current ($F_{(4, 85)}$=5.352, $p < 0.001$) and a main effect of recording site (***, $F_{(1, 85)}$=35.89, $p < 0.0001$), but no interaction ($F_{(4,85)}$=0.5921, $p > 0.05$) was observed. *, $p < 0.05$; **, $p < 0.01$; Bonferroni post-hoc analysis. *(e)* Results are shown of experiments in which PPTg stimulus duration was varied while intensity (150 μA) and frequency (60 Hz) were fixed and recordings made in the NAc core or shell. Repeated measures two-way ANOVA shows a main effect of stimulus duration ($F_{(5, 102)}$=12.74, $p < 0.0001$), a main effect of recording site (***, $F_{(1, 102)}$=47.80, $p < 0.0001$), and an interaction ($F_{(5, 102)}$=3.092, $p < 0.05$). *, $p < 0.05$; ***, $p < 0.001$; Bonferroni post-hoc analysis. *(f)* Results are shown of experiments in which MFB stimulus frequencies were varied while intensity (150 μA) and duration (30 pulses) were fixed and recordings made in the NAc core or shell. Repeated measures two-way ANOVA shows a main effect of stimulus frequency ($F_{(4, 85)}$=9.811, $p < 0.0001$) and a main effect of recording site (***, $F_{(1, 85)}$=28.71, $p < 0.0001$). A borderline interaction ($F_{(4, 85)}$=2.479, p=0.0500) was also observed. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Bonferroni post-hoc analysis. *(g)* Results are shown of experiments in which the half-peak strength of current intensity ($S_{1/2}$) was compared across stimulation and recording sites. Two-way ANOVA shows a main effect of stimulation site (*, $F_{(1, 29)}$=5.430, $p < 0.05$), but no effect of recording site ($F_{(1, 29)}$=0.3952, $p > 0.05$) and no interaction ($F_{(1, 29)}$=1.194, $p > 0.05$). *(h)* Results are shown of experiments in which the half-peak strength of stimulus duration ($S_{1/2}$) was compared across stimulation and recording sites. Two-way ANOVA shows a main effect of stimulation site (***,$F_{(1, 29)}$=90.68, $p < 0.0001$), but no effect of recording site ($F_{(1, 29)}$=0.02140, $p > 0.05$) and no interaction ($F_{(1, 29)}$=0.5386, $p > 0.05$). *(i)* Results are shown of experiments in which the half-peak strength of stimulus frequency ($S_{1/2}$) was compared across stimulation and recording sites. Two-way ANOVA shows a main effect of stimulation site (**,$F_{(1, 29)}$=8.911, $p < 0.01$), but no effect of recording site ($F_{(1, 29)}$=2.953, $p > 0.05$) and no interaction ($F_{(1, 29)}$=0.01420, $p > 0.05$). Error bars represent SEM.

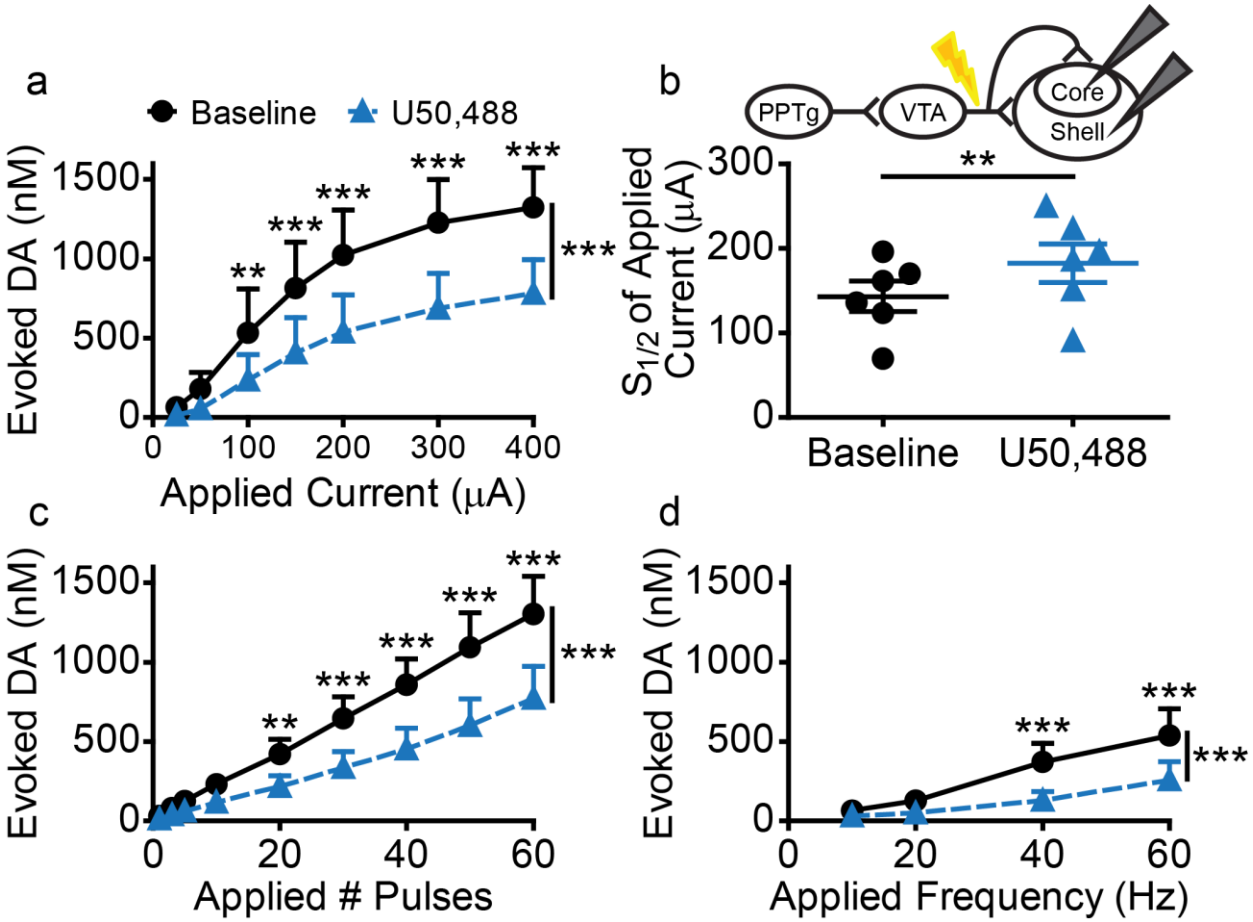

Figure 2.6. Inhibition of dopamine release caused by KOR activation varies by stimulus intensity applied to MFB.

To characterize the ability of KOR activation at nerve terminals to inhibit evoked NAc dopamine responses at different stimulus intensities, a sweep of stimulus parameters was performed before and after administration of U50,488 (5 mg/kg, *i.p.*) with bipolar stimulating electrodes placed in the MFB. U50,488 consistently decreased levels of evoked dopamine regardless of stimulus parameter. However, for each stimulus parameter repeated measures two-way ANOVA found an interaction between the effect of U50,488 and the intensity of the stimulus parameter. One-way ANOVAs performed to analyze the percent change from the recordings taken prior to U50,488 administration showed a significant effect for the stimulus current intensity but not for stimulus duration or stimulus frequency. Finally, the half-peak strength of stimulus intensity was consistently increased after administration of U50,488. Given that this data set consistently suggested the ability of KOR activation to induce changes in dopamine release that varied by stimulus intensity, and further showed a shift in the half-peak strength of stimulus intensity after KOR activation, we chose to utilize stimulus parameters for future MFB experiments that modeled the physiological burst firing of dopamine neurons (Hyland *et al*, 2002).

(a) Results of experiments in which MFB stimulus current intensity was varied while duration (60 pulses) and frequency (60 Hz) were fixed and recording from the NAc before and after administration of U50,488 (5 mg/kg, *i.p.*). Repeated measures two-way ANOVA shows a significant decrease in evoked NAc dopamine after administration of U50,488 (***, $F_{(1, 35)}$=135.0, $p < 0.0001$), a main effect of applied current intensity ($F_{(6, 35)}$=3.745, $p < 0.01$), and an interaction ($F_{(6, 35)}$=6.244, $p < 0.001$). One-way ANOVA showed that MFB stimulus current intensity significantly altered the percent change from baseline caused by treatment with U50,488 (data not shown; $F_{(6, 30)}$=2.470, $p < 0.05$). **, $p < 0.01$; ***, $p < 0.001$; Bonferroni post-hoc analysis. *(b)* Results are shown of experiments in which the half-peak strength of MFB stimulus current intensity ($S_{1/2}$) was compared before and after administration of U50,488 (5 mg/kg, *i.p.*). T test shows a significant increase in $S_{1/2}$ after administration of U50,488 ($t_{(5)}$=6.796, $p < 0.01$). *(c)* Results are shown of experiments in which MFB stimulus duration was varied while current intensity (150 μA) and frequency (60 Hz) were fixed and recording from the NAc before and after administration of U50,488 (5 mg/kg, *i.p.*). Repeated measures two-way ANOVA shows a significant decrease in evoked NAc dopamine after administration of U50,488 (***, $F_{(1, 45)}$=207.4, $p < 0.0001$), a main effect of stimulus duration ($F_{(8, 45)}$=9.731, $p < 0.0001$), and an interaction ($F_{(8, 45)}$=15.54, $p < 0.0001$). One-way ANOVA did not show a significant effect of MFB stimulus duration on the percent change from baseline caused by treatment with U50,488 (data not shown; $F_{(8, 40)}$=1.183, $p > 0.05$). **, $p < 0.01$; ***, $p < 0.001$; Bonferroni post-hoc analysis. *(d)* Results are shown of experiments in which MFB stimulus frequency was varied while current intensity (150 μA) and duration (30 pulses) were fixed and recording from the NAc before and after administration of U50,488 (5 mg/kg, *i.p.*). Repeated measures two-way ANOVA revealed a significant decrease in evoked NAc dopamine after administration of U50,488 (***, $F_{(1, 20)}$=40.04, $p < 0.0001$), a main effect of stimulus frequency ($F_{(3, 20)}$=3.796, $p < 0.05$), and an interaction ($F_{(3, 20)}$=5.748, $p < 0.01$). One-way ANOVA analyzing the percent change from baseline did not show a significant effect of MFB stimulus frequency on the percent change from baseline caused by treatment with U50,488 (data not shown; $F_{(3, 15)}$=2.255, $p > 0.05$). ***, $p < 0.001$; Bonferroni post-hoc analysis. Error bars represent SEM.

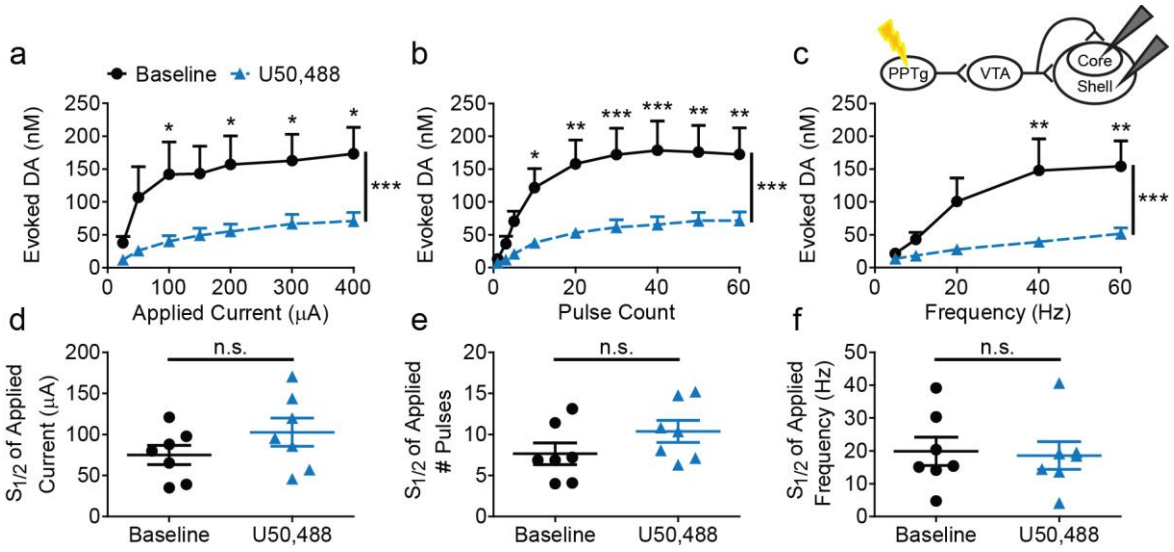

Figure 2.7. Inhibition of dopamine release caused by KOR activation does not vary by stimulus intensity applied to PPTg.

To characterize the ability of somatodendritic KOR activation to inhibit evoked NAc dopamine responses at different stimulus intensities, a sweep of stimulus parameters was performed before and after administration of U50,488 (5 mg/kg, *i.p.*) with bipolar stimulating electrodes placed in the PPTg. U50,488 consistently decreased levels of evoked dopamine regardless of stimulus parameter. In contrast to the effects found during MFB stimulation, for PPTg stimulation repeated measures two-way ANOVA only found an interaction between the effect of U50,488 and the stimulus duration. One-way ANOVA performed to analyze the percent change from the recordings taken prior to U50,488 administration revealed no significant effect of any stimulus parameter. Administration of U50,488 also did not significantly alter the half-peak strength of stimulus intensity. Given that this data set consistently suggested that combined activation of terminal and somatodendritic KORs induced changes in dopamine release that did not vary by intensity of PPTg stimulation, we chose to utilize PPTg stimulus parameters that induced basal levels of dopamine release comparable to those observed after MFB stimulation.

(a) Results are shown of experiments in which PPTg stimulus current intensity was varied while duration (60 pulses) and frequency (60 Hz) were fixed and recording from the NAc before and after administration of U50,488 (5 mg/kg, *i.p.*). Repeated measures two-way ANOVA shows a significant decrease in evoked NAc dopamine after administration of U50,488 (***, $F_{(1, 42)}$=45.59, $p < 0.0001$), but no interaction ($F_{(6, 42)}$=0.6736, $p > 0.05$) was observed. One-way ANOVA showed that PPTg stimulus current intensity did not significantly alter the percent change from baseline caused by treatment with U50,488 (data not shown; $F_{(6, 36)}$=0.6767, $p > 0.05$). *, $p < 0.05$; Bonferroni post-hoc analysis. *(b)* Results are shown of experiments in which PPTg stimulus duration was varied while current intensity (150 μA) and frequency (60 Hz) were fixed and recording from the NAc before and after administration of U50,488 (5 mg/kg, *i.p.*). Repeated measures two-way ANOVA shows a significant decrease in evoked NAc dopamine after administration of U50,488 (***, $F_{(1, 54)}$=79.19; $p < 0.0001$) and an interaction ($F_{(8, 54)}$=2.399; $p < 0.05$). One-way ANOVA showed that PPTg stimulus duration did not significantly alter the percent change from baseline caused by treatment with U50,488 (data not shown; $F_{(8, 48)}$=0.9991; $p > 0.05$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Bonferroni post-hoc analysis. *(c)* Results are shown of experiments in which PPTg stimulus frequency was varied while current intensity (150 μA) and duration (30 pulses) were fixed and recording from the NAc before and after administration of U50,488 (5 mg/kg, *i.p.*). Repeated measures two-way ANOVA shows a significant decrease in evoked NAc dopamine after administration of U50,488 (***, $F_{(1, 30)}$=25.87, $p < 0.0001$), but no interaction ($F_{(4, 30)}$=2.668, $p > 0.05$) was observed. One-way ANOVA showed that PPTg stimulus frequency did not significantly alter the percent change from baseline caused by treatment with U50,488 (data not shown; $F_{(4, 24)}$=1.554, $p > 0.05$). **, $p < 0.01$; Bonferroni post-hoc analysis. *(d)* Results are shown of experiments in which the half-peak strength of PPTg stimulus current intensity ($S_{1/2}$) was compared before and after administration of U50,488 (5 mg/kg, *i.p.*). T test showed that U50,488 failed to alter $S_{1/2}$ ($t_{(6)}$=1.980, $p > 0.05$). *(e)* Results are shown of experiments in which the half-peak strength of PPTg stimulus duration ($S_{1/2}$) was compared before and after administration of U50,488 (5 mg/kg, *i.p.*). T test showed that U50,488 failed to alter $S_{1/2}$ ($t_{(6)}$=2.362, $p > 0.05$). (f) Results are shown of experiments in which the half-peak strength of PPTg stimulus duration ($S_{1/2}$) was compared before and after administration of U50,488 (5 mg/kg, *i.p.*). T test showed that U50,488 did not significantly alter $S_{1/2}$ ($t_{(6)}$=0.2172, $p > 0.05$). Error bars represent SEM.

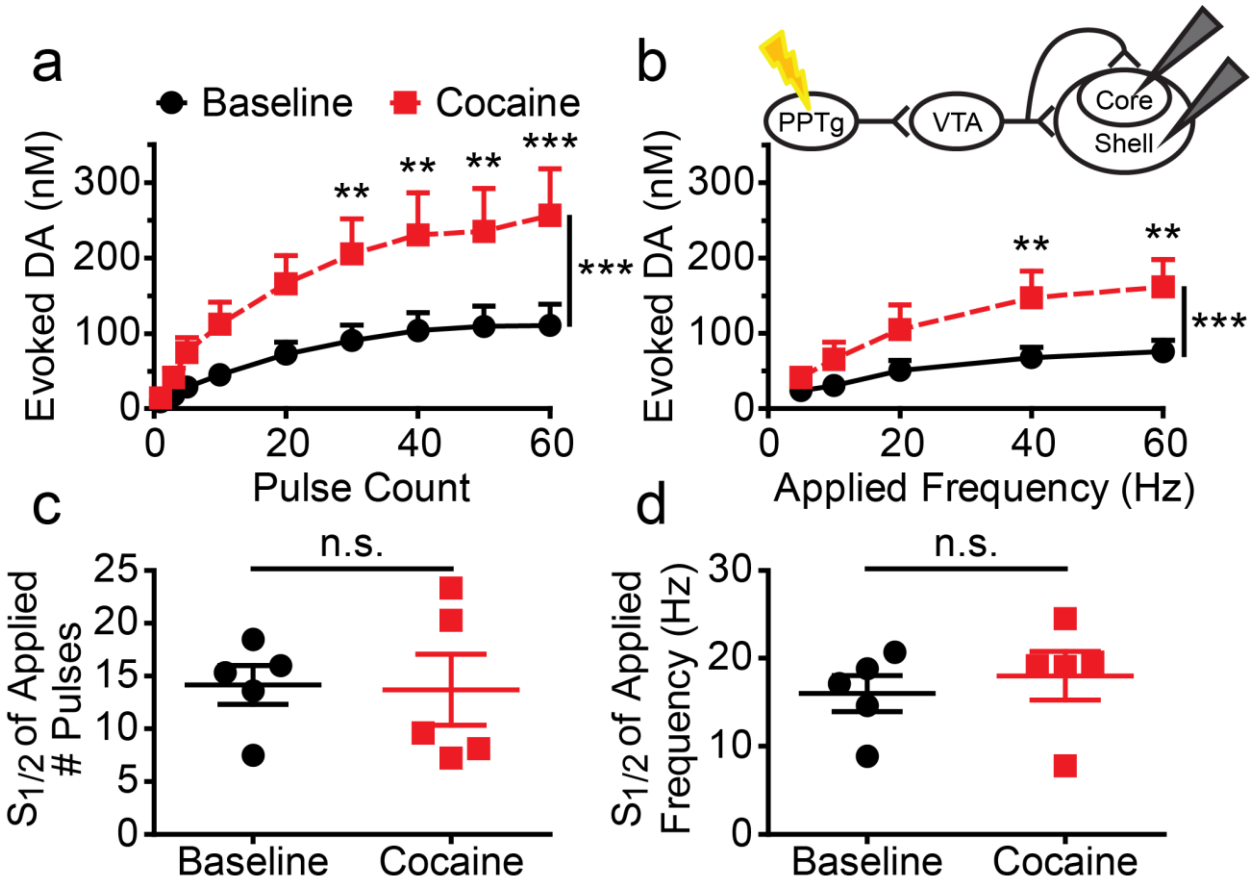

Figure 2.8. The effects of cocaine on evoked dopamine responses in both core and shell of NAc after PPTg stimulation do not vary by stimulus intensity.

To characterize the ability of cocaine to increase evoked NAc dopamine responses at different stimulus intensities, a sweep of stimulus duration and frequency was performed before and after before and after administration of cocaine (15 mg/kg, *s.c.*) with bipolar stimulating electrodes placed in the PPTg. Repeated measures two-way ANOVA showed a significant interaction between the effect of cocaine and stimulus duration but not stimulus frequency. One-way ANOVA performed to analyze the percent change from the recordings taken prior to cocaine administration revealed no significant effect of stimulus pulse duration or stimulus frequency. Administration of cocaine also consistently failed to alter the half-peak strength of stimulus intensity. Given that this data set consistently suggested that administration of cocaine induced changes in evoked dopamine that did not vary by intensity of PPTg stimulation, we chose to utilize PPTg stimulus parameters that induced basal levels of dopamine release comparable to those observed after MFB stimulation.

(a) Results are shown of experiments in which PPTg stimulus duration was varied while current intensity (150 μA) and frequency (60 Hz) were fixed and recording from the NAc before and after administration of cocaine (15 mg/kg, *s.c.*). Repeated measures two-way ANOVA shows a significant increase in evoked NAc dopamine after administration of cocaine (***, $F_{(1, 36)}$=60.03, $p < 0.0001$) and an interaction ($F_{(8, 36)}$=2.417, $p < 0.05$) between administration of cocaine and stimulus duration. One-way ANOVA showed that PPTg stimulus duration did not significantly alter the percent change from baseline caused by treatment with cocaine (data not shown; $F_{(8, 32)}$=0.6854, $p > 0.05$). **, $p < 0.01$; ***, $p < 0.001$; Bonferroni post-hoc analysis. *(b)* Results are shown of experiments in which PPTg stimulus frequency was varied while current intensity (150 μA) and duration (30 pulses) were fixed and recording from the NAc before and after administration of cocaine (15 mg/kg, *s.c.*). Repeated measures two-way ANOVA shows a significant increase in evoked NAc dopamine after administration of cocaine (***, $F_{(1, 20)}$=32.19, $p < 0.0001$) but no interaction ($F_{(4, 20)}$=1.831, $p > 0.05$) was observed. One-way ANOVA showed that PPTg stimulus frequency did not significantly alter the percent change from baseline caused by treatment with cocaine (data not shown; $F_{(3, 12)}$=0.1495, $p > 0.05$). **, $p < 0.01$; Bonferroni post-hoc analysis. *(c)* Results are shown of experiments in which the half-peak strength of PPTg stimulus duration ($S_{1/2}$) was compared before and after administration of cocaine (15 mg/kg, *s.c.*). T test shows that administration of cocaine failed to alter $S_{1/2}$ ($t_{(4)}$=0.1802, $p > 0.05$). *(d)* Results are shown of experiments in which the half-peak strength of PPTg stimulus duration ($S_{1/2}$) was compared before and after administration of cocaine (15 mg/kg, *s.c.*). T test shows that administration of cocaine failed to alter $S_{1/2}$ ($t_{(4)}$=1.427, $p > 0.05$). Error bars represent SEM.

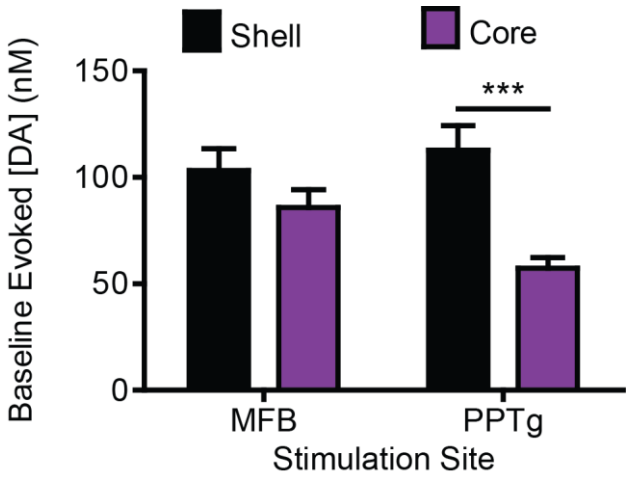

Figure 2.9. PPTg and MFB stimulation evoked similar dopamine responses in NAc.

The baseline data from all experiments used in figures 2, 3, and 5 (excluding reduced stimulation experiments, which were only collected from PPTg-Core) were collected and analyzed. Two-way ANOVA showed a significant interaction between stimulation site and recording site ($F_{(1, 109)}$=4.176, $p < 0.05$) and a main effect of recording site ($F_{(1, 109)}$=15.32, $p < 0.001$). However, Bonferroni post-hoc analysis showed that although the difference between core and shell was significant for PPTg-stimulated dopamine responses ($t = 4.355$, $p < 0.001$), there was no significant difference between core and shell for MFB-stimulated dopamine responses ($t = 1.282$, $p > 0.05$), suggesting the main effect was driven by differences in PPTg-stimulated dopamine response. Further, no main effect was observed for stimulation site ($F_{(1, 109)}$=1.053, $p > 0.05$), suggesting that the stimulation parameters used successfully evoked similar concentrations of released dopamine in the NAc for both MFB and PPTg stimulations. Error bars represent SEM.

A follow-up experiment was performed to determine how reducing stimulation intensity of PPTg affected cocaine-induced increases in the dopamine response recorded in the NAc core. For this experiment, after the recording and stimulation electrodes were lowered to their final locations, two sweeps of stimulus current intensity were performed in 25 μA increments, from 0-150 uA, first in an ascending then descending intensity. Duration was set at 30 pulses and frequency at 60 Hz. The results of these two sweeps were averaged together and the stimulus current at which the [DA] evoked was half of that evoked by 150 μA was determined. The recordings then began with a stimulus of 30 pulses at 60 Hz and 150 μA until baseline was recorded as in other experiments. After baseline was reached, stimulus current was changed to the previously determined reduced current. Recordings then continued as normal, with cocaine administered 60 min after switching to the reduced current and recordings continuing for another 60 min. Data was analyzed as in other experiments.

Analysis. Data were compiled in Microsoft Excel and statistical analyses were performed using Prism 5.01 for Windows (GraphPad, San Diego, CA). Dopamine concentrations were determined by recording the peak current within 1 sec of the termination of electric stimulation and calibrating as described above. FSCV data were analyzed using linear regression, t test, one-way ANOVA, two-way ANOVA, and two-way repeated measures ANOVA as described in the text. Because t1/2 data were nonparameteric, no analysis was performed on raw t1/2 data. Instead, the arithmetic shift between baseline t1/2 and t1/2 after drug administration was analyzed. This was found to pass a Kolmogorov-Smirnov normality test in 15/16 cases ($p > 0.05$). When ANOVA found significant results, Bonferroni post-hoc analyses were applied where appropriate; in certain cases Bonferroni post-hoc analysis were applied after no interaction was seen to confirm negative results. Data are presented as mean ± SEM. Significance was placed at $p < 0.05$.

RESULTS

Prior studies have shown that kappa receptor agonists can inhibit cocaine reward measured in rodents either by CPP or self-administration methods (McLaughlin *et al*, 2006a; Schenk *et al*, 1999). An example of that result is shown (Figure 2.1A) where the kappa agonist U50,488 (5mg/kg) administered 15 min prior to cocaine (15 mg/kg) significantly reduced the CPP of male C57Bl/6 mice. In contrast, U50,488 administered 60 min prior to cocaine resulted in a significant potentiation of the subsequent cocaine-CPP (McLaughlin *et al*, 2006; Figure 2.1A). We rationalize this KOR-induced potentiation of cocaine CPP as a consequence of KOR-induced dysphoria leading to an increased magnitude of cocaine reward (Figure 2.1B) (Bruchas *et al*, 2010). One mechanism of this interaction could be if prior treatment with KOR agonist caused an increase in the effects of cocaine on dopamine release in the NAc, but another could be that cocaine-induced increases in dopamine are proportionately larger after inhibition by KOR agonist. To test this model, we measured dopamine release in anesthetized mice using fast-scan cyclic voltammetry (FSCV). As shown in Figure 2.1C, stimulation of the MFB (6 pulses, 30 Hz, 300 μA) evoked a stereotyped cyclic voltammagram typical of dopamine (inset and colorplot Figure 2.1C). The peak amplitude of the evoked current recorded using a calibrated carbon fiber electrode in the NAc core is shown. KOR activation by U50,488 (5 mg/kg *i.p.*) reduced amplitude of the evoked response whereas cocaine (15 mg/kg *s.c.*) increased the amplitude and slowed the clearance, measured as $t_{1/2}$ (Figure 2.1C). These doses were the same as those used in the CPP drug interaction study (Figure 2.1A). In a representative experiment, evoked responses were measured every 300 sec. After establishing a stable baseline, U50,488 was injected and the time course of response was recorded. Subsequent administration of cocaine increased the evoked dopamine response and the $t_{1/2}$ (Figure 2.1D).

Basal responses are consistent across stimulation and recording sites

In this series of experiments, we stimulated either the MFB or PPTg and measured evoked responses in either the NAc core or shell. Stimulation of MFB directly drives release at dopamine terminals, whereas PPTg stimulation presynaptically drives burst firing in dopaminergic neurons in the ventral tegmental area (VTA) (Floresco *et al*, 2003; Zweifel *et al*, 2009). The extent of inhibition of evoked dopamine release caused by U50,488 did not depend on the stimulation or recording site, suggesting that KOR regulates evoked dopamine release consistently in both pathways and in both projection sites (Figure 2.10A). Two-way ANOVA failed to find any effect of recording site ($F_{(1, 27)}=0.002972$, $p > 0.05$), stimulation site ($F_{(1, 27)}=0.07009$, $p > 0.05$), or any interaction between the two ($F_{(1, 27)}=2.449$, $p > 0.05$). Further, no effect of U50,488 was found on t1/2 for any combination of stimulation and recording site, with one-way ANOVA showing that recordings from MFB-shell ($F_{(2, 17)}=0.3507$, $p > 0.05$), MFB-core ($F_{(2, 19)}=0.003610$, $p > 0.05$), PPTg-shell ($F_{(2, 22)}=0.4929$, $p > 0.05$), and PPTg-core ($F_{(2, 17)}=0.09620$, $p > 0.05$) all fail to show a significant effect of U50,488 relative to the two saline-pretreated groups (data not shown). In addition, evoked dopamine responses were increased by cocaine to the same extent in both pathways and in both projection sites (Figure 2.10B). Two-way ANOVA failed to find any effect of recording site ($F_{(1, 26)}=0.4639$, $p > 0.05$), stimulation site ($F_{(1, 26)}=0.01971$, $p > 0.05$), or any interaction between the two ($F_{(1, 26)}=4.130$, $p > 0.05$).

Terminal effects of KOR activation are region-dependent and time-independent whereas somatodendritic effects are both region- and time-dependent

As diagrammed in Figure 2.11A, mice were treated with U50,488 or saline prior to cocaine or saline administration 15 or 60 min later. While stimulating in the MFB and recording from the NAc shell, changes in evoked dopamine were recorded (Figure 2.11B). Evoked responses were stable during the 2 hr course of the experiment for each pairing of stimulation and recording sites (black circles, saline-saline). Further, baseline levels of dopamine were similar for each treatment group within each combination of stimulation and recording site, demonstrating that observed differences between groups are not driven by basal differences in

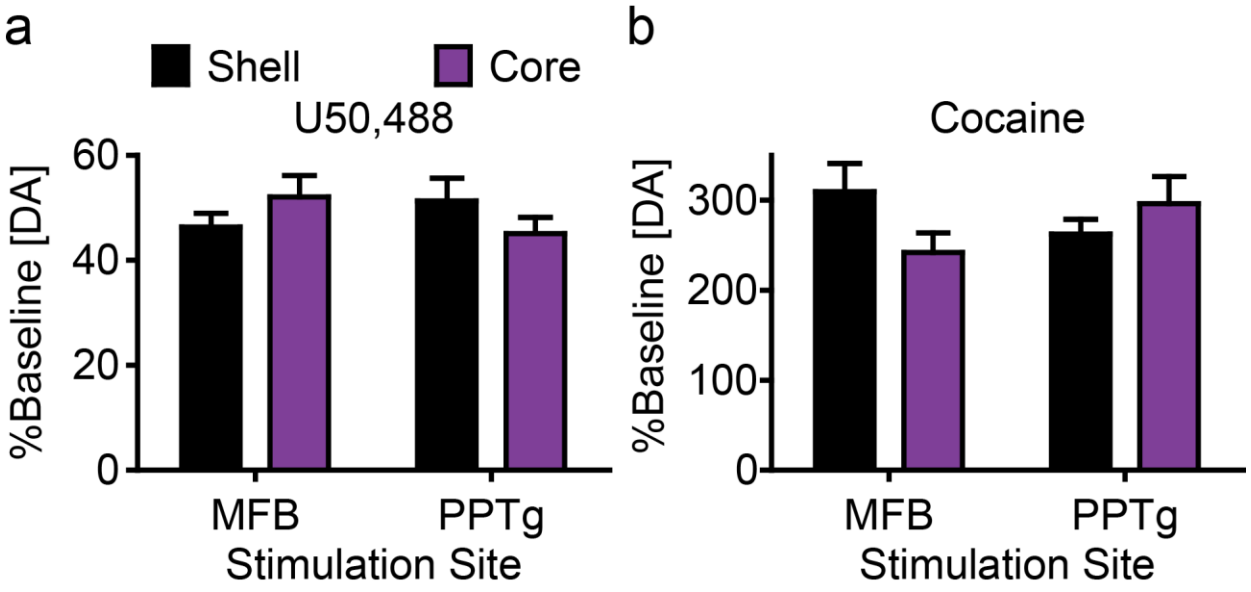

Figure 2.10. The dopaminergic response to U50,488 alone and cocaine alone is consistent across stimulation and recording sites.

(a) The response to U50,488 (shown as percent baseline [dopamine]) in animals injected with U50,488 60 min prior to cocaine shows that administration of U50,488 uniformly inhibits dopamine release in the NAc regardless of recording site (core vs. shell) or stimulation site (MFB vs. PPTg) (n=6-9). *(b)* The response to cocaine (shown as percent baseline [dopamine]) in animals injected with saline 60 min prior to cocaine shows that administration of cocaine uniformly elevates dopamine in the NAc regardless of recording site (core vs. shell) or stimulation site (MFB vs. PPTg) (n=6-9). Error bars represent SEM.

the concentration of evoked dopamine (Figure 2.12). The time course of the responses to U50,488 and cocaine are shown (Figure 2.11, panels C,F,I,L). MFB-evoked dopamine responses recorded from the NAc shell shows that pretreatment with U50,488 at either time point inhibited the response to cocaine relative to saline-pretreated animals (Figure 2.11D). This is confirmed by a two-way repeated measures ANOVA comparing only the cocaine-treated groups before and after administration of cocaine (data not shown), which showed a main effect of time ($F_{(1, 15)}$=154.4, $p < 0.0001$) and of treatment prior to cocaine ($F_{(2, 15)}$=12.98, $p < 0.001$), but no interaction ($F_{(2,15)}$=2.492, $p > 0.05$). One-way ANOVA showed a significant difference between the four groups after treatment with cocaine or saline (Figure 2.11D, $F_{(3, 20)}$=16.45, $p < 0.0001$). Although cocaine increased the evoked dopamine response over baseline, subsequent Bonferroni post-hoc analysis demonstrated that prior administration of U50,488 significantly inhibited the response to cocaine relative to saline-pretreated animals (Figure 2.11D) after pretreatment either 15 min (t=2.939, $p < 0.05$) or 60 min (t=3.806, $p < 0.01$) prior.

By contrast, MFB-evoked dopamine responses recorded from the NAc core show that pretreatment with U50,488 failed to alter the response to cocaine relative to saline-pretreated animals after pretreatment at either 15 min (t=0.4444, $p > 0.05$) or 60 min (t=0.7870, $p > 0.05$) (Figure 2.11G). A two-way repeated measures ANOVA comparing only cocaine-treated groups before and after administration of cocaine (data not shown) showed a main effect of time ($F_{(1, 19)}$=223.2, $p < 0.0001$) and a significant interaction ($F_{(2, 19)}$=3.538, $p < 0.05$), but no main effect of treatment prior to cocaine ($F_{(2, 19)}$=0.2956, $p > 0.05$). A one-way ANOVA showed a significant difference between the four groups after treatment with cocaine or saline (Figure 2.11G, $F_{(3, 25)}$=15.11, $p < 0.0001$). These results suggest that although the KOR regulation of dopamine release was comparable in core and shell, the interaction between KOR and cocaine results in greater inhibition in the shell. Surprisingly, there was no difference in the response between 15 and 60 min pretreatment in either region (shell: t=0.2587, $p > 0.05$; core: t=1.246, $p > 0.05$), which suggests that the ability of KOR agonists to block cocaine CPP was not mediated by terminal effects on dopamine release in the NAc.

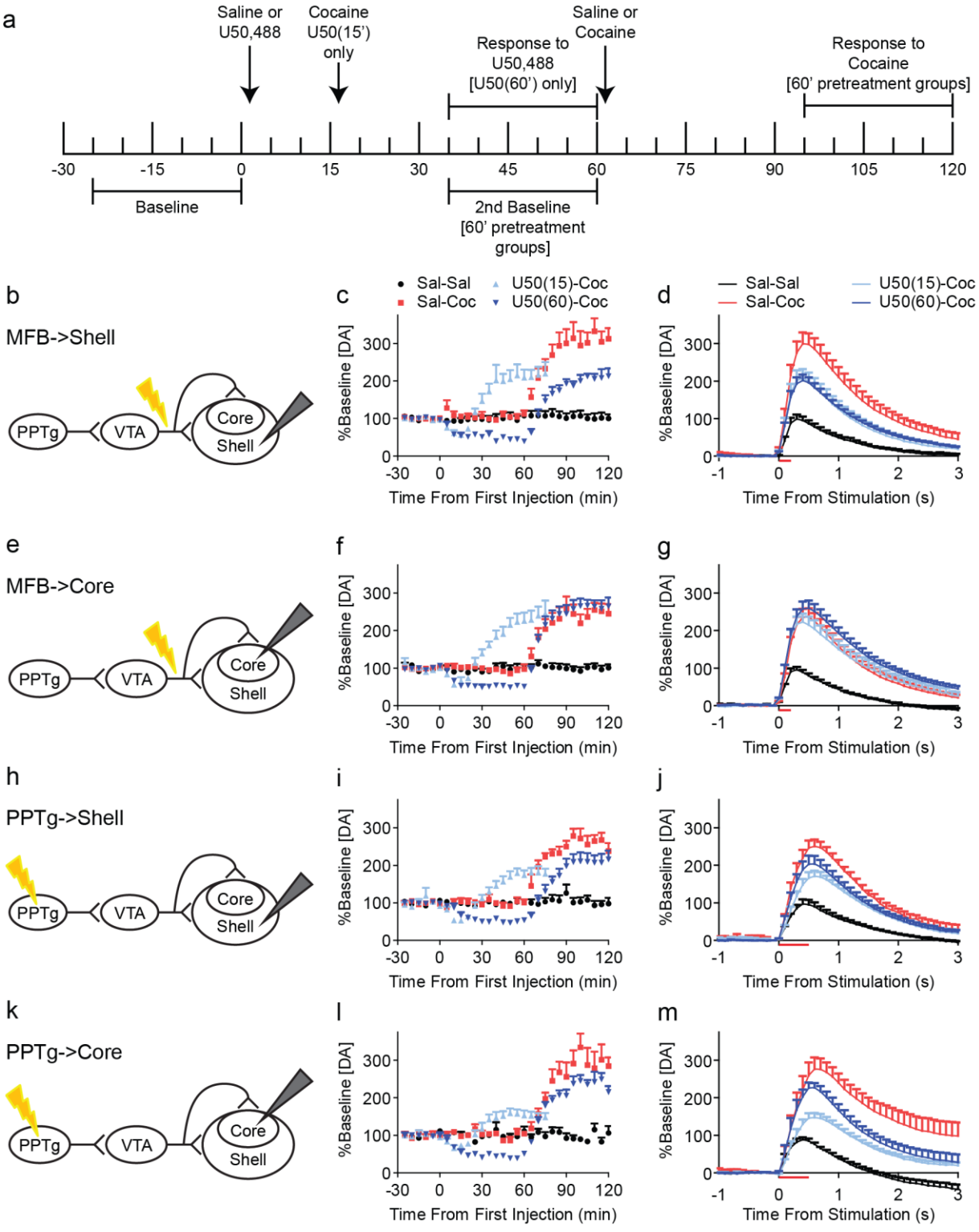
a
Saline or U50,488
Cocaine U50(15') only
Response to U50,488 [U50(60') only]
Saline or Cocaine
Response to Cocaine [60' pretreatment groups]
-30 -15 0 15 30 45 60 75 90 105 120
Baseline
2nd Baseline [60' pretreatment groups]
b
MFB->Shell
PPTg VTA Core Shell
c
Sal-Sal
Sal-Coc
U50(15)-Coc
U50(60)-Coc
%Baseline [DA]
Time From First Injection (min)
d
Time From Stimulation (s)
e
MFB->Core
f
g
h
PPTg->Shell
i
j
k
PPTg->Core
l
m

Figure 2.11. The effects of KOR activation on the dopaminergic response to cocaine at different pretreatment time points are differentially regulated depending on recording site and stimulation site.

(a) Diagram showing time points of treatment for each experiment. Stimulations occurred every 5 min as marked by vertical lines. The average of the first six stimulations (0-25 min before the first injection) was used as baseline for this figure; the average of the six stimulations comprising the period from 35-60 min after the second injection were used as the response to cocaine or saline. *(b)* Cartoon depicting stimulation of MFB and recording of dopamine released in NAc shell. *(c)* Timeline of MFB-evoked dopamine release recorded from the NAc shell from all groups; showing responses to saline, U50,488 (5 mg/kg *i.p.*) and/or cocaine (15 mg/kg *s.c.*). Administration of U50,488 induced an inhibition of dopamine release in both groups, and administration of cocaine consistently elevated dopamine levels (n=4-8). *(d)* The response of MFB-evoked dopamine recorded from the NAc shell to cocaine or saline from all four groups, shown as averaged evoked dopamine currents. Red bar marks time of stimulation. U50,488 pretreatment significantly inhibited the response to cocaine in a time-independent manner relative to saline-pretreated animals; however, cocaine still elevated dopamine to levels greater than those recorded from animals treated with saline alone (n=4-8). *(e)* Cartoon depicting stimulation of MFB and recording of dopamine released in NAc core. *(f)* Timeline of MFB-evoked dopamine release recorded from the NAc core from all groups; showing responses to saline, U50,488 (5 mg/kg *i.p.*) and/or cocaine (15 mg/kg *s.c.*). Administration of U50,488 inhibited dopamine release in both groups, and administration of cocaine consistently elevated dopamine levels (n=7-9). *(g)* The response of MFB-evoked dopamine recorded from the NAc core to cocaine or saline from all four groups, shown as averaged evoked dopamine currents. U50,488 pretreatment failed to alter the response to cocaine relative to saline-pretreated animals (n=7-9). *(h)* Cartoon depicting stimulation of PPTG and recording of dopamine released in NAc shell. *(i)* Timeline of PPTG-evoked dopamine release recorded from the NAc shell from all groups; showing responses to saline, U50,488 (5 mg/kg *i.p.*) and/or cocaine (15 mg/kg *s.c.*). Administration of U50,488 inhibited dopamine release in both groups, and administration of cocaine consistently elevated dopamine levels (n=7-9). *(j)* The response of PPTG-evoked dopamine recorded from the NAc shell to cocaine or saline from all four groups, shown as averaged evoked dopamine currents. U50,488 pretreatment at 15 min significantly inhibited the response to cocaine, whereas U50,488 at 60 min shows a trend towards an inhibited response to cocaine; in both cases relative to saline-pretreated animals. In all animals receiving cocaine, dopamine levels were elevated to an extent greater than that recorded from animals treated with saline alone (n=7-9). *(k)* Cartoon depicting stimulation of PPTG and recording of dopamine released in NAc core. *(l)* Timeline of PPTG-evoked dopamine release recorded from the NAc core from all groups; showing responses to saline, U50,488 (5 mg/kg *i.p.*) and/or cocaine (15 mg/kg *s.c.*). Administration of U50,488 inhibited dopamine release in both groups, and administration of cocaine consistently elevated dopamine levels (n=6-8). *(m)* The response of PPTG-evoked dopamine recorded from the NAc core to cocaine or saline from all four groups, shown as averaged evoked dopamine currents. U50,488 pretreatment at 15 min significantly inhibits the response to cocaine relative to both saline-pretreated animals and animals receiving U50,488 pretreatment at 60 min; further, cocaine after 15 min pretreatment with U50,488 failed to significantly alter dopamine levels relative to animals treated with saline alone. By contrast, U50,488 pretreatment at 60 min failed to alter the response to cocaine relative to saline-pretreated animals, while elevating dopamine to levels significantly greater than those recorded from animals treated with saline alone (n=6-8). Error bars represent SEM.

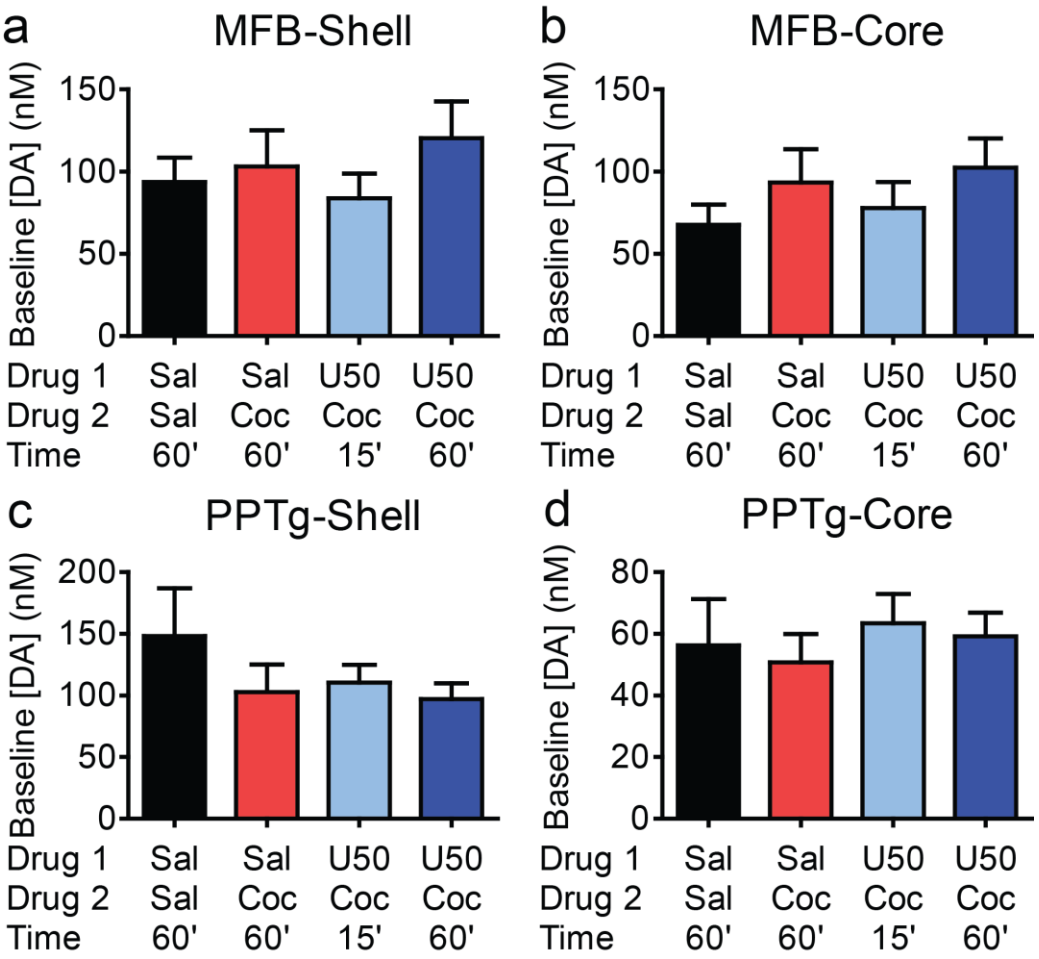

Figure 2.12. Reported effects of U50,488 and cocaine were not a consequence of differences in baseline dopamine response.

To ensure that the reported effects of each experiment were not due to changes in the level of dopamine in any group prior to treatment, one-way ANOVAs were performed analyzing the basal concentration of evoked dopamine for each combination of stimulation and recording site. In each case, no significant effect was found nor did Bonferroni post-hoc analysis reveal any significant differences between groups. This shows that differences between each treatment group were not mediated by differences in baseline [dopamine] prior to drug administration.

(a) Summary of baseline levels of MFB-evoked dopamine for experiments recorded from the NAc shell. One-way ANOVA revealed no differences between groups ($F_{(3, 20)}$=0.5702, $p > 0.05$). *(b)* Summary of baseline levels of MFB-evoked dopamine for experiments recorded from the NAc core. One-way ANOVA revealed no differences between groups ($F_{(3, 25)}$=0.8545, $p > 0.05$). *(c)* Summary of baseline levels of PPTg-evoked dopamine for experiments recorded from the NAc shell. One-way ANOVA revealed no differences between groups ($F_{(3, 28)}$=0.9282, $p > 0.05$). *(d)* Summary of baseline levels of PPTg-evoked dopamine for experiments recorded from the NAc core. One-way ANOVA revealed no differences between groups ($F_{(3, 24)}$=0.2960, $p > 0.05$). Error bars represent SEM.

Similar results were evident after PPTg stimulation. Pretreatment with U50,488 did not increase the amplitude of the cocaine response measured in either shell or core (Figure 2.11J,M). A two-way repeated measures ANOVA comparing only cocaine-treated groups before and after administration of cocaine in recordings from the NAc shell again showed a main effect of time ($F_{(1, 22)}$=241.8, $p < 0.0001$) and a main effect of treatment prior to cocaine ($F_{(2, 22)}$=9.262, $p < 0.01$), but no interaction ($F_{(2, 22)}$=2.593, $p > 0.05$). One-way ANOVA performed on the evoked dopamine response measured in the NAc shell showed a significant difference between the four groups after treatment with cocaine or saline (Figure 2.11J, $F_{(3, 28)}$=15.22, $p < 0.0001$). Bonferroni post-hoc analysis showed that in the NAc shell, prior administration of U50,488 reduced the response to cocaine relative to saline-pretreated animals after pretreatment at 15 min (t=3.482, $p < 0.01$), with a similar trend seen after 60 min pretreatment (t=2.288, $p > 0.05$). Similar to MFB recordings, there was no separation between pretreatment at 15 min and at 60 min (t=1.342, $p > 0.05$).

Two-way repeated measures ANOVA comparing the evoked dopamine response in the NAc core following PPTg stimulation from only cocaine-treated groups before and after administration of cocaine showed results slightly different from those observed following MFB stimulation: this analysis showed a main effect of time ($F_{(1, 19)}$=170.8, $p < 0.0001$), a main effect of treatment prior to cocaine ($F_{(2, 19)}$=14.16, $p < 0.001$), and a significant interaction ($F_{(2, 19)}$=11.20, $p < 0.001$). Intriguingly, this was the only combination of recording and stimulation site to show both a main effect of treatment prior to cocaine and an interaction between time and treatment. One-way ANOVA performed on the dopamine response in the NAc core following PPTg stimulation showed a significant difference between the four groups after treatment with cocaine or saline (Figure 2.11M, $F_{(3, 24)}$=20.08, $p < 0.0001$). Interestingly, unlike the other combinations of stimulation and recording site, the PPTg-core combination showed a unique separation between the effects of U50,488 at the two pretreatment times. Bonferroni post-hoc analysis shows that the response to cocaine after pretreatment 60 min prior was significantly greater than that seen after pretreatment at 15 min (t=2.972, $p < 0.05$). Further, the response to

cocaine 15 min after prior treatment with U50,488 failed to induce significant elevations of dopamine relative to that seen in animals treated with saline alone ($t=2.054$, $p > 0.05$), unlike the responses seen after similar pretreatment in MFB-shell ($t=3.340$, $p < 0.05$), MFB-core ($t=4.789$, $p < 0.001$), and PPTg-shell ($t=2.958$, $p < 0.05$).

The difference in response between the MFB and PPTg stimulation sites suggests that action of kappa receptors in the somatodendritic region of the VTA dopaminergic neurons rather than at their terminals in the NAc may have a key role in the kappa opioid-induced potentiation of the cocaine response. To test this hypothesis, an experiment was performed in which after the baseline was established, current intensity was reduced to a level evoking [DA] half of that observed after 150 μA stimulation. T test found that reduction of baseline dopamine levels after reduction of stimulation current intensity was not significantly different from inhibition of dopamine induced by U50,488 administration (data not shown; $t_{(10)}=0.1824$, $p > 0.05$). One-way ANOVA performed on the dopamine response in the NAc core comparing this group to the other groups receiving cocaine showed a significant difference between the four groups after treatment with cocaine (Figure 2.13B, $F_{(3, 24)}=9.253$, $p < 0.001$). Further, Bonferroni post-hoc showed a significant reduction in the response to cocaine after reduction of stimulus intensity compared to saline-pretreated animals ($t=4.035$, $p < 0.01$) but a roughly equivalent response to that seen after fifteen min pretreatment with U50,488 ($t=0.3370$, $p > 0.05$). These findings support the hypothesis that the reduced response of PPTg-stimulated dopamine to cocaine observed in the NAc core after systemic U50,488 administration is due to somatodendritic KOR activation inhibiting the firing of NAc core-projecting dopamine neurons.

KOR-induced potentiation of cocaine reward can be modeled by the altered baseline of evoked dopamine responses

During CPP training, mice are placed in the preference chamber directly following administration of cocaine. As such, it seems likely that the relevant component of the evoked dopamine response is that immediately preceding the cocaine injection. When we reanalyze the

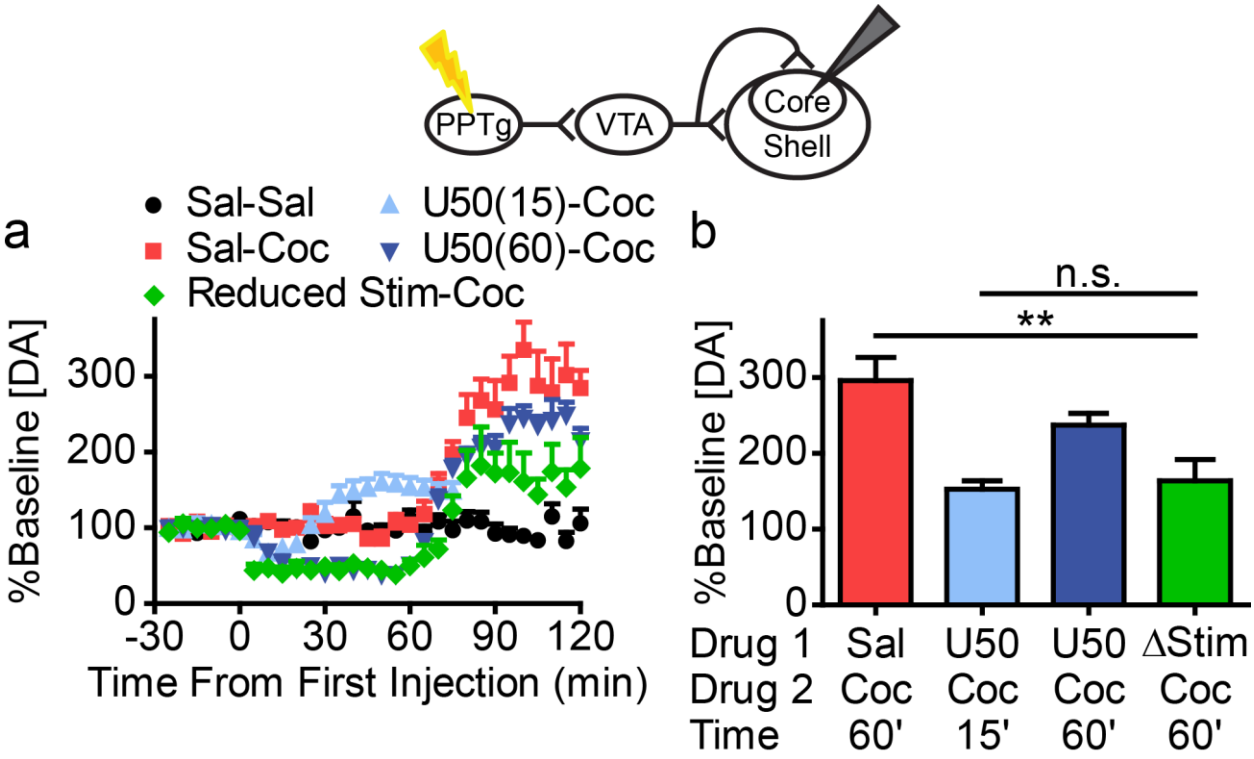

Figure 2.13. The potentiation of the evoked dopamine response to cocaine caused by prior treatment with U50,488 was not solely a consequence of the reduced baseline recorded in the NAc core following PPTg stimulation.

In this analysis, evoked dopamine responses were normalized to the initial baseline prior to first injection (saline and drug-treated groups) or changes in stimulation current intensity (reduced stimulation group).

(a) Timeline of PPTg-evoked dopamine release recorded from the NAc core from all groups is summarized for groups of recordings showing the effects of saline and U50,488 (5 mg/kg *i.p.*) treatment, the reduction in stimulus current intensity, and the effects of cocaine (15 mg/kg *s.c.*). T test showed that the reduction in stimulus current intensity reduced the dopamine response to a similar extent as U50,488 treatment (data not shown; $t_{(10)}=0.1824$, $p > 0.05$). Cocaine consistently increased dopamine responses (n=6-8). Sal-Sal, Sal-Coc, U50(15)-Coc, and U50(60)-Coc data are the same as shown in Figure 2.11 panel (l). *(b)* One-way ANOVA showed that pretreatment significantly altered the percent change from baseline caused by treatment with cocaine ($F_{(3, 24)}=9.253$, $p < 0.001$). Further, Bonferroni post-hoc tests showed that reduction of stimulus current intensity reduced the response to cocaine relative to saline pretreatment (**, $t=4.035$, $p < 0.01$), but not relative to U50,488 administered 15 minutes prior to cocaine (n.s., $t = 0.3370$, $p > 0.05$).

evoked dopamine response for each group using the baseline immediately prior to cocaine injection, the dopamine responses in the NAc all showed broadly similar results (Figure 2.14A-D). For MFB-stimulated dopamine responses recorded in the NAc shell, one-way ANOVA showed a significant effect of treatment (Figure 2.14A; $F_{(3, 20)}=24.33$, $p < 0.0001$). Pretreatment with U50,488 sixty min prior to cocaine led to significantly greater elevations in evoked dopamine response than those observed in saline-pretreated animals ($t=3.762$, $p < 0.01$) or animals pretreated with U50,488 at 15 min ($t=3.972$, $p < 0.01$). However, pretreatment with U50,488 fifteen min prior to cocaine showed no difference from saline-pretreated animals ($t=0.6211$, $p > 0.05$) and led to increases in dopamine response significantly different from that seen in animals treated with saline alone ($t=3.345$, $p < 0.05$).

MFB-stimulated dopamine recorded from the NAc core showed a similar and also significant effect of cocaine or saline (Figure 2.14B; $F_{(3, 25)}=56.68$, $p < 0.0001$). Pretreatment with U50,488 sixty min prior to cocaine led to relatively greater elevations in dopamine than those observed in saline-pretreated animals ($t=7.864$, $p < 0.001$) or animals pretreated with U50,488 at 15 min ($t=7.177$, $p < 0.001$). However, pretreatment with U50,488 at 15 min showed no difference from saline-pretreated animals ($t=0.6648$, $p > 0.05$) and increases in dopamine significantly greater than that seen in animals treated with saline alone ($t=5.492$, $p < 0.001$) were recorded.

PPTg-stimulated dopamine recorded from the NAc shell showed a significant effect ($F_{(3, 28)}=45.52$, $p < 0.0001$) similar to that observed after MFB stimulation in either NAc subregion (Figure 2.14C). Pretreatment with U50,488 with U50,488 sixty min prior to cocaine led to relatively greater elevations in dopamine than those observed in saline-pretreated animals ($t=5.295$, $p < 0.001$) or animals pretreated with U50,488 at 15 min ($t=7.198$, $p < 0.001$). However, pretreatment with U50,488 at 15 min showed no difference from saline-pretreated animals ($t=2.245$, $p > 0.05$) and led to increases in dopamine significantly different from that seen in animals treated with saline alone ($t=3.949$, $p < 0.01$).

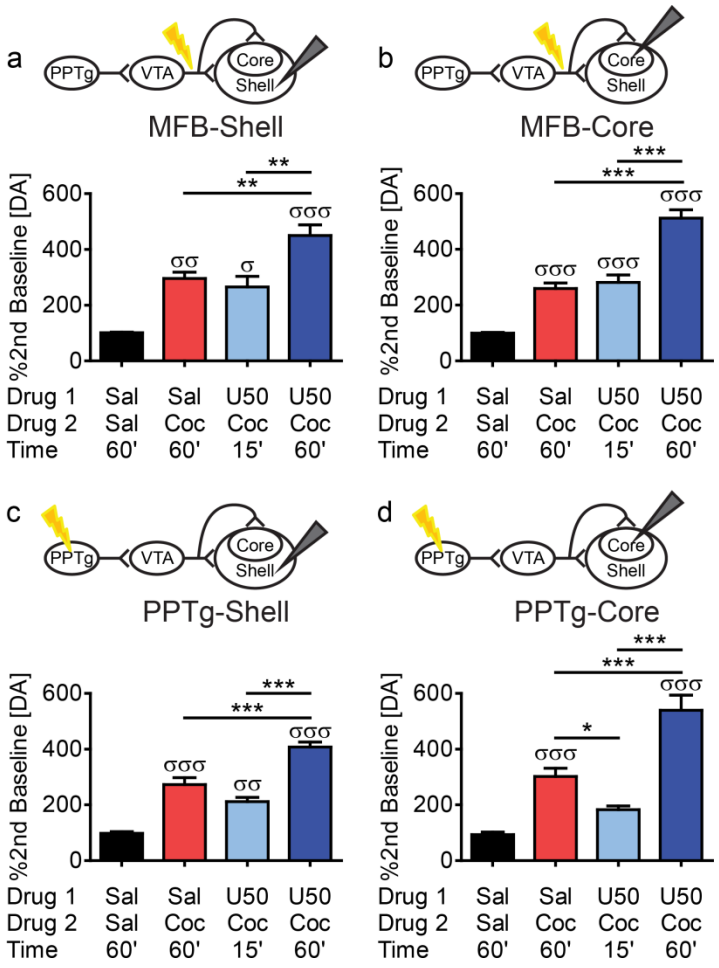

Figure 2.14. Using the time prior to cocaine administration as a baseline shows that 60 min pretreatment with U50,488 consistently potentiates cocaine's ability to elevate dopamine concentration regardless of stimulation or recording site.

By contrast, only PPTg-stimulated dopamine release recorded from the NAc core shows a block of the response to cocaine after 15 min pretreatment with U50,488. *(a)* 60 min pretreatment with U50,488 prior to cocaine potentiated MFB-evoked dopamine recorded from the NAc shell relative to both saline-pretreated animals and 15 min pretreatment with U50,488 prior to cocaine (n=4-8). *(b)* 60 min pretreatment with U50,488 prior to cocaine potentiated MFB-evoked dopamine recorded from the NAc core relative to both saline-pretreated animals and 15 min pretreatment with U50,488 prior to cocaine (n=7-9). *(c)* 60 min pretreatment with U50,488 prior to cocaine potentiates PPTg-evoked dopamine recorded from the NAc shell relative to both saline-pretreated animals and 15 min pretreatment with U50,488 prior to cocaine (n=7-9). *(d)* 60 min pretreatment with U50,488 prior to cocaine potentiated PPTg-evoked dopamine recorded from the NAc core relative to both saline-pretreated animals and 15 min pretreatment with U50,488 prior to cocaine. Further, 15 min pretreatment with U50,488 significantly inhibited the response to cocaine relative to saline-pretreated animals; and cocaine after 15 min pretreatment with U50,488 failed to alter dopamine levels significantly relative to animals treated with saline alone (n=6-8). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between groups as marked; σ $p < 0.05$, σσ $p < 0.01$, σσσ $p < 0.001$ labeled group vs saline. Error bars represent SEM.

PPTg-stimulated dopamine release recorded in the NAc core also showed a significant effect ($F_{(3, 24)}$=36.84, $p < 0.0001$) of treatment (Figure 2.14D). Similar to the results seen with the other stimulation-recording pairings, Bonferroni post-hoc showed that 60 min pretreatment with U50,488 led to significantly greater increases in evoked dopamine relative to animals treated with saline prior to cocaine ($t=5.566$, $p < 0.001$) and relative to animals treated with U50,488 fifteen min prior to cocaine ($t=8.345$, $p < 0.001$). However, Bonferroni post-hoc analyses also showed that the response to cocaine was significantly smaller after pretreatment with U50,488 fifteen min prior to cocaine compared to pretreatment with saline ($t=3.002$, $p < 0.05$). Further, cocaine administered 15 min after U50,488 failed to elevate NAc core dopamine to levels significantly greater than animals treated with only saline ($t=2.103$, $p > 0.05$), while animals that received cocaine 60 min after either saline or U50,488 both showed significant increases relative to saline-only animals (sal-coc: $t=4.883$, $p < 0.001$; U50(60)-coc: $t=9.773$, $p < 0.001$). Finally, when using one-way ANOVA to compare saline- and U50,488-pretreated animals to animals receiving a reduction in stimulus current, a similar pattern emerged (Figure 2.15A, $F_{(3, 24)}$=18.99, $p < 0.0001$). Bonferroni post-hoc comparing this alternate baseline showed no difference between saline-pretreated animals animals receiving a reduction in stimulus prior to cocaine ($t=0.9607$, $p > 0.05$). This confirms the results of the experiment shown in Figure 2.8, demonstrating that for PPTg stimulation the response to cocaine as percent baseline is independent of the stimulus intensity. As such, similar to saline-pretreated animals, animals receiving a reduced stimulus current showed a significantly greater response to cocaine than animals pretreated with U50,488 fifteen min prior to cocaine ($t=3.450$, $p < 0.01$) and a significantly weaker response to cocaine than animals treated with U50,488 60 min after U50,488 ($t=3.764$, $p < 0.01$). The unique difference between the responses to 15 min and 60 min pretreatment suggests that NAc core-projecting dopaminergic neurons play a key role in the ability of KOR agonists to both decrease and increase the rewarding properties of cocaine.

Analysis of the interaction between U50,488 and cocaine by recording and stimulation sites reveals different effects at different pretreatment times

The difference between U50,488 pretreatment 15 min and 60 min prior to cocaine was also evident when the MFB and PPTg stimulated responses were directly compared (Figure 2.15). Two-way ANOVA showed that pretreatment with U50,488 fifteen min prior to cocaine induced a significant effect of stimulation site (Figure 2.15B, $F_{(1, 22)}=9.823$, $p < 0.01$) while showing no effect of recording site ($F_{(1, 22)}=0.03423$, $p > 0.05$) and no interaction ($F_{(1, 22)}=1.302$, $p > 0.05$). As described previously, a two-way ANOVA showed that in saline-pretreated animals the response to cocaine was consistent across recording and stimulation site (Figure 2.10B); as such, we would expect any effect of recording or stimulation site seen in U50,488-pretreated animals to be a result of KOR activation. These results show that the effect of U50,488 was greater on the PPTg stimulated response than on MFB, suggesting that there was an effect of somatodendritic KOR activation at this time point that is only evident in the presence of cocaine.

By contrast, two-way ANOVA showed that pretreatment with U50,488 sixty min prior to cocaine induced a significant effect of recording site (Figure 2.15C,D, $F_{(1, 27)}=5.102$, $p < 0.05$) but no effect of stimulation site ($F_{(1, 27)}=0.2767$, $p > 0.05$) and no interaction ($F_{(1, 27)}=0.6487$, $p > 0.05$). The presence of a main effect of stimulation site in 15 min, but not 60 min, pretreated animals supports the interpretation that there was a time-dependent somatodendritic effect of KOR activation on the response to cocaine. The presence of a main effect of recording site in 60 min-pretreated animals suggests that there was a region-dependent effect underlying the inhibition of the response to cocaine seen in shell but not core that was dependent on activation of terminal KORs. Further, the presence of a main effect of recording site in the 60 min U50,488-pretreated animals but not saline-pretreated animals demonstrates that the presence of an inhibited response to cocaine in shell but not core (as clearly seen after MFB-stimulated dopamine release) was not due to a ceiling effect in the overall response to cocaine in these two brain regions.

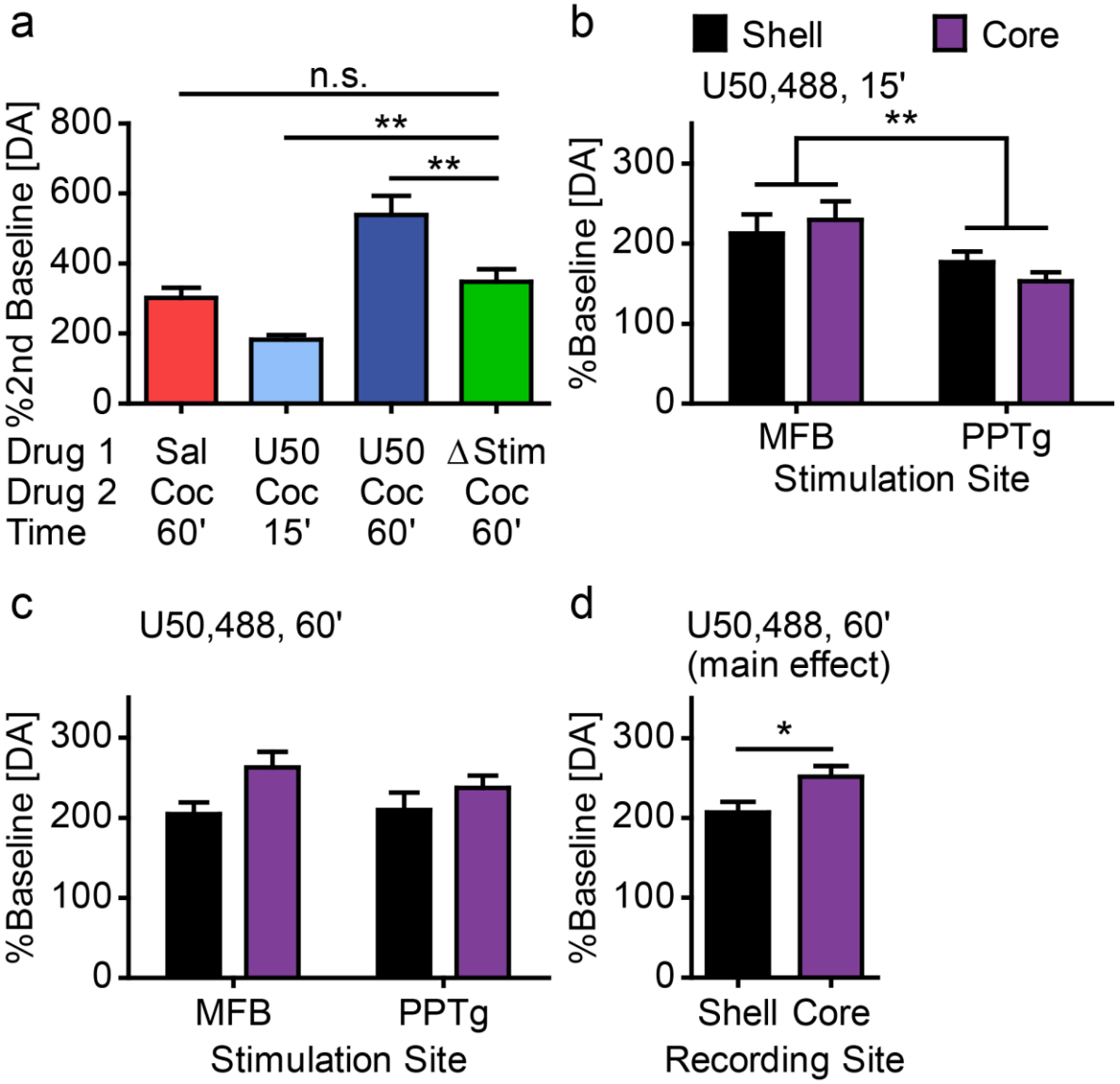

Figure 2.15.

(a) Using the time prior to cocaine as a baseline shows that reduced stimulus intensity prior to and after cocaine administration has a reduced response to cocaine relative to 60 min pretreatment with U50,488 or 15 min pretreatment with U50,488, suggesting that the firing of dopaminergic neurons is not inhibited 60 minutes after U50,488 administration. (n=6-8). *(b-d)* Analysis by pretreatment time shows that after 15 min pretreatment with U50,488, the response to cocaine is determined by stimulation site. By contrast, after 60 min pretreatment with U50,488, the response to cocaine is determined by recording site. *(b)* 15 min after pretreatment with U50,488, the response of PPTg-stimulated dopamine to cocaine is significantly inhibited relative to MFB-stimulated dopamine (n=4-8). *(c)* 60 min after pretreatment with U50,488, the response of PPTg-stimulated dopamine to cocaine was significantly inhibited in the NAc shell relative to the NAc core (n=6-9). *(d)* The main effect of the two-way ANOVA shown in panel (c). n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ between groups as marked. Error bars represent SEM.

DISCUSSION

The principal findings of this study are first, that KOR-induced potentiation of cocaine reward can be modeled by KOR-induced changes in NAc dopamine at the time of cocaine administration. Second, that the ability of KOR activation to block cocaine reward cannot be explained solely by terminal inhibition of dopamine release in the NAc, but must also incorporate effects of somatodendritic KOR activation on dopaminergic neurons. Third, that activation of terminal KOR on dopaminergic neurons significantly inhibits the response to cocaine in NAc shell but not in NAc core. Fourth, our findings suggest a key role for dopaminergic neurons that project to the NAc core in KOR-mediated effects on cocaine reward.

KOR agonist-induced block of cocaine reward is not mediated through terminal inhibition of NAc dopamine release

Acute pretreatment (5-20 min) with KOR agonists blocks the induction of cocaine CPP (McLaughlin *et al*, 2006a; Zhang *et al*, 2004a, 2004b) and the reward-sensitizing properties of cocaine (Shippenberg *et al*, 1996), as well as blocking cocaine-induced decreases in ICSS thresholds (Tomasiewicz *et al*, 2008). Previous attempts to explain this phenomenon have focused on terminal effects of KOR activation in dopaminergic neurons (Carlezon and Thomas, 2009; Carroll and Carlezon, 2013; Tejeda *et al*, 2012). This is consistent with the knowledge that although both intra-NAc and intra-VTA microinjection of U50,488 result in conditioned place aversion (Bals-Kubik *et al*, 1993a), intra-NAc but not intra-VTA perfusion of U69,593 induces a reduction in NAc dopamine (Devine *et al*, 1993; Margolis *et al*, 2006a; Spanagel *et al*, 1992). Similarly, our results show that a consistent inhibition of basal dopamine concentrations was seen in both subregions regardless of whether MFB or PPTg are stimulated, suggesting that somatodendritic KOR activation did not induce an additive reduction in dopamine release above and beyond terminal inhibition of release. This is despite the fact that previous experiments have shown that in mice NAc-projecting dopaminergic neurons are directly inhibited by the KOR agonist U69,593 (Ford *et al*, 2006), presumably via activation of G-protein

coupled inwardly rectifying potassium channels (Margolis *et al*, 2003). However, our results also show a time-dependent reduction in the ability of cocaine to increase PPTg-evoked but not MFB-evoked NAc dopamine after pretreatment with U50,488. Further, our results show that fifteen min pretreatment with U50,488 inhibits the response of PPTg-evoked dopamine to cocaine in the NAc core in a manner similar to that seen after reducing the intensity of the stimulus current. This suggests that cocaine administration exposes an obscured somatodendritic component of KOR activation in NAc-projecting dopaminergic neurons.

Activation of terminal KOR on dopaminergic neurons inhibits the response to cocaine in NAc shell but not core

Intriguingly, our experiments found that although pretreatment with U50,488 inhibited the response of MFB-stimulated dopamine to cocaine in the NAc shell, no such effect was seen in the NAc core. This suggests that activation of KORs on dopamine terminals in the NAc shell and the NAc core lead to activation of different signal transduction pathways. Given that the inability of U50,488 to inhibit the dopaminergic response to cocaine was paired with a selective increase in the half-life of released dopamine relative to saline-pretreated animals, a role for effects on clearance of released dopamine seems likely. One mediator which could potentially play a role is p38 MAPK, which plays a key role in mediating the stress-induced dysphoric properties of KOR activation. U50,488 CPA and swim stress-induced increases in immobility are blocked by i.c.v. administration of the p38 inhibitor SB203580 (Bruchas *et al*, 2007a). Activation of p38 MAPK by anisomycin has been shown to inhibit dopamine uptake in transfected CHO cells (Zhu *et al*, 2005), and inhibition of p38 by SB202190 has been shown to increase the Vmax of the dopamine transporter (DAT) after mutation of multiple N-terminal serine phosphoacceptor sites (Lin *et al*, 2003). P38 MAPK is also capable of phosphorylating threonine 53 in the N-terminal domain of DAT (Gorentla *et al*, 2009), although mutations intended to block phosphorylation at this site lead to a reduction in DAT Vmax, suggesting that phosphorylation would increase dopamine clearance (Foster *et al*, 2012). Further, previous

research has demonstrated that KOR-induced activation of p38 MAPK can lead to target-specific changes in monoamine clearance, as repeated forced swim stress induces increases in SERT surface expression in terminals in the ventral striatum but not dorsal striatum, prefrontal-cortex, hippocampus, amygdala, or dorsal raphe (Schindler *et al*, 2012).

Also consistent with the idea that terminal KOR activation may act to differently regulate the response to cocaine in different brain regions, a previous experiment has shown using microdialysis that direct perfusion of the KOR agonist dynorphin A(1-17) into the dorsal striatum 5 min prior to systemic cocaine blocked cocaine-induced elevations of dopamine in the same region (Zhang *et al*, 2004b). This is in contrast with our data, which shows that KOR agonists inhibit (but do not block) cocaine-induced elevations of dopamine in the NAc shell and fail to alter the response to cocaine in the NAc core after MFB stimulation. However, data collected with microdialysis will primarily reflect changes in tonic dopamine release whereas the increased temporal resolution of FSCV renders it more sensitive to individual phasic responses (Robinson *et al*, 2003). As such, it is also possible that KOR regulates tonic release by different mechanisms than phasic release events.

NAc core-projecting dopaminergic neurons are uniquely responsive to KOR agonist pretreatment time

Our data show that although U50,488 administration inhibits the response of mesolimbic dopamine in the NAc shell to cocaine, after the 15 min pretreatment cocaine still elevates NAc shell dopamine relative to animals treated with saline alone regardless of stimulation site. By contrast, in the NAc core the response of PPTg-stimulated dopamine to cocaine is inhibited by 15 min pretreatment with U50,488 relative to both saline-pretreated and 60 min U50,488-pretreated animals, while also blocking cocaine from elevating dopamine to levels significantly greater than animals treated with saline alone regardless of baseline used. This suggests that NAc core-projecting dopaminergic neurons may play a unique role in

mediating stress-induced modulation of the magnitude of drug reward. This finding is seemingly inconsistent with theories that dopaminergic projections to the medial NAc shell play a key role in learning stimulus-outcome association, whereas dopaminergic projections to the lateral NAc—including the core—are key to generating or selecting appropriate responses to conditioned responses (Ikemoto, 2007). However, it is potentially consistent with observations that the NAc core plays a key role in drug seeking, with activation in this region being required for both cocaine-primed and stress-primed reinstatement to drug self-administration while activation of the shell is required only for stress-induced reinstatement (McFarland and Kalivas, 2001; McFarland *et al*, 2004). Additionally, the fact that the NAc core seems to play a key role in both acquisition/consolidation and expression of responses to conditioned rewards (Ikemoto, 2007) is consistent with the observation that both stress and KOR agonists can potentiate drug reward when administered prior either to drug training or to a place preference test (McLaughlin *et al*, 2003a, 2006a; Schindler *et al*, 2010). As such, this suggests that future experiments examining interactions between stress and drug reward should focus on this subregion as a site for integrating these signals.

MFB and PPTg stimulation can be used to isolate separate contributions to dopamine signaling

Electrical stimulation of the MFB directly activates dopamine fibers and elicits terminal release of dopamine; by contrast, electrical stimulation of the PPTg induces glutamate and acetylcholine release at terminals in the VTA, leading to a selective increase in burst firing of dopaminergic neurons and terminal release of dopamine which is dependent on activation of NMDA receptors on VTA dopamine neurons (Floresco *et al*, 2003; Zweifel *et al*, 2009). Phasic dopamine release induced by burst firing is more responsive to dopamine uptake inhibitors than tonic release (Floresco *et al*, 2003); and phasic release has been proposed to play a unique role in the rewarding properties of drugs of abuse (Wanat *et al*, 2009). The PPTg itself encodes reward information in a context-dependent manner (Norton *et al*, 2011). Given that KOR

agonists can presynaptically inhibit glutamate release on to VTA dopaminergic neurons (Margolis *et al*, 2005a), it is possible that the results of experiments examining PPTg-stimulated dopamine release reflect effects of KOR activation on PPTg axon terminals. However, as the Allen Mouse Brain Atlas fails to show KOR mRNA in the vicinity of PPTg nuclei (Lein *et al*, 2007), this suggests that the results are more likely mediated by direct KOR effects on dopaminergic neurons. This is also consistent with the prior observation that KOR expression on dopaminergic neurons is required for KOR-induced inhibition of NAc dopamine (Chefer *et al*, 2013b), indicating that KOR activation on VTA-projecting terminals is not sufficient to alter dopamine signaling at the level of the NAc. Further, this would indicate that the time-dependent nature of the response to cocaine after U50,488 pretreatment, seen in PPTg-stimulated dopamine release but not MFB-stimulated dopamine release, is a response specific to KOR activation in the somatodendritic compartment of dopaminergic neurons.

Conclusion

Our results from recordings of MFB-stimulated dopamine release show that activation of terminal KORs alter the response to cocaine in a manner that is dependent on NAc-subregion but not time, with recordings from the shell but not core showing an inhibited response to cocaine after pretreatment with the KOR U50,488 at both time points. However, in both cases the response to cocaine still elevates dopamine concentrations to levels greater than that seen in animals treated with saline alone. Our results further suggest that the inability of inhibition of dopamine release to alter the response to cocaine in the NAc core may be due to an increased inhibition of uptake after pretreatment with U50,488. Our results from recordings of PPTg-stimulated dopamine release show that activation of somatodendritic KORs alter the response to cocaine in a manner that is primarily dependent on time and potentially partially dependent on subregion, with recordings 15 min but not 60 min after pretreatment with U50,488 showing an inhibited response to cocaine. In particular, recordings from the core 15 min after pretreatment fail to show a significant increase in dopamine relative to animals

pretreated with saline animals, while animals pretreated with U50,488 sixty min prior to cocaine fail to show any difference from animals pretreated with saline 60 min prior to cocaine. Further analysis indicates that tracking the response to cocaine at the time of cocaine administration shows that the response of PPTg-stimulated dopamine release in the NAc core mirrors the behavioral response, suggesting that the subpopulation of VTA dopaminergic neurons which project to the core may play a unique role in the ability of stress to increase the rewarding properties of psychostimulants and further suggesting that behavioral and pharmacological manipulations of dopamine must incorporate an understanding of dopamine signaling as a dynamic phenomenon.

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Chapter 3

Inhibition of nucleus accumbens dopamine is not sufficient to support aversion for kappa opioid receptor-mediated dysphoria

This chapter was formatted from the following article: "Presynaptic inhibition of dopamine release is not required for kappa opioid receptor mediated aversion." Ehrich JM, Messinger DI, Kuhar JR, Jarvie BC, Blaszka DJ, Schattauer SS, Bruchas MR, Zweifel LS, Kieffer BL, Phillips PE, Chavkin C. Submitted to *Nature Neuroscience* in October 2014.

J.M.E. designed all of the experiments in this chapter, collected data for all figures, and wrote the chapter. D.I.M. designed animal breeding protocols for experiments using transgenic mice. J.R.K. developed AAV-DIO KOR and AAV-DIO KSA viruses used in generating Figure 3.4 with assistance from L.S.Z. B.C.J. contributed behavioral data to Figure 3.4d. D.J.B. performed immunohistochemistry for Figure 3.3f. S.S.S. developed the lenti-KOR, lenti-KSA, and lenti-eGFP viruses used in generating Figure 3.3. M.R.B. contributed behavioral data to Figure 3.6a and assisted in designing experiments. B.L.K. generated fKOR mice for use in Figure 3.2. P.E.M.P. and C.C. assisted in designing experiments and in writing the chapter.

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ABSTRACT

Changes in dopamine signaling within the nucleus accumbens (NAc) play a key role in theories of aversion and reward. Using kappa opioid receptor (KOR) agonists as a model, we found that terminal inhibition of dopamine release is insufficient to drive contextual aversion. Instead, aversion to KOR agonists was dependent on phosphorylation of the KOR on dopamine neurons and subsequent activation of p38 MAPK. Conditional knockout of p38 MAPK from dopamine neurons blocked the aversive properties of KOR agonists but failed to alter inhibition of dopamine release in the NAc. Stress-evoked release of dynorphins, the endogenous KOR agonists, has been implicated in the dysphoric and proaddictive effects of stress, and these regulatory mechanisms establishing the balance between serotonin and dopamine transmission in NAc are likely to contribute to the stress-vulnerabilities underlying depression and drug abuse.

Introduction

Kappa opioid receptor (KOR) activation produces dysphoria in humans (Pfeiffer *et al*, 1986) and aversion in rodents (Mucha and Herz, 1985). Similarly, stress-induced release of the endogenous KOR agonist, dynorphin (Chavkin *et al*, 1982), encodes the dysphoric properties of stress (Land *et al*, 2008). Considerable evidence suggests that these aversive/dysphoric effects result from KOR activation on ventral tegmental area (VTA) neurons that project to the nucleus accumbens (NAc) (Bals-Kubik *et al*, 1993b; Shippenberg *et al*, 1993). KOR expression is evident in dopaminergic axon terminals in the NAc (Meshul and McGinty, 2000; Svingos *et al*, 2001), and KOR agonists inhibit dopamine release in the NAc (Di Chiara and Imperato, 1988), dorsal striatum (Schlösser *et al*, 1995), and prefrontal cortex (Margolis *et al*, 2006a; Tejeda *et al*, 2013). KOR activation also directly inhibits VTA dopamine neuron firing (Ford *et al*, 2006; Margolis *et al*, 2003), and acutely increases dopamine uptake (Thompson *et al*, 2000). Direct administration of the KOR agonist U50,488 to the VTA or to dopaminergic terminal regions in the NAc and medial prefrontal cortex (mPFC) also induces conditioned place aversion (CPA) (Bals-Kubik *et al*, 1993b). Further, stereotaxic administration of the long-acting KOR antagonist nor-binaltorphimine (norBNI) (Bruchas *et al*, 2007b) in the NAc blocks CPA to systemically administered U50,488 (Land *et al*, 2009). Conditional knockout of KOR from dopamine neurons blocks both CPA and inhibition of dopamine release, and intra-VTA injection of a virus directing expression of KOR rescues both in the same mice (Chefer *et al*, 2013b). Finally, injection of either the dopaminergic neurotoxin 6-OHDA or the D1 antagonist SCH23390 in the NAc blocks the ability of KOR agonists to induce CPA (Shippenberg *et al*, 1993). Given that exposure to aversive stimuli can inhibit dopamine neurons (Brischoux *et al*, 2009; Ungless *et al*, 2004) and that synaptic inhibition of dopamine neurons is also aversive in animal models (Tan *et al*, 2012), the prevailing model proposes that KOR agonists and stress-induced release of dynorphin produce aversion by reducing dopamine release in the NAc (Carroll and Carlezon, 2013; Di Chiara and Imperato, 1988; Spanagel *et al*, 1990; Tejeda *et al*, 2012). Further, as intra-

VTA injection of KOR agonists fails to alter NAc dopamine concentrations (Devine *et al*, 1993; Margolis *et al*, 2006a; Spanagel *et al*, 1992) and local administration of KOR agonists robustly inhibits NAc dopamine concentrations (Lemos *et al*, 2012b; Werling *et al*, 1988), it is proposed that inhibition of NAc dopamine is mediated by direct activation of terminal KORs (Carroll and Carlezon, 2013; Schwarzer, 2009; Tejeda *et al*, 2012). However, the evidence supporting this mechanism is largely correlative, and it should also be noted that KOR activation in NAc also inhibits the release of glutamate, GABA, and serotonin (Hjelmstad and Fields, 2003; Margolis *et al*, 2005b; Tao and Auerbach, 2002).

Recent evidence supports an alternative model of KOR-mediated dysphoria centered around KOR-induced activation of the mitogen-activated protein kinase p38 (p38α MAPK; also called Mapk14 or SAPK, for stress-activated protein kinase) and the serotonergic neurons of the dorsal raphe nucleus (DRN). Local administration of the KOR agonist U50,488 decreases extracellular serotonin in both the DRN and the NAc(Tao and Auerbach, 2002). The KOR agonists U69,593 and Salvinorin A both inhibit K^+-evoked release of [^{3}H]5-HT in hippocampal synaptosomes (Grilli *et al*, n.d.). Phosphorylation of KOR by G-protein receptor kinase 3 (GRK3) on serine 369 is necessary for arrestin-dependent activation of p38 MAPK (Figure 3.1), and U50,488 fails to induce CPA in GRK3 knockout mice (Bruchas *et al*, 2006) or after administration of the p38 inhibitor SB203580 (*i.c.v.*) (Bruchas *et al*, 2007a). Inactivation of KOR within the DRN by stereotaxic administration of norBNI prevents KOR agonist-induced CPA (Land *et al*, 2009). Further, targeted lentiviral restoration of KOR signaling to the DRN rescues KOR agonist-induced CPA; this rescue was dependent on both phosphorylation of the receptor by GRK3 and activation of KOR on serotonergic terminals in the NAc (Land *et al*, 2009). Finally, it has been shown that KOR agonist-induced CPA is dependent on the expression of p38α MAPK in serotonergic neurons and on p38-dependent increases in plasma membrane serotonin transporter surface expression in the terminals of NAc-projecting serotonin neurons (Bruchas *et al*, 2011; Schindler *et al*, 2012). This alternative data set suggests that KOR-mediated dysphoria is controlled by actions of p38α MAPK in NAc-projecting serotonergic neurons,

potentially independent of KOR activity in dopaminergic neurons. Understanding how the dynorphin/KOR system regulates the neuronal circuit underlying dysphoria is key to developing better treatments for stress-induced depression and addiction behaviors, but our comprehension of how KOR-regulation of the serotonergic and dopaminergic inputs to the NAc interact to encode aversion is still incomplete. For example, we do not yet know whether KOR regulation of serotonergic inputs affects dopamine synaptic transmission.

To address these questions, we directly assessed the role of p38 MAPK in VTA dopamine neurons. Using a combination of local receptor inactivation, cre-dependent viral re-expression, and transgenic techniques, we found that ablation of p38α MAPK in dopaminergic neurons blocked the aversive properties of KOR activation, but that KOR-induced inhibition of dopamine release in the NAc was insufficient to induce aversion.

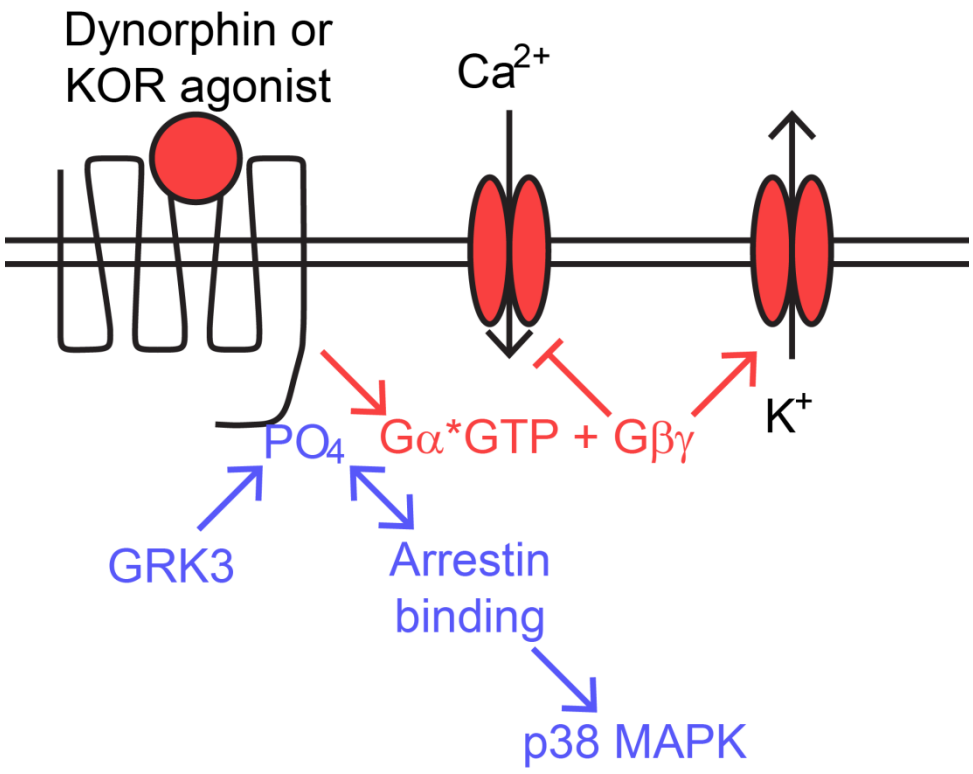


Figure 3.1 Receptor phosphorylation is required for arrestin-dependent activation of p38 MAPK, but not acute g protein-mediated signaling.

Diagram of the signaling events following kappa opioid receptor activation. Agonist binding to receptor activates g protein signaling, and subsequent phosphorylation of the receptor enables arrestin-dependent signaling. Arrestin binding blocks Gβγ release (desensitization), but acts as a kinase scaffold to increase p38 MAPK phosphorylation.

METHODS

Animals. Male C57Bl/6 mice (Charles River, Wilmington, MA) >50 days were used and given *ad libitum* access to food and water. Conditional knockout of p38α in dopaminergic neurons was achieved using p38α floxed (*Mapk14*$^{lox/+}$) mice on C57Bl/6 background as previously described (Bruchas *et al*, 2011) and crossing them with mice that express Cre recombinase under the control of the dopamine transporter (DAT, *Slc6a3*$^{Cre/+}$), a unique marker for dopaminergic neurons (Zhuang *et al*, 2005). Global gene deletion of KOR (*Oprk1*$^{-/-}$) on C57Bl/6 background was accomplished as previously described (Hough *et al*, 2000). In some instances conventional KOR gene knockout mice were bred to also carry the DAT-Cre transgene. Conditional gene knockout of KOR was achieved using a floxed KOR line generated by the Institut Clinique de la Souris, in which Exon 1 of KOR was flanked by loxP sites. This exon was then selectively excised in dopaminergic neurons by crossing to the DAT-Cre line (Zhuang *et al*, 2005) and in serotonergic neurons by crossing with the ePet-Cre line (Scott *et al*, 2005). All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Genotyping. Genotyping was performed as previously described (Bruchas *et al*, 2011). PCR screening was performed using the following primers: A3 (5'-ATGAGATGCAGTACCCTTGGAGACCAGAAG-3') and A4 (5'-AGCCAGGGCTATACAGAGAAAAACCCTGTG-3') for the floxed and wild type (+) p38α alleles, giving bands of 230 and 180-bp, respectively. Primers A1 (5'-CCACAGAAGAGATGGAGCTATATGGATCTC-3') and A4 were used to detect the null p38α$^{\Delta}$ allele as a 420-bp PCR product. The DAT-Cre and ePet-Cre transgenes were detected using 5'-AGCGTTCGAACGCACTGATTTCG-3' and 5'-CGCCGTAAATCAATCGATGAGTTG-3', yielding a 330-bp band. The ROSA26-yellow fluorescent protein (ROSA-YFP) reporter gene was screened for with 5'-AAGACCGCGAAGAG TTTGTC-3', 5'-AAAGTCGCTCTGAGTTGTTAT-3', and 5'-GGAGCGGGAGAAATGGATATG-3', giving a 320-bp EYFP and 600-bp wild type band. For

screening of global KOR knockouts, the wild-type KOR allele was amplified using 5'-AATTGTCTTTTGGCCACTGC-3' and 5'-GTTGGTTGCGGTCTTCATCT-3' giving a 510–bp band, while the null KOR knockout allele yielded a 450-bp band when screened in a separate reaction with 5'-AGGAAGCAAAAAGCCTCTCC-3' and 5-'CTCGTCCTGCAGTTCATTCA-3'. For the floxed KOR mice, 5'-GCACCAAAGTCAGGGAAGGT-3' and 5'CAGGGCATATACTCTTCTCGCT-3' were used to screen for the wild type and floxed KOR alleles, giving bands of 317 and 427-bp, respectively.

Stereotaxic Injection. Mice were anesthetized with isoflurane and mounted on a Model 1900 stereotaxic alignment system (David Kopf Instruments). For VTA injections, a 32 gauge needle with a 45 degree beveled tip (Hamilton Company) was lowered bilaterally (A/P = -3.30mm, M/L = +/- 0.30mm from bregma) and 500 nL was injected at D/V -4.70-4.75mm and at -4.40-4.45mm from bregma. For DRN injections, a 25 gauge needle with beveled tip (Hamilton Company) was lowered unilaterally (A/P = -4.65mm, M/L = 0.00mm from bregma) and 1000 nL was injected at D/V -3.90-4.00mm from bregma. Injection locations were confirmed via postmortem immunohistochemistry.

Vector design and production. Lenti-KOR and Lenti-KOR(S369A) (Lenti-KSA) were developed and produced as previously described (Land *et al*, 2009). AAV-DIO KOR-GFP and AAV-DIO KSA-GFP were developed using the AM/CBA-DIO-poly vector (Cardin *et al*, 2009). Rat KOR-GFP or KSA-GFP was subcloned into the AAV-DIO vector using AgeI and NotI in the reverse coding orientation so that expression would only be driven in the presence of Cre recombinase under the chicken beta-actin promoter.

After confirming sequence fidelity, HEK293T cells were transfected with 10ug AAV and 20ug pDG1 vector per 10 cm plate. Three days post-transfection cells were harvested and subjected to three freeze thaw cycles. The supernatant was transferred to a Beckman tube containing a 40% sucrose cushion and spun at 27,000rpm overnight at 4˚C. Pellets were resuspended in CsCl at a density of 1.37g/ml and spun at 5,000rpm overnight at 4˚C. The following day, 1ml CsCl

fractions were run on an agarose gel and genome containing fractions were selected and spun again at 5000rpm overnight at 4˚C. 1mL fractions were collected again and genome containing fractions were dialyzed overnight. The filtered solution was transferred to a Beckman tube and spun at 27,000rpm overnight at 4˚C. The pellet (containing purified AAV) was resuspended in 100µl 1xHBSS.

Conditioned Place Preference (CPP). Mice were trained in a balanced three-chamber apparatus consisting of chambers of equal size with horizontal or vertical stripes, and a third smaller chamber in the center. The paradigm consisted of tests on day one and four and training on days two and three. On test days the mice were placed in the center chamber then allowed to roam freely through all three chambers for 30 min. Time spent in each chamber was recorded with a video camera (Canon DC310) and analyzed using Ethovision (Noldus, Wageningen, The Netherlands). On training days, mice received saline vehicle (10 mL/kg, i.p.) while restricted to one chamber in the morning and U50,488 (2.5-5.0 mg/kg at 10 mL/kg, i.p.) or cocaine (15 mg/kg at 10 mL/kg, s.c.) in the other chamber in the afternoon. The drug-paired chamber was assigned based on the day one pre-test to ensure that the average time spent in each chamber was the same for all groups. The final preference score was determined based on the difference in the amount of time spent in the drug-paired chamber on the day four post-test and the day one pre-test. All mice were handled 1-2 times a day for four days the week prior to the day one pre-test. For cocaine CPP experiments, locomotor activity was recorded and analyzed.

U50,488 Locomotor Experiments. Following the four day U50,488 CPA experiment, on days five and six a subset of mice were injected with either saline or U50,488 (10 mg/kg) and allowed to roam the CPP chamber freely. To allow for a within subjects comparison, injections were administered in an alternating fashion such that half of the mice in a given cohort received each injection on a given day.

Tail-flick analgesia. Mice were held while the tip of their tail was submerged in 52 +/- 1 ˚C water, and the tail withdrawal latencies were timed. Each mouse was exposed twice, once as a baseline and a second time 30 min after administration of 15 mg/kg U50,488.

Immunohistochemistry. Mice were anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and intracardially perfused with ice-cold phosphate-buffered saline (PBS) and 4% paraformaldehyde. Brains were stored overnight in 4% paraformaldehyde, then cryoprotected in 30% sucrose (wt/vol) in phosphate buffer (PB) solution. At least 48 hours later, brains were sliced at 30 μm using a microtome (Leica). Slices were stored at 4˚C in PB with 0.1% sodium azide. VTA and DRN slices were then taken and washed for 3 x 10 minutes in PBS before blocking in PBS containing 5% normal goat serum and 0.3% Triton X-100. Slices were then incubated for 36-48 hours in blocking solution containing pairs of the following primary antibodies: chicken anti-tyrosine hydroxylase (1:1000, AB9702, Millipore), rabbit anti-KOR (1:1000, from an affinity purified 1.32 μg/mL stock) (Drake *et al*, 1996), chicken anti-GFP (1:3000, AB13970, Abcam), mouse anti-tyrosine hydroxylase (1:500, T1299, Sigma), mouse anti-tryptophan hydroxylase (1:500, T0678, Sigma), and mouse anti-GAD67 (1:1000, MAB5406, Millipore). Slices were then washed for 6 x 15 minutes in PBS and incubated in blocking solution containing Alexa 488 and Alexa 555 (1:500, Life Technologies) tagged to IgG of the appropriate species. The slices were then washed for 3 x 10 minutes in PBS and 2 x 10 minutes in PB. Slices were then mounted onto Superfrost Plus slides (Fisher) with Vectashield mounting media and sealed with nail polish for microscopy. All slices were imaged at the W.M. Keck Center for Advanced Studies in Neural Signaling (Seattle, WA) on either a Leica SL confocal or a Nikon Upright with QImaging Camera.

Rotarod. Mice were placed on a 4 cm wide rotating rod (Rotarod, San Diego Instruments), which accelerated from 4 to 40 rpm over 4 min. The latency to fall off of or clasp onto the rotating rod was recorded.

***In vitro* voltammetry.** Mice were rapidly decapitated and the brain was dissected and slices prepared as previously described (Lemos *et al*, 2012b). Microelectrodes were fabricated by pulling carbon fiber through a glass pipette using a Sutter P-97 puller to form a glass seal. The fiber was then cut to approximately 100-120 μm past the seal. The potential at the working electrode was held at -0.4V versus an Ag/AgCl reference electrode, then ramped to +1.3V and then back to -0.4V at 400V/s at a rate of 10Hz. A single biphasic electric pulse was applied using a platinum/iridium parallel bipolar stimulating electrode (FHC; Bowdoin, ME) at 300-500 μA to evoke dopamine release. Each animal contributed 1-3 slices to each group. Each slice was counted as an individual replicate.

***In vivo* voltammetry.** Mice were anesthetized with 1.5 g/kg urethane. Sixty minutes later, depth of anesthesia was confirmed and mice were mounted on a Model 900 stereotaxic alignment system (David Kopf instruments). A carbon fiber microelectrode (manufactured as described above) was implanted in the NAc (A/P = +1.52mm, M/L = +/-1.15mm, D/V = -3.9-4.3mm from bregma) and a silver chloride reference electrode was implanted contralaterally. Bipolar parallel stimulation electrodes (Plastics One; Roanoke, VA) were implanted either in the medial forebrain bundle (MFB) (A/P = -2.40mm, M/L = +/-1.10mm, D/V = -4.5-5.20mm from bregma) or the pedunculopontine tegmental area (PPTg) (A/P = -0.68mm from lambda, M/L = +/- 0.70mm, D/V = -2.50-3.20mm from bregma). For MFB recordings, a stimulus of 6 pulses was applied at 30 Hz and 300 μA to evoke dopamine release. For PPTg recordings, a stimulus of 30 pulses was applied at 60 Hz and 150 μA. Stimuli were applied every 300 sec. After 60 min, mice were injected with U50,488 (5 mg/kg, *i.p.*) and recording continued for 60 min. The six stimuli prior to drug administration were averaged to define the baseline. At the end of each experiment, the remaining urethane was injected into the mouse prior to inducing an electrolytic lesion at the site of the working electrode. The mouse was then intracardially perfused with ice-cold PBS 4% paraformaldehyde. After cryoprotection with 30% sucrose, brains were sliced at 40 μm and then stained with cresyl violet to confirm the recording site (and, for PPTg stimulation experiments, the stimulation site) as previously described (Ehrich *et al*, 2014).

Drugs. (+/-) U50,488H, (+/-) U69,593, nor-binaltorphimine dihydrochloride (norBNI), and cocaine hydrochloride were provided by the National Institute of Drug Abuse Drug Supply Program. SB203580 was obtained from Millipore (Billerica, MA). Urethane was obtained from Sigma-Aldrich (St. Louis, MO). Sodium pentobarbital and Beuthanasia Special-D were obtained from University of Washington Medical Center Drug Services. All drugs were dissolved in saline for use *in vivo* or aCSF for use *in vitro*.

Analysis. Data was compiled in Microsoft Excel and data analyses were performed in Prism 5.01 for Windows (GraphPad, San Diego, CA). CPP data was analyzed by Student's t test or one-way ANOVA where appropriate. Locomotor, tail-flick analgesia, and voltammetry data were analyzed using two-way repeated measures ANOVA. For *in vivo* voltammetry, the last six stimuli of each treatment (baseline vs. U50,488) were averaged for each animal and this average was used for statistical comparisons. For *in vitro* voltammetry, the last four stimuli of each treatment (baseline, SB203580/Vehicle, U69,593, and norBNI) were averaged for each slice. When ANOVA revealed statistical significance, Bonferroni post-hoc analyses were applied where appropriate; in certain cases Bonferroni post-hoc analyses were applied after no interaction was seen to confirm negative results. Data are presented as mean ± SEM. Significance was set at $p < 0.05$.

RESULTS

KOR Activation in Both Dopamine and Serotonin Neurons is Required for Place Aversion

To assess the role of KOR activation in dopamine neurons and serotonin neurons in aversion, we generated a conditional knockout of KOR in dopaminergic (DAT-Cre-expressing) neurons and serotonergic (ePet-Cre-expressing) neurons using a Cre-induced excision of a floxed KOR allele (KOR CKO^{DAT} and KOR CKO^{Pet}, respectively). Male $KOR^{lox/lox}$ littermate controls from both colonies showed CPA to U50,488, whereas neither KOR CKO^{DAT} nor KOR CKO^{Pet} showed U50,488 CPA (Figure 3.2a, one-way ANOVA, $F_{(2,40)}=7.338$, $p < 0.01$; post-hocs: $KOR^{lox/lox}$ vs. KOR CKO^{DAT}, $t = 2.340$, $p < 0.05$; $KOR^{lox/lox}$ vs. KOR CKO^{Pet}, $t = 3.623$, $p < 0.01$). These results demonstrated that selective genetic ablation of KOR within either class of neurons is sufficient to block U50,488 CPA.

KOR CKO From Dopaminergic Neurons, But Not Serotonergic Neurons, Blocks Inhibition of Dopamine Signaling

Prior studies suggest that the aversion response to KOR activation is caused by inhibition of dopamine release in the NAc (Shippenberg *et al*, 1993), which requires expression of KOR on dopamine neurons (Chefer *et al*, 2013b; Tejeda *et al*, 2013). To determine if KOR CKO^{Pet} blocked U50,488 CPA by altering the response of NAc dopamine signaling to KOR activation, we used fast-scan cyclic voltammetry (FSCV) to record changes in evoked dopamine release from midbrain slices containing the NAc (Figure 3.2b). $KOR^{lox/lox}$ and KOR CKO^{Pet} mice both showed that the KOR agonist U69,593 (1 μM) produced robust inhibition of evoked dopamine (to 52.7% and 54.5% below baseline, respectively) that could be reversed upon washout or addition of the KOR antagonist norBNI (1 μM) (Figure 3.2c-e). In contrast, evoked dopamine response in slices from KOR CKO^{DAT} showed no significant change in the presence of either drug (Figure 3.2c-e). A two-way repeated measures ANOVA showed a significant interaction ($F_{(2,11)}=18.10$, $p < 0.001$). Further, post-hoc analyses demonstrated that both $KOR^{lox/lox}$

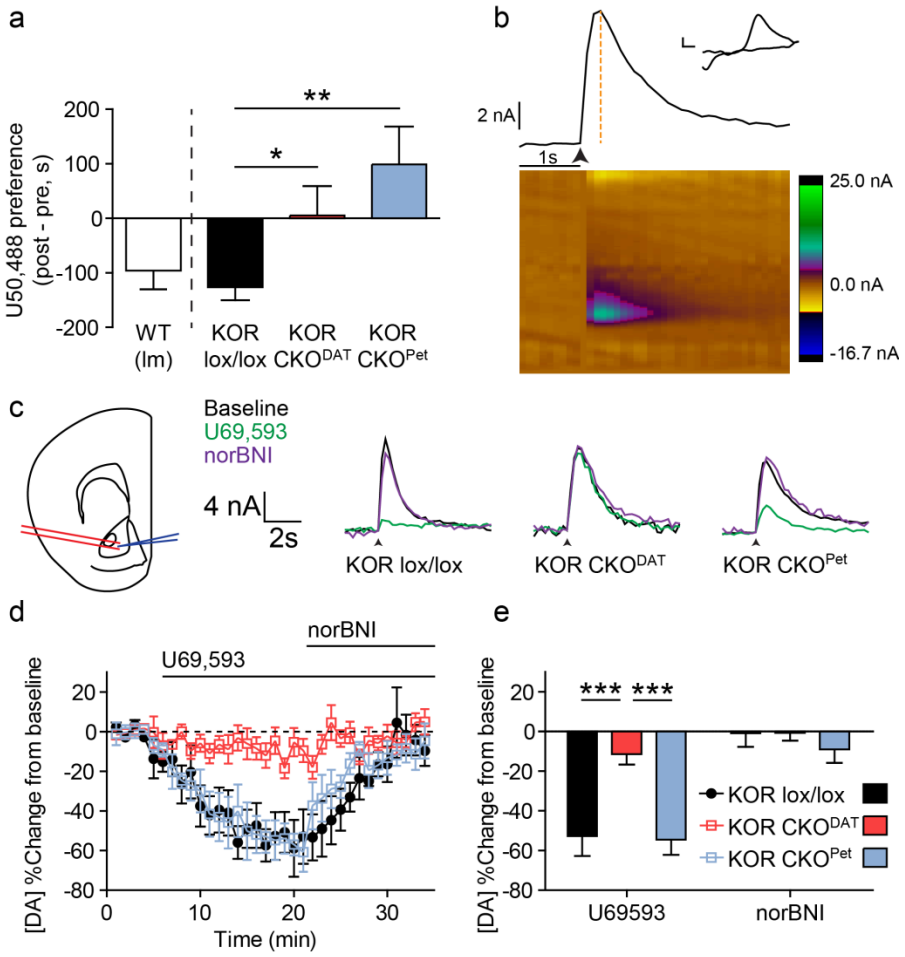


Figure 3.2. Conditional knockout of KOR from either dopaminergic or serotonergic neurons blocks U50,488-CPA, but only KOR CKO from dopaminergic neurons blocks inhibition of dopamine release.

(a) Preference scores show that both CKO KOR^{DAT} and CKO KOR^{Pet} lack U50,488 CPA relative to KOR$^{lox/lox}$ littermates (n=9-22) (* $p < 0.05$, ** $p < 0.01$, Bonferroni post-hoc after one-way ANOVA). *(b)* Representative image showing analysis of slice FSCV data. Bottom: heat map shows current generated by oxidation and reduction at the site of the working electrode during application of the triangle waveform. Top: current by time plot shows evoked dopamine release and uptake. Orange dashed line shows magnitude of peak current, which was recorded for each stimulation (marked by arrow: 1 pulse, 300-500 μA). Inset: cyclic voltammagram from the time of peak current confirms that the current recorded was induced by oxidation and reduction of dopamine; scale bar shows 2 nA, 0.1 V. *(c)* Left: diagram depicting placement of bipolar stimulating electrode (red) and recording electrode (blue) during slice FSCV experiments (modified from Franklin and Paxinos). Right: representative traces showing raw current responses of KOR$^{lox/lox}$, KOR CKO^{DAT} and KOR CKO^{Pet} to 1 μM U69,593 (green) and 1 μM norBNI (purple). Scale bars show 4 nA, 2s; stimulation marked by arrow. *(d)* Change from baseline locally stimulated peak [DA] during baseline and administration of 1 μM U69,593 and 1 μM norBNI. Each drug was bath-applied during the interval signified by the bar. *(e)* Summary data shows that KOR CKO^{DAT} blocked U69,593-induced inhibition of dopamine relative to KOR$^{lox/lox}$ littermates; whereas inhibition of dopamine was unaltered in KOR CKO^{Pet} (n=4-5 slices from 2-3 animals) (*** $p < 0.001$, Bonferroni post-hoc after one-way ANOVA). All graphs show mean ± SEM.

($t = 4.309$, $p < 0.001$) and KOR CKO^{Pet} ($t = 4.242$, $p < 0.001$) mice showed significant inhibition of dopamine release relative to KOR CKO^{DAT} mice after U69,593. Thus, while KOR excision from either dopamine neurons or serotonin neurons blocks CPA, KOR excision from serotonin neurons blocks CPA despite continued inhibition of NAc dopamine release.

KOR Activation in the VTA is Required for Place Aversion

To further assess the role of KOR activation in VTA dopamine neurons, we first injected the long-acting KOR antagonist norBNI (2.5 μg/μL) into the VTA. NorBNI has previously been demonstrated to block KOR activation up to 21 days after administration (Bruchas *et al*, 2007b), and we have previously shown that after local injection of norBNI into the DRN, KOR activation is unaltered in serotonergic projections from the DRN to the NAc (Land *et al*, 2009). Mice injected with saline in the VTA showed CPA to U50,488, whereas mice injected with norBNI in the VTA failed to show U50,488 CPA (Figure 3.3a; unpaired t test, $t_{(7)} = 3.306$, $p < 0.05$). This demonstrated that activation of KORs within the VTA is necessary to induce U50,488 CPA, and by extension this also demonstrates that activation of KOR on the terminals of dopamine neurons within the NAc is not sufficient to induce U50,488 CPA.

Restoration of Full KOR Signaling to the VTA of $KOR^{-/-}$ Mice Rescues the Aversive Properties of KOR Activation

We next isolated the effect of KOR activation on neurons located within the VTA by stereotaxically injecting the VTA of $KOR^{-/-}$ (*Oprk1*$^{-/-}$) mice with a lentiviral vector directing expression of the wild type KOR (lenti-KOR) (viral design as previously shown (Land *et al*, 2009)). As a control, separate cohorts of mice were injected with lentiviral vectors directing expression of a KOR with a serine to alanine substitution at serine 369 (lenti-KSA) or of enhanced green fluorescent protein alone (lenti-eGFP). Phosphorylation of KOR by GRK3 at serine 369 is necessary for arrestin-dependent activation of p38 MAPK while allowing for

arrestin-independent signaling, such as activation of extracellular signal-regulated kinase (ERK1/2) (Bruchas *et al*, 2006). After a three-week recovery to allow time for receptor expression, mice were tested for U50,488 CPA. Lenti-KOR injection in the VTA selectively restored the aversive properties of KOR activation (one-way ANOVA, $F_{(2,33)} = 5.140$, $p < 0.05$) (Figure 3.3b). $KOR^{-/-}$ mice expressing lenti-KOR in the VTA showed U50,488-induced aversion while $KOR^{-/-}$ mice expressing lenti-KSA ($t = 2.891$, $p < 0.05$) or lenti-eGFP ($t = 2.644$, $p < 0.05$) did not (Figure 3.3b). $KOR^{-/-}$ mice injected with lenti-KOR in the DRN as a positive control also showed a restoration of U50,488 CPA (data not shown, one-sample t test, $p < 0.01$, $t_{(14)} = 3.538$), replicating our previous findings (Land *et al*, 2009).

Systemic injection of 10 mg/kg U50,488 induces locomotor hypoactivity, an effect that is mediated by KOR actions at substantia nigra dopamine neurons (Schnur and Walker, 1990). For two days following the CPA post-test, a subset of animals were alternately injected with either saline or U50,488 (10 mg/kg, i.p.) and allowed to freely explore the CPP chambers. Two-way ANOVA showed a significant interaction ($F_{(4,37)}=8.480$, $p < 0.0001$) and $KOR^{+/+}$ mice showed a significant inhibition in locomotor activity after U50,488 relative to saline ($t = 7.259$, $p < 0.001$). Neither $KOR^{-/-}$ mice nor any virally injected group showed an effect of U50,488 on locomotor activity ($p > 0.05$) (Figure 3.3c), demonstrating that the restoration of KOR expression was restricted.

We previously reported that injection of lenti-KOR or lenti-KSA into the DRN restored U50,488-induced analgesia, leading to an increase in warm water tail-flick latency (Land *et al*, 2009). Two-way ANOVA showed a significant interaction ($F_{(4,57)} = 8.827$, $p < 0.0001$), and subsequent post-hoc analysis demonstrated that only $KOR^{+/+}$ mice show a significant increase in their tail-flick latency 30 min after injection with U50,488 (15 mg/kg, i.p.) ($KOR^{+/+}$, $p < 0.001$). Neither $KOR^{-/-}$ mice without viral injection nor any group receiving lentiviral injections into the VTA showed an effect of U50,488 ($p > 0.05$, all groups) (Figure 3.3d). As a positive control, we performed a tail-flick analgesia test on $KOR^{-/-}$ mice that had received lenti-KOR injected into the DRN. A paired t test showed that the lenti-KOR injection into the DRN restored U50,488-

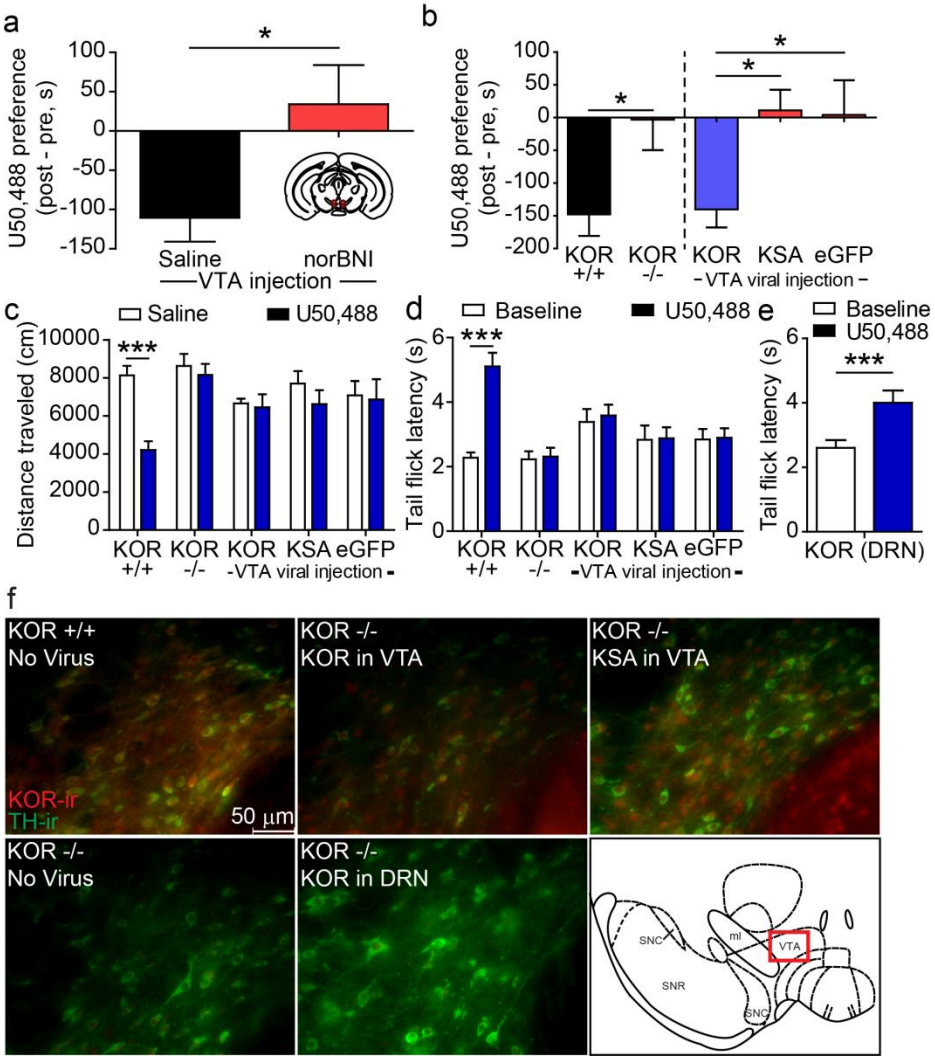


Figure 3.3. KOR activation within the VTA is both necessary and sufficient for U50,488-induced CPA.

(a) Place preference scores for WT mice injected with either saline or 2.5 μg/μL norBNI in the VTA prior to U50,488 conditioning (n = 8) (* $p < 0.05$, t test, saline vs. norBNI). *(b)* Place preference scores for $KOR^{+/+}$ mice and $KOR^{-/-}$ mice receiving either no injection, lenti-KOR, lenti-KSA, or lenti-eGFP in the VTA prior to U50,488 CPA (n = 11-19) (left: * $p < 0.05$, t test; right: * $p < 0.05$, Bonferroni post-hoc analysis after one-way ANOVA). *(c)* Locomotor activity after saline or 10 mg/kg U50,488 (n = 6-11) (*** $p < 0.001$, Bonferroni post-hoc analysis after two-way ANOVA) (x-axis labels are the same groups as in panel b). *(d)* Latency to tail-flick after submersion in warm water. Scores before and 30 min after 15 mg/kg U50,488 (n = 11-19) (*** $p < 0.001$, Bonferroni post-hoc after two-way ANOVA). (x-axis labels are the same groups as in panel b). *(e)* Latency to tail-flick after submersion in warm water for $KOR^{-/-}$ mice injected with lenti-KOR in DRN. Scores before and 30 min after 15 mg/kg U50,488 (n = 13) (*** $p < 0.001$, paired t test). *(f)* Representative IHC images showing restoration of KOR-immunoreactivity to the VTA of $KOR^{-/-}$ after injection of lenti-KOR or lenti-KSA into the VTA, but not after injection of lenti-KOR into the DRN. Bottom-right inset shows location of the section of the parabrachial pigmented nucleus of the VTA from which images were taken (modified from Franklin and Paxinos(Paxinos George, 2008)).

induced analgesia in these mice ($t_{(12)} = 4.409$, $p < 0.001$) (Figure 3.3e). This demonstrated that the restoration of U50,488 CPA in lenti-KOR VTA mice was not due to viral diffusion to the nearby DRN.

After the behavioral analyses were concluded, mice were euthanized and immunohistochemistry (IHC) was performed to assess KOR expression in the VTA. KOR-immunoreactivity (ir) was evident in the VTA of wild-type mice, as well as $KOR^{-/-}$ mice injected with lenti-KOR or lenti-KSA in the VTA; but not in uninjected $KOR^{-/-}$ mice (Figure 3.3f). KOR-ir was not evident in the VTA after lenti-KOR injection into the DRN (Figure 3.3g), thus further demonstrating that the aversion seen after DRN injection was not due to viral diffusion to the VTA. KOR expression was not seen outside of the VTA in mice injected intra-VTA with either lenti-KSA or lenti-KOR (data not shown).

Selective restoration of KOR Activation to VTA Dopamine Neurons of $KOR^{-/-}$ Mice Rescues Aversion in a Phospho-dependent Manner

Although the previous experiment demonstrates that KOR activation and phosphorylation within VTA neurons was sufficient to support U50,488 CPA, it did not clarify which cell types are required. To determine if KOR expression within dopaminergic neurons of the VTA alone was sufficient to support KOR-CPA, we developed an adeno-associated double-floxed inverse open reading frame (AAV-DIO) viral construct (Cardin *et al*, 2009) directing expression of GFP-tagged KOR or KSA selectively in Cre-expressing neurons (Figure 3.4a; DIO-KOR-GFP or DIO-KSA-GFP). The number of cells showing colocalization between GFP-ir and immunoreactivity for tyrosine hydroxylase (TH-ir), a selective marker for dopamine neurons, was significantly increased in $DAT^{Cre/+}$mice relative to $DAT^{+/+}$mice (Figure 3.4b,c; two-way ANOVA, main effect of genotype, $F_{(1,43)}=110.7$, $p < 0.0001$), demonstrating selectivity of expression. Although WT control mice showed a normal CPA to U50,488, $DAT^{Cre/+}$ $KOR^{-/-}$ mice showed U50,488 CPA after intra-VTA injection with DIO-KOR-GFP but not DIO-KSA-GFP (Figure

3.4d; unpaired t test, $t(29)=2.767$, $p < 0.01$). This demonstrates that KOR activation in VTA dopamine neurons alone is sufficient to support CPA, but this requires an intact GRK3 phosphorylation site.

Expression of p38α MAPK in Dopamine Neurons is Selectively Required for KOR CPA

To test the hypothesis that p38 MAPK activation in dopaminergic neurons is required for the aversive properties of KOR activation, we generated a conditional knockout of p38α MAPK in DAT-expressing neurons, using a Cre-induced recombination of a floxed p38α MAPK allele (Bruchas *et al*, 2011). A subset of animals carried a floxed-STOP ROSA-YFP reporter allele to ensure that Cre-induced recombination only occurred in dopamine neurons. Subsequent IHC demonstrated that YFP highly co-expressed with tyrosine hydroxylase (TH-ir) within the VTA only in Cre-expressing animals (Figure 3.5a), but did not co-label with the 67 kDa isoform of glutamic acid decarboxylase, a marker for GABAergic somata (Figure 3.5b). The DRN also contains dopaminergic neurons (Fu *et al*, 2010; Hasue and Shammah-Lagnado, 2002; Stratford and Wirtshafter, 1990), and we confirmed that Cre-driven YFP expression co-labeled with TH-ir neurons in the A10c and A10dc populations within the DRN. However, no overlap was observed between YFP-ir cells and immunoreactivity for tryptophan hydroxylase (TPH), a marker for serotonergic neurons (Figure 3.5c). Experiments were performed on three genotypes: WT ($DAT^{+/+}$; $p38\alpha^{+/lox}$), Cre Δ ($DAT^{Cre/+}$; $p38\alpha^{-/+}$), and p38α CKO^{DAT} ($DAT^{Cre/+}$; $p38\alpha^{-/lox}$). The Cre Δ mice were utilized as controls to address any concerns regarding potential reductions in p38α or DAT expression (Giros *et al*, 1996).

As expected, WT and Cre Δ mice showed U50,488-induced CPA (Figure 3.6a). However, p38α CKO^{DAT} mice failed to show CPA to the drug-paired context (one-way ANOVA, $F_{(2,31)} = 3.800$, $p < 0.05$; post-hoc vs. WT, $t = 2.366$, $p < 0.05$; post-hoc vs. Cre Δ, $t = 2.496$, $p < 0.05$) (Figure 3.6a). These results suggest that the aversive properties of KOR activation are dependent on the

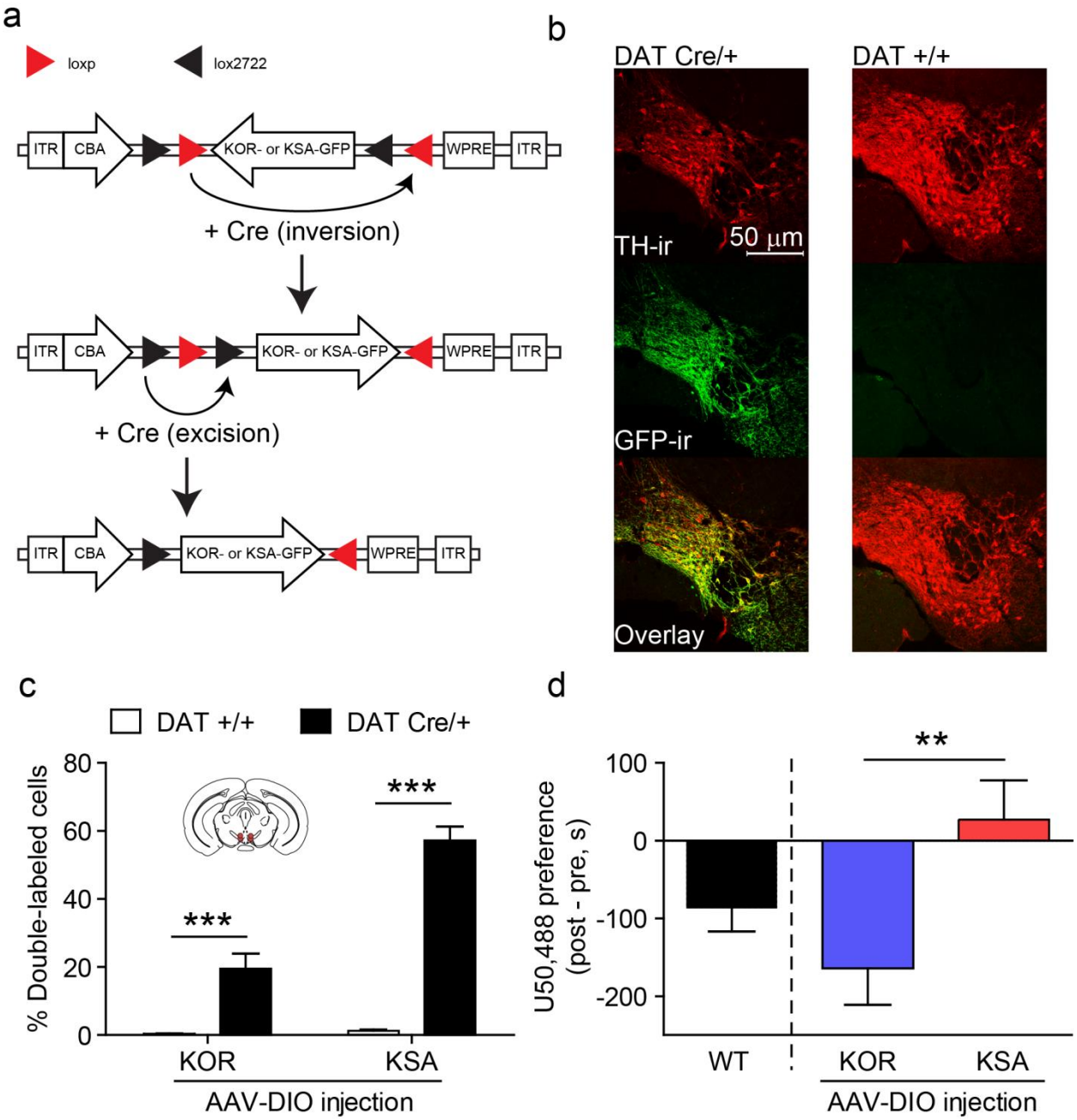


Figure 3.4. KOR activation selectively in VTA dopamine neurons is sufficient for KOR CPA.

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

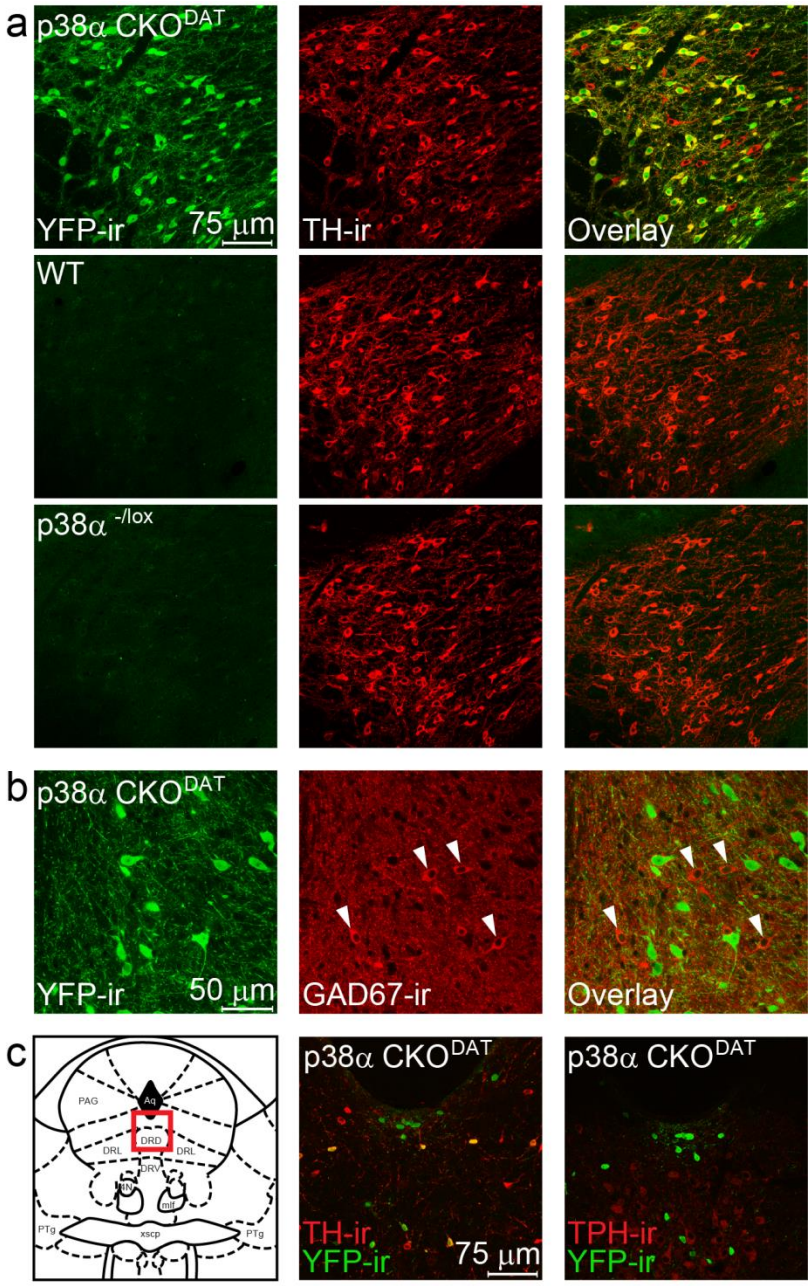


Figure 3.5. ROSA-YFP reporter shows that DAT-Cre selectively expresses in dopaminergic neurons.

(a) Representative IHC images showing that YFP-ir in an animal with DAT-Cre-directed ROSA-YFP expression extensively co-labels with TH-ir throughout the VTA, yet YFP expression is absent in sections from mice lacking Cre. *(b)* Representative IHC images showing that YFP-ir in an animal with DAT-Cre-directed YFP expression in the VTA does not co-label with GAD67-ir. Arrows point to GABAergic neurons. *(c)* Representative IHC images showing that YFP-ir in an animal with DAT-Cre-directed YFP expression in the DRN co-labels with TH-ir but not TPH-ir. Left panel highlights the imaged section of the DRN (modified from Franklin and Paxinos (Paxinos George, 2008)).

expression of p38α MAPK in dopaminergic neurons.

We next tested mice with repeated exposure to the rotarod performance assay, a dopamine-dependent test of motor coordination in which mice learn to avoid a negative outcome over repeated trials (Zhou and Palmiter, 1995). p38α CKO^{DAT} mice show no deficit in this task, as demonstrated by a statistically significant main effect of trial (Figure 3.6b; two-way repeated measures ANOVA, $F_{(8, 36)}$=55.55, $p < 0.0001$) and no effect of genotype ($F_{(2,36)} = 1.059$, $p = 0.3573$). Although a statistically significant interaction was seen ($F_{(16,36)} = 2.098$, $p < 0.01$), post-hoc analyses showed no significant difference between the performance of p38α CKO^{DAT} mice and WT ($p > 0.05$) or Cre Δ ($p > 0.05$) mice during any trial. In fact, p38α CKO^{DAT} mice show a trend towards improved performance relative to WT, suggesting that the lack of U50,488-induced CPA is not due to a deficit in avoidance learning.

To determine whether conditional knockout of p38α affected Pavlovian contextual learning, we measured cocaine CPP. p38α CKO^{DAT} mice developed robust cocaine CPP and there was no significant difference between the three genotypes (one-way ANOVA, $F_{(2,25)} = 1.385$, $p = 0.2688$) (Figure 3.6c). Locomotor activity was recorded and analyzed during the cocaine conditioning sessions, and significant locomotor sensitization was evident in all three genotypes (Figure 3.6d), as demonstrated by a main effect of training session ($F_{(1,28)} = 52.12$, $p < 0.0001$), no interaction ($F_{(2,28)} = 0.1836$, $p = 0.8333$) and no effect of genotype ($F_{(2,28)} = 1.314$, $p = 0.2849$). Further, post-hoc for all groups showed a statistically significant increase in locomotor activity on the second day of cocaine administration relative to the first (WT: $t = 3.867$, $p < 0.01$; Cre Δ: $t = 3.863$, $p < 0.01$; p38α CKO^{DAT}: $t = 4.808$, $p < 0.001$). Because acute sensitization to the locomotor effects of cocaine has been hypothesized to be mediated by induction of glutamatergic long-term potentiation (LTP) in dopaminergic neurons (Borgland *et al*, 2006), these results suggest that inactivation of p38α MAPK does not affect the LTP induction underlying this response.

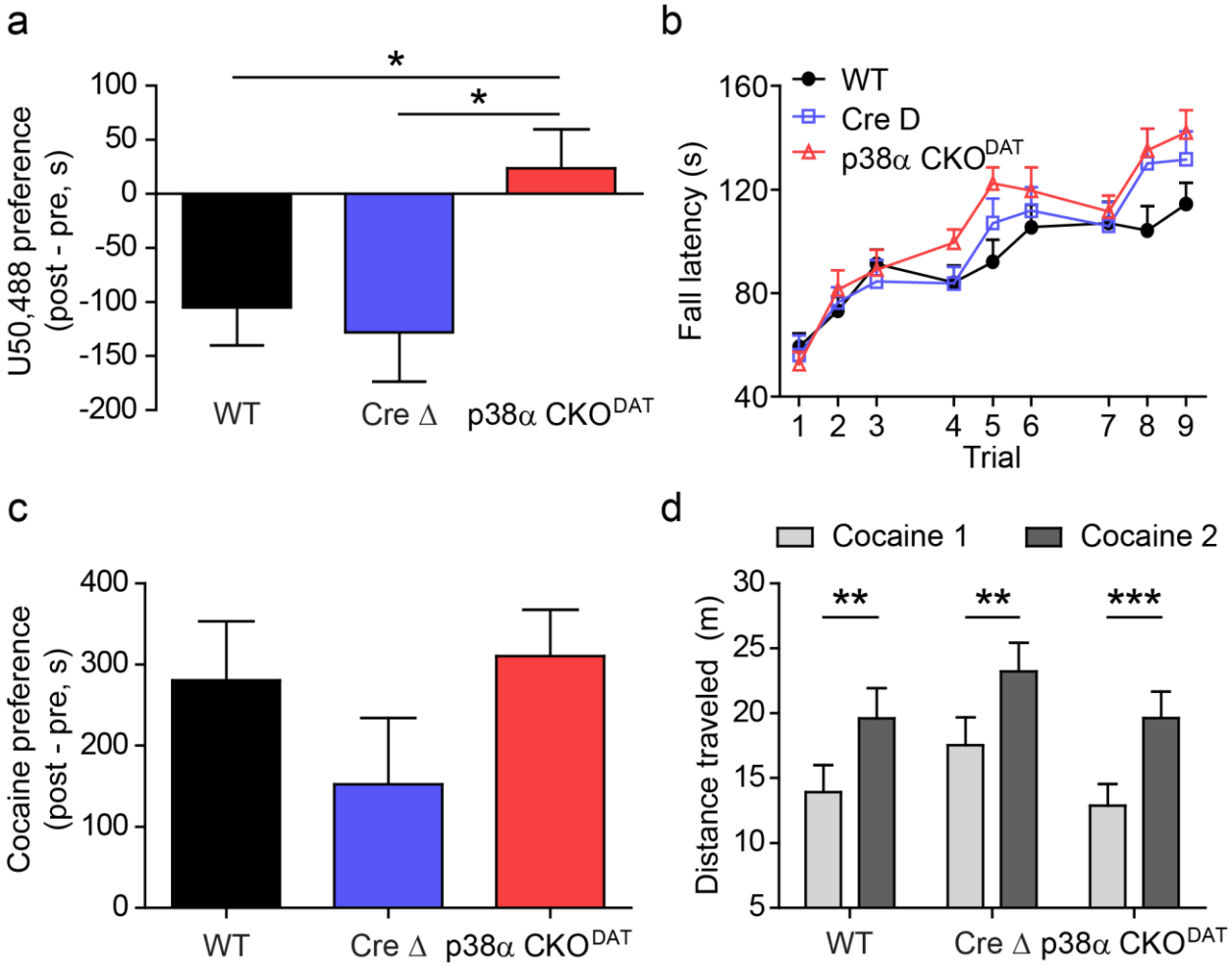


Figure 3.6 Conditional knockout of p38 MAPK from dopaminergic neurons selectively blocks the aversive properties of KOR activation.

(a) Preference scores show that p38α CKO^{DAT} mice do not show U50,488 CPA, unlike WT and Cre Δ littermates (n=12-20) (* $p < 0.05$, Bonferroni post-hoc after one-way ANOVA). *(b)* Fall latency for each trial from rotarod performance assay shows no effect of p38α CKO^{DAT} (n=10-16). *(c)* WT, Cre Δ, and p38α CKO^{DAT} mice all show normal cocaine CPP (n=8-11). *(d)* WT, Cre Δ, and p38α CKO^{DAT} mice all show normal acute locomotor sensitization to cocaine (n=8-11) (** $p < 0.01$, *** $p < 0.001$, Bonferroni post-hoc after two-way ANOVA).

Conditional Knockout of p38α from Dopamine Neurons Fails to Alter KOR-induced Inhibition of Dopamine Release *in vivo*

To determine if p38α MAPK was also required for the inhibition of dopamine release, we used FSCV to measure subsecond changes in the release of dopamine in anesthetized mice (Figure 3.7a). U50,488 (5 mg/kg, i.p.) inhibited dopamine release evoked by medial forebrain bundle (MFB) stimulation. U50,488 reduced evoked dopamine release equivalently in both NAc core (54.5% +/- 4.2% inhibition from baseline) and shell (50.7% +/- 2.3% inhibition from baseline) of wild type mice, but did not do so in $KOR^{-/-}$ mice (Figure 3.7b; two-way ANOVA, main effect of genotype: $F_{(1,16)}=205.3$, $p < 0.0001$).

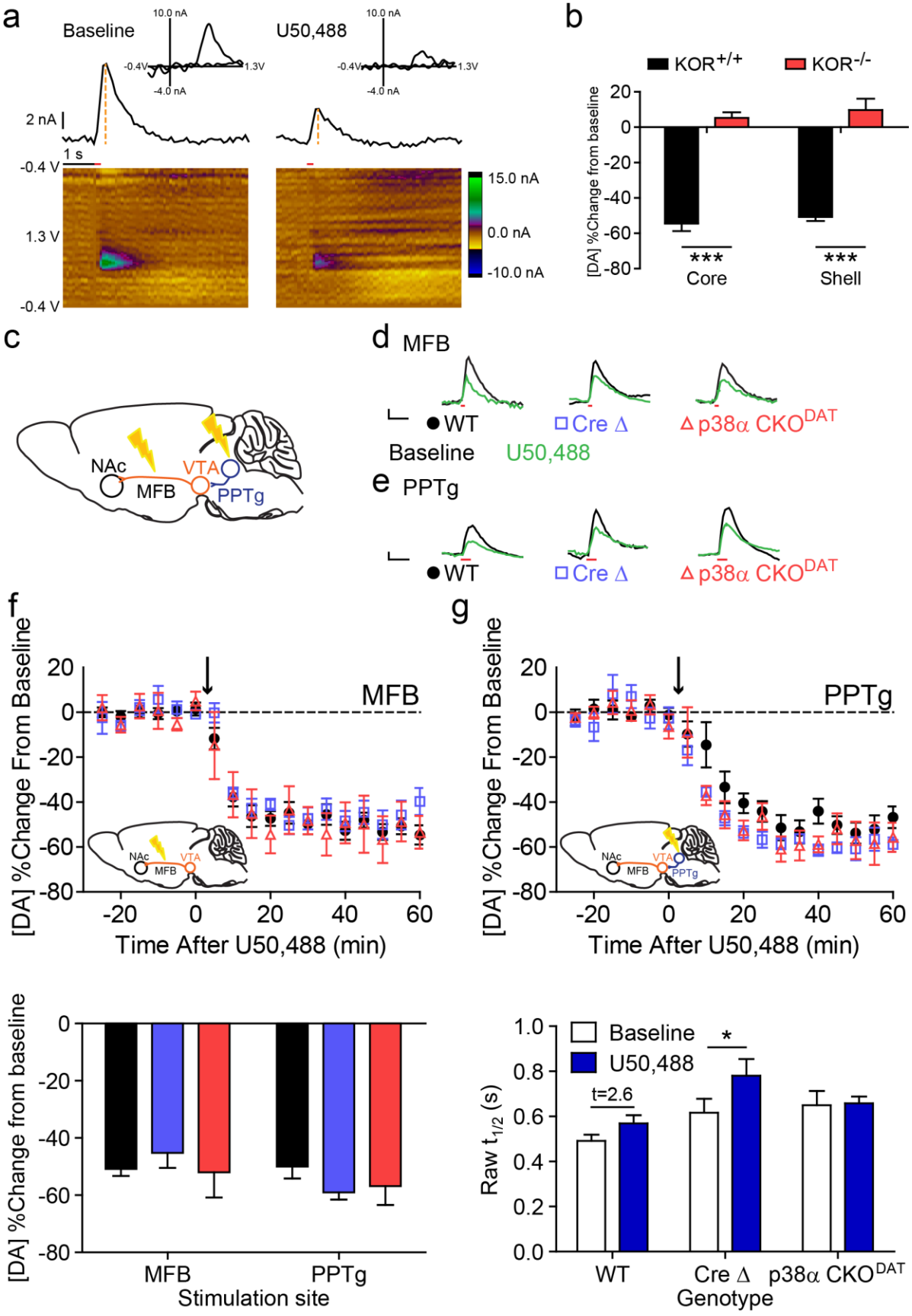
Because there was no trend towards a main effect of NAc subregion on the inhibition of dopamine release ($F_{(1,16)} = 0.9631$, $p = 0.3410$) and no interaction ($F_{(1,16)}=0.006200$, $p = 0.9382$), time-resolved data collected from the NAc core and shell was collapsed together for comparison between MFB and pedunculopontine tegmental nucleus (PPTg) stimulation. MFB stimulation allows for direct stimulation of the fibers projecting to the NAc and minimizes the potential impact of somatodendritic KOR activation, whereas PPTg stimulation synaptically activates VTA neurons (Zweifel *et al*, 2009) and may reveal somatodendritic effects of KOR activation on dopamine release in the NAc (Ehrich *et al*, 2014; Ford *et al*, 2006, 2007; Margolis *et al*, 2006a). Positioning of the stimulating electrodes as shown (Figure 3.7c), resulted in robust dopamine signal following either MFB or PPTg stimulation (inset Figure 3.7d,e, respectively). Release evoked by either MFB or PPTg stimulation was significantly inhibited by U50,488 (5 mg/kg, i.p.); however, conditional knockout of p38α from dopamine neurons did not affect U50,488-induced inhibition of dopamine release following either MFB (Figure 3.7d,f,h; one-way ANOVA showed no effect: $F_{(2,23)}=0.4620$, $p = 0.6358$) or PPTg (Figure 3.7e,g,h; one-way ANOVA: $F_{(2,18)}=1.206$, $p = 0.3226$) stimulation. Given the role of p38 in aversion and the prediction that the inhibition of dopamine release should be intrinsically aversive, the lack of effect of p38α deletion on evoked dopamine release was a surprise. This suggested that the aversive effects of

KOR activation were not caused by either presynaptic or somatic inhibition of dopamine release within the NAc.

Because both KOR activation and direct p38 MAPK activation by anisomycin have been shown to alter the rate of dopamine uptake (Thompson *et al*, 2000; Zhu *et al*, 2005), we analyzed the half-life ($t_{1/2}$) of MFB-evoked dopamine. Although two-way ANOVA showed no interaction, a main effect of U50,488 administration was observed ($F_{(1,22)}=9.776$, $p < 0.01$). This effect appeared to be driven by a strong effect in the Cre Δ mice ($t=3.134$, $p < 0.05$), although a trend towards an effect was also observed in WT mice ($t=2.569$, $p < 0.10$). A clear lack of an effect was observed in p38α CKO^{DAT} mice ($t=0.1589$, $p > 0.05$). As such, it is possible that KOR may regulate uptake of dopamine released in the nucleus accumbens in a p38 MAPK-dependent fashion. This is also surprising, as if this p38 MAPK-dependent on uptake is real, it is observed in the absence of any effect on actual dopamine concentrations. Intriguingly, there was also a clear main effect of genotype ($F_{(2,22)}=5.089$, $p < 0.05$). This suggests that the presence of $DAT^{Cre/+}$ may lead to a general inhibition of uptake, presumably due to a decrease in DAT expression.

Pharmacological Inhibition of p38 MAPK by SB203580 Fails to Alter KOR-induced Inhibition of Dopamine Release *in vitro*

To validate the results of the FSCV experiments performed *in vivo* in transgenic mice, we applied the selective p38 inhibitor SB203580 in an *in vitro* slice FSCV assay. Similar to results obtained *in vivo*, electrically-evoked dopamine release detected in NAc slices was significantly reduced by the KOR agonist U69,593, and the inhibition was blocked by pretreatment with norBNI (10μM) (Figure 3.8a,b). Bath application of 10μM SB203580 failed to alter basal dopamine release (two-way repeated measures ANOVA showed no main effect of SB203580: $F_{(1,14)}=1.297$; $p = 0.7794$). SB203580 also failed to alter either inhibition of dopamine release by U69,593 or the reversal of inhibition by norBNI (main effect of KOR drug: $F_{(2,14)}=51.95$; $p < 0.0001$; no interaction: $F_{(2,14)}=1.067$; $p = 0.3576$). Despite the lack of a significant interaction, a post-hoc was performed to confirm that SB203580 had no effect on U69,593-induced


a
Baseline
U50,488
10.0 nA
-4.0 nA
-0.4V
1.3V
2 nA
1 s
-0.4 V
1.3 V
-0.4 V
15.0 nA
0.0 nA
-10.0 nA
b
KOR+/+
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20
0
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-80
***
Core
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PPTg
Stimulation site
Baseline
U50,488
*
t=2.6
Raw t1/2 (s)
WT
Cre Δ
p38α CKODAT
Genotype

Figure 3.7. Conditional knockout of p38α MAPK from dopaminergic neurons fails to alter inhibition of dopamine release by U50,488 recorded *in vivo*.

(a) Representative images demonstrating analysis of FSCV data before and after administration of U50,488. Bottom: Heat maps showing current generated by oxidation and reduction at the site of the working electrode during application of the triangle waveform. Red line shows time of MFB stimulation (6 pulses at 30 Hz, 300 μA) Middle: current by time plot shows evoked dopamine release and uptake. Orange dashed lines show magnitude of peak current, which was recorded for each stimulation. Top: cyclic voltammagram from the time of peak current confirms that the current recorded is induced by oxidation and reduction of dopamine. *(b)* Change from baseline MFB-stimulated peak [DA] after administration of U50,488 in KOR +/+ and $KOR^{-/-}$ mice, as recorded from the NAc core and shell. (n=4-6) (*** $p < 0.001$, Bonferroni posthoc after two-way ANOVA). *(c)* Cartoon demonstrating MFB- and PPTg-evoked dopamine release. *(d) & (e)* Representative traces showing raw current responses of MFB-stimulated (d) and PPTg-stimulated (e) dopamine in WT, Cre Δ, and p38α CKO^{DAT} to 5 mg/kg U50,488 (green). Scale bars show 2 nA, 1s; stimulation marked by red line. *(f)* Change from baseline MFB-stimulated peak [DA] after administration of U50,488 (marked by arrow) in WT, Cre Δ, and p38α CKO^{DAT} mice. *(g)* Change from baseline PPTg-stimulated peak [DA] after administration of U50,488 (marked by arrow) in WT, Cre Δ, and p38α CKO^{DAT} mice. *(h)* Summary data shows that p38α CKO^{DAT} fails to alter U50,488-induced inhibition of dopamine relative to WT and Cre Δ littermates (n=5-16). *(i)* Despite the lack of a significant interaction, activation of KOR may inhibit dopamine uptake after MFB stimulation in a p38-dependent fashion, leading to an increase in $t_{1/2}$. (n=5-16) (*, $p < 0.05$; $t=2.6$, $p > 0.05$; Bonferroni post-hoc after two-way ANOVA).

inhibition of dopamine release (t=0.7348, p > 0.05). To see if SB203580 had any effect on the velocity of the inhibition of dopamine release, we analyzed the slope of the linear phase during U69,593 onset and found no difference between the two (data not shown: t test, $t_{(14)}$=0.1010, p = 0.9210; Vehicle=-6.0±2.1, SB203580=-6.2±1.5).

We also analyzed the half-life ($t_{1/2}$) of released dopamine to determine if there was any effect of either p38 MAPK inhibition or KOR activation in this assay (Figure 3.8c). No effect of U69,593 was found on $t_{1/2}$ of released dopamine (no main effect of KOR drug: $F_{(2,14)}$=1.018, p = 0.3745), which was also unaltered by SB203580 (main effect: $F_{(2,14)}$=0.0003, p = 0.9871). Despite the lack of a significant interaction, two post-hoc analyses were performed to confirm that SB203580 had no effect on dopamine uptake either with or without the presence of U69,593 (SB203580 or vehicle alone: t=0.6755, p > 0.05; U69,593: t=0.9288, p > 0.05). This may be interpreted as suggesting that the previously reported effects on dopamine uptake (Figure 3.7i) were a false positive. However, given that the effect reported *in vivo* was based on the response from 35-60 minutes after U50,488 administration, p38 MAPK may be more elevated during those experiments than the period <15 min after U69,593 administration utilized for *in vitro* data(Schattauer *et al*, 2012). As such, the *in vitro* slice data may be from a time point at which p38-dependent effects on uptake cannot be observed. These results indicate that the KOR-induced inhibition of dopamine release in the NAc did not require p38 activation either *in vivo* or *in vitro*, and thus that inhibition of dopamine release in the NAc is not sufficient to drive behavioral aversion.

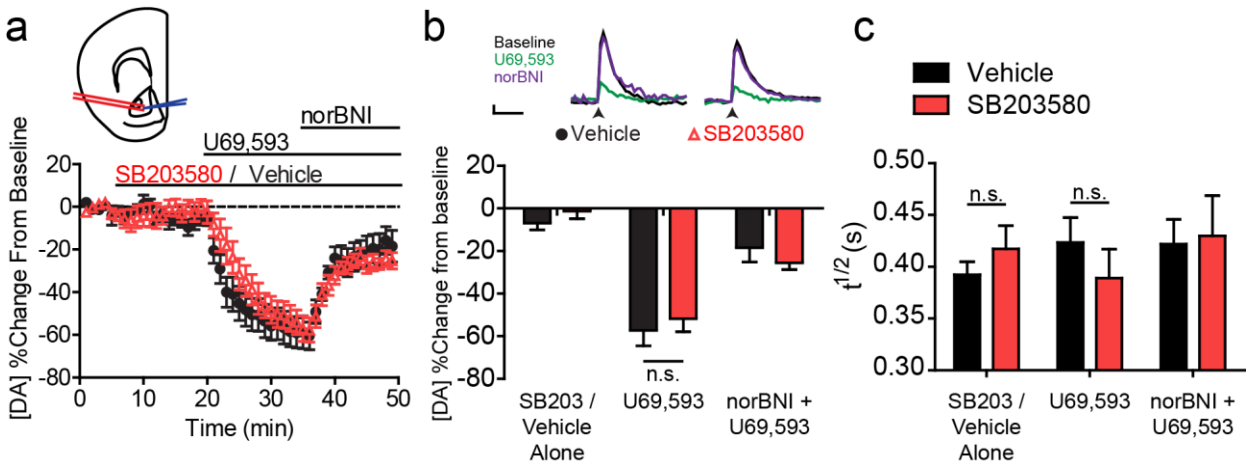

Figure 3.8. Pharmacological inhibition of p38 MAPK by SB203580 fails to alter KOR-mediated inhibition of dopamine release.

(a) Change from baseline locally stimulated peak [DA] during baseline and administration of 10 μM SB203580 or aCSF vehicle, 1 μM U69,593 and 1 μM norBNI. Each drug was bath-applied during the interval signified by the bar. Inset: diagram depicting placement of bipolar stimulating electrode (red) and recording electrode (blue) during slice FSCV experiments (modified from Franklin and Paxinos). *(b)* Summary data shows that the p38 inhibitor SB203580 (10 μM) fails to alter U69,593-induced inhibition of dopamine release. (n=8 slices from 7 animals) (n.s. $p > 0.05$, Bonferroni post-hoc after two-way repeated measures ANOVA despite lack of interaction). Inset: Locally stimulated dopamine release during administration of SB203580 or vehicle, U69,593, and norBNI. Scale bars show 1 s, 2 nA; stimulation marked by arrow. *(c)* Half-life of locally stimulated dopamine from peak during administration of 10 μM SB203580 or aCSF vehicle, 1 μM U69,593, and 1 μM norBNI (n = 8 slices from 7 animals) (n.s. $p > 0.05$, Bonferroni post-hoc after two-way repeated measures ANOVA despite lack of interaction).

DISCUSSION

The principal findings of this study are that KOR activation of p38 MAPK within the VTA is required for the aversive properties of KOR activation. Second, the aversive properties of KOR activation are rescued in $KOR^{-/-}$ mice by viral restoration of KOR signaling to VTA dopamine neurons. This restoration is dependent on the phosphorylation of KOR at serine 369, which is required for p38 activation but not for activation of ERK (Land *et al*, 2009). Third, the aversive properties of KOR activation are dependent on the expression of p38α in dopaminergic neurons. Fourth, the ability of KOR agonists to inhibit dopamine release is not dependent on p38 MAPK activation within dopaminergic neurons. Together, these findings suggest that activation of p38 MAPK by KOR in dopaminergic neurons is required to elicit the aversive properties of KOR agonists, and this process occurs independently of KOR's ability to inhibit dopamine release.

Exposure to behavioral stressors is a well-established risk factor for triggering episodes of depression or relapse to drug abuse in humans (Kendler *et al*, 1999; Sinha *et al*, 2006), and the neural circuits mediating these stress responses have been partially identified using rodent models of these behaviors (Kalivas and McFarland, 2003; Lammel *et al*, 2014). Notably, stress-induced activation of dynorphin / KOR systems in rodent brain potentiates the rewarding valence of drugs of abuse (cocaine, nicotine, and ethanol) (McLaughlin *et al*, 2003b; Schindler *et al*, 2012; Smith *et al*, 2012; Sperling *et al*, 2010), encodes the dysphoric properties of uncontrolled stress (Land *et al*, 2008), mediates prodepressive behaviors (Bruchas *et al*, 2007a), and mediates stress-induced reinstatement of drug-seeking behaviors (Redila and Chavkin, 2008). The present study suggests that activation of the dynorphin / KOR system encodes these responses by activating p38α MAPK in both serotonergic and dopaminergic pathways. Although KOR activation inhibits dopamine release, surprisingly this inhibition was not sufficient to produce aversion.

KOR agonists induce dysphoria in humans (Pfeiffer *et al*, 1986), aversion in animal models (Mucha and Herz, 1985; Shippenberg and Herz, 1986), and inhibition of dopamine signaling in the NAc in animal models (Di Chiara and Imperato, 1988; Spanagel *et al*, 1990). As such, it has been proposed that these effects are causally related: the aversive properties of KOR agonists are mediated by inhibition of NAc dopamine signaling (Carroll and Carlezon, 2013; Di Chiara and Imperato, 1988; Spanagel *et al*, 1990; Tejeda *et al*, 2012). However, this hypothesis and the data that support it are highly correlative. It was driven by the then-prevalent theory that NAc dopamine signaling mediated hedonic responses (Wise, 2008), whereas current theories focus more on the role of dopamine signaling in effort (Salamone *et al*, 2003), reward prediction error (Schultz, 2013), reinforcement or incentive salience (Montague *et al*, 2004). The precise role played by reduction of dopamine in these models is varied and not entirely clear, as the experiments performed have focused on pharmacological or behavioral responses which increase NAc dopamine or directly affect dopamine signaling at or downstream of the receptor.

KOR-CPA is blocked by 6-OHDA lesioning of NAc-projecting dopamine neurons (Shippenberg *et al*, 1993). In addition, optogenetic inhibition of dopamine neurons (Ilango *et al*, 2014; Tan *et al*, 2012) or activation of dopamine neuron-inhibiting GABAergic neurons (Tan *et al*, 2012) were shown to produce aversion. However, dopaminergic neurons corelease neurotransmitters including glutamate (Stuber *et al*, 2010) and GABA (Tritsch *et al*, 2012), as well as cholecystokinin (Artaud *et al*, 1989) and neurotensin (Binder *et al*, 2001). Further, it was recently demonstrated that optogenetic excitation of VTA cells projecting to the NAc induces a state of stress susceptibility, while optogenetic inhibition of these same cells induces a state of stress resilience (Chaudhury *et al*, 2013). As such, it may be the case that inhibition of NAc dopamine signaling actually opposes the dysphoric properties of KOR activation by rendering stress-paired cues less salient. Thus, how the components of these VTA neurons are involved and the extent to which presynaptic inhibition of dopamine release uniquely contributes to aversion are not clear and warrant further study.

Conditional knockout of p38α MAPK from Dopaminergic Neurons Blocks KOR CPA Without Altering Inhibition of Dopamine Release

Inhibition of dopamine release by terminal KORs in the NAc has been previously proposed to mediate the aversive properties of KOR agonists (Carroll and Carlezon, 2013; Ebner *et al*, 2010). The mechanism of KOR-induced inhibition of dopamine release is currently unclear. KOR activation inhibits GABA release in the NAc by inhibiting N-type calcium channels (Hjelmstad and Fields, 2003), and p38 MAPK activation is required for bradykinin-induced inhibition of N-type calcium channels (Wilk-Blaszczak *et al*, 1998). If activation of p38α MAPK were required for KOR-inhibition of dopamine release, this would explain why VTA-only expression of wild-type KOR (which can activate p38 MAPK) but not KSA (which is incapable of activating p38 MAPK (Bruchas *et al*, 2006) but still capable of activating Gβ/γ and G protein-coupled inwardly-rectifying potassium channels in transfected AtT-20 cells (McLaughlin *et al*, 2003b)) restores U50,488-induced CPA. Similarly, it would argue that conditional knockout of p38α MAPK from dopaminergic neurons blocks U50,488-induced CPA by blocking KOR-mediated inhibition of dopamine release. The present study demonstrates that inhibition of dopamine release occurred independently of p38 MAPK activation, indicating that the aversive properties of KOR agonists are separable from their ability to inhibit dopamine release.

Potential Downstream Effects of p38 MAPK in Dopaminergic Neurons

The p38 MAPK inhibitor SB202190 has been shown to increase dopamine uptake in multiple DAT mutants but not wild type rat DAT when expressed in COS cells (Lin *et al*, 2003). p38 MAPK can also phosphorylate threonine 53 at the N-terminal region of DAT in *E. coli* T7 express cells, an event likely to induce changes in the DAT secondary structure (Gorentla *et al*, 2009). Anisomycin-stimulated p38 MAPK activation has also been shown to inhibit dopamine uptake in an SB203580-dependent manner in CHO cells. Given that KOR-induced p38 MAPK activation is capable of inducing increases in serotonin uptake (Bruchas *et al*, 2011) in a manner

dependent on increased SERT translocation into the membrane of NAc-projecting serotonin neurons (Schindler *et al*, 2012), this could suggest an analogous role for p38 MAPK in KOR-induced changes in dopamine uptake. In the present study, we observed some evidence towards a p38-dependent inhibition of uptake. Previous reports examining the effects of KOR activation on dopamine uptake have been inconsistent, finding either no effect (Ebner *et al*, 2010; Schindler *et al*, 2012), or an increase in uptake (Thompson *et al*, 2000). However, if there are effects of KOR-induced p38 MAPK activation on dopamine uptake, then the magnitude and direction of the effect observed may be both dependent on the time after agonist administration and the site studied (Schindler *et al*, 2012). As such, a more in-depth understanding of this interaction may require further study.

P38 MAPK activation can also alter synaptic plasticity. Although the observed lack of effect on acute locomotor sensitization to cocaine suggests intact glutamatergic LTP in dopamine neurons, p38 MAPK has been shown to have effects on long-term depression (Bolshakov *et al*, 2000; Boudreau *et al*, 2007; Zhong *et al*, 2008). In addition, KOR- or stress-induced activation of p38 MAPK can inhibit GIRK channels in both AtT-20 cells (Clayton *et al*, 2009) and serotonergic neurons of the DRN (Lemos *et al*, 2012a). The results from the present study suggest that p38α MAPK-mediated changes in somatic excitability through changes in either LTP/LTD mechanisms or channel function may underlie the aversive response.

Reinterpretation of the KOR/Dynorphin Aversive Circuit

Previous experiments have focused on the role of a single monoaminergic circuit as mediating the aversive properties of KOR activation, predominately via effects on dopamine signaling (Shippenberg *et al*, 1993) or via effects on serotonergic signaling (Bruchas *et al*, 2011; Land *et al*, 2009). Other experiments have focused on the ability of KOR agonists to alter glutamatergic or GABAergic input onto dopamine neurons (Margolis *et al*, 2005b; Polter *et al*, 2014). The current results, however, suggest a key role for both populations (Figure 3.9).

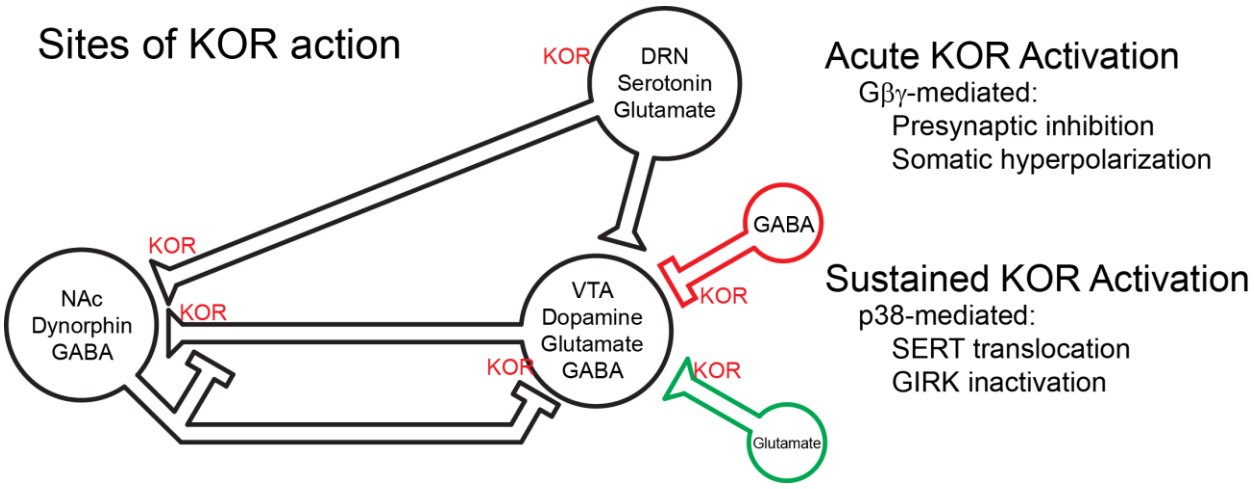

Figure 3.9: Diagrammatic summary of possible mechanisms of dynorphin regulation of mood.

The principal finding of this study is that activation of p38α MAPK within dopamine neurons of the VTA is required for the aversive properties of KOR activation, while presynaptic inhibition of dopamine release is not. This finding complements our earlier study demonstrating that activation of p38α MAPK within the serotonin neurons of the dorsal raphe nucleus (DRN) is also required for KOR aversion (Bruchas *et al*, 2011). Having both serotonin and dopamine regulation required for aversion can be reconciled if they each encode different components of the aversion response. For example, if KOR regulation of serotonin mediates the affective response (e.g. dysphoria) while KOR regulation of dopamine enables the associative learning (e.g. salience) necessary to link the affective response with the associative cues. KOR-dependent p38α MAPK activation in the DRN neurons causes SERT translocation resulting in a transient hyposerotonergic state in the ventral striatum (Bruchas *et al*, 2011; Schindler *et al*, 2012). How p38α MAPK activation in the VTA mediates KOR actions is not completely clear; however, whole-cell voltage clamp recordings of serotonergic neurons (TPH-ir) in the DRN (Lemos *et al*, 2012a) demonstrated that KOR activation acutely increased post-synaptic G-protein gated inwardly rectifying potassium (GIRK) currents and presynaptically inhibited excitatory synaptic inputs. Sustained KOR activation increased postsynaptic p38α MAPK activation in the TPH-ir neurons of the DRN resulting in a block of KOR increases in GIRK without affecting presynaptic EPSCs (Lemos *et al*, 2012a). Thus, KOR activation reduces DRN excitability acutely (increase GIRK, decrease Glut), while sustained KOR activation enhances TPH-ir neuron somatic excitability. KOR activation in VTA similarly regulates potassium currents (Margolis *et al*, 2003), and conditional knockout of p38α MAPK in VTA dopamine neurons is similarly expected to block this adaptive response. How this effects the neuronal circuit is not clear, but changes in VTA circuit functioning is presumed to underlie the loss of the aversion response in CKO mice lacking p38α MAPK in VTA dopamine neurons as diagrammed in the cartoon.

In $KOR^{+/+}$ animals p38 MAPK activation is necessary in both classes of neurons; and further, activation of KORs is necessary within the NAc and DRN (Land *et al*, 2009), the VTA (present results), and in the mPFC (Tejeda *et al*, 2013), although it is not clear whether or not those receptors are only located on serotonergic and dopaminergic neurons (Figure 3.2a). This implies the potential presence of a circuit of KOR activation, requiring either simultaneous or feedforward activation of KORs and subsequently of p38 MAPK in an extended circuit in order to reach aversion. Alternately, it is also possible that KOR activation in different circuits could mediate different modes of aversion, operationally mediated by disparate mood states such as depression, dysphoria, or anxiety. The CPA model used in this and other studies may be incapable of parsing between these different mechanisms. Future experiments should focus on trying to distinguish between these different states to determine if each are disparately effected by manipulation of KOR and p38 MAPK activation in these circuits.

These experiments suggest that p38 MAPK in dopamine neurons plays a key role in the aversive properties of KOR activation. The actions of dynorphin and KOR-induced activation of p38 MAPK have been proposed to mediate the dysphoric component of stress (Land *et al*, 2008) and play a key role in depressive behaviors (Bruchas *et al*, 2011). As such, this study demonstrates that further research is necessary for a clear understanding of the role p38 MAPK plays as a potential target for future therapeutics.

Chapter 4

Conclusions and Future Directions

The principal findings of these studies demonstrate that KOR-induced alteration of the activity could mediate opposing effects on drug reward, while also establishing a role for KOR-induced activation of p38 MAPK in VTA dopamine neurons in mediating the dysphoric component of stress. We show that coadministration of cocaine and the KOR agonist U50,488 acutely block each other's effects on dopamine signaling in the NAc core, but this effect is dependent on inhibition of dopamine neurons by somatodendritic KOR activation. In the same series of experiments, we show that this inhibition is no longer present if cocaine is administered sixty minutes after U50,488, leading to a dopaminergic response to cocaine in the NAc that parallels the behavioral response to these treatments as well. Additionally, we show that KOR signaling in the dopaminergic neurons of the VTA is both necessary and sufficient for the aversive properties of KOR activation. This is the first demonstration that the aversive properties of KOR activation are dependent on phosphorylation of the receptor and subsequent activation of p38 MAPK, but not on the ability of KOR activation to inhibit dopamine release in the NAc.

These experimental results seem to suggest a contradiction: we have shown that KOR-induced changes in dopamine signaling can model a bidirectional alteration of the rewarding properties of cocaine, but also that the dysphoric/aversive properties of KOR activation itself seem not to be mediated by changes in dopamine signaling. Given that KOR-induced changes in drug reward are hypothesized to be mediated by the dysphoric properties of KOR activation (Bruchas *et al*, 2010), a common mechanism should underlie both phenomena. A pair of follow-up experiments would contribute to our understanding of this question. The first would be to determine if conditional knockout of p38α MAPK from dopamine neurons also blocks the

ability of KOR activation to block or potentiate drug reward. The results of our experiments on KOR-CPA would suggest that this is the case. However, although it has also been demonstrated that activation of p38α MAPK in serotonergic neurons of the dorsal raphe nucleus are critical for both KOR-CPA and potentiation of drug reward, the idea that KOR-CPA and KOR-induced block of cocaine reward are mediated by identical mechanisms has been presumed rather than directly tested. Another experiment would be to determine if conditional knockout of p38α MAPK from dopamine neurons alters the effects on the dopaminergic response to cocaine reported in the first chapter. We were unable to perform this latter experiment using our conditional knockout, as we found that $DAT^{Cre/+}$ animals have a basal inhibition of dopamine uptake (as reported in the second chapter), presumably due to reductions in DAT expression. Further, this seems to inhibit the ability of cocaine to increase dopamine concentrations in the NAc (unpublished data), potentially obscuring the results of this experiment. However, were this experiment to be performed, if KOR-mediated alterations of drug reward are blocked by p38α MAPK CKO we would further predict that if changes in NAc dopamine signaling are essential then these animals would not show the inhibited response to cocaine after coadministration of cocaine and U50,488 or the potentiated response to cocaine after 60 minute pretreatment with U50,488. Instead, at each time point these animals would show a normal response to cocaine relevant to the baseline at the time of cocaine administration.

This would seem to suggest that KOR-mediated aversion and reward alteration are mediated by disparate mechanisms. However, although we have demonstrated that the inhibition of dopamine release is not sufficient to induce aversion to KOR activation, we have not determined whether or not it is necessary. It is possible that both p38α MAPK activation and the inhibition of dopamine release are necessary to mediate aversion. If this is so, it could be similarly possible that both the inhibition of dopamine release and p38α MAPK-mediated alterations in the response to cocaine are required for the block or potentiation of cocaine reward. One piece of evidence that suggests this could be the case is that we found that the response to cocaine of PPTg-stimulated dopamine but not MFB-stimulated dopamine in the NAc

core was inhibited if cocaine was administered 15 minutes after U50,488 pretreatment. This argues that somatodendritic KOR activation inhibited the activity of dopamine neurons above and beyond the effects of terminal inhibition of dopamine release. However, we also found that in the absence of cocaine, inhibition of dopamine release was equivalent regardless of stimulation or recording site. Further of note is that this effect was gone 60 minutes after U50,488 pretreatment, even though the inhibition of dopamine release plateaued 15 minutes after administration. This suggests the possibility that cocaine-induced changes in dopamine signaling reveal an inhibition of dopamine neuron activity that is somehow obscured by terminal effects on dopamine release. This is consistent with the observation that uptake inhibitors like cocaine increase dopamine signaling stimulated by burst firing of dopamine neurons but not tonic firing (Floresco *et al*, 2003). If KOR activation preferentially inhibits the transition from tonic to burst firing without directly altering the overall firing rate of dopamine neurons, then it is plausible that this effect might only be observed in our paradigm after administration of cocaine. This is consistent with the knowledge that KOR can activate GIRK channels in dopamine neurons (Margolis *et al*, 2003), which seem to play a key role in the transition between the two states (Lalive *et al*, 2014).

Another possibility is that our recordings were not conducted at the right time point to observe the effect. In both a heterologous expression model and in the serotonergic neurons of the DRN, it has been shown that KOR-stimulated activation of p38α MAPK mediates a heterologous desensitization of GIRK channels (Clayton *et al*, 2009; Lemos *et al*, 2012a). It is possible that this desensitization event is required for properly-timed termination of the inhibition of dopamine release, or for termination of GIRK-induced hyperpolarization of dopamine neurons. If the timing of offset of the inhibition is altered, this could prevent animals from properly pairing the contextual cue of the drug-paired chamber in the CPP paradigm with a dysphoric state induced by inhibition of dopamine release. Given that our recordings were terminated at a time point at which the inhibition of dopamine release was still maximal, we do not know if the offset is altered in the absence of p38α MAPK.

One final option is that the effects are mediated by a change in the signaling of dopamine neurons, but not by changes in the signaling of dopamine itself. Two obvious possibilities are that there exist populations of VTA dopamine neurons which corelease the neurotransmitters glutamate and GABA in the NAc (Stuber *et al*, 2010; Tritsch *et al*, 2012). It is possible that p38 MAPK alters signaling by these neurotransmitters in a manner independent of effects on dopamine release. Another possibility is that KOR-induced p38 MAPK activation could mediate changes in brain-derived neurotrophic factor (BDNF) signaling. It has been shown that increases in BDNF signaling play a key role in mediating social avoidance after repeated social defeat stress (Krishnan *et al*, 2007). More specifically, it has been shown that social defeat stress induces large increases in BDNF in the NAc in social avoidance-susceptible mice relative to unstressed mice or mice that are resilient to social avoidance (Berton *et al*, 2006). Further, intra-NAc injections of BDNF induce susceptibility to social avoidance in mice receiving a submaximal exposure to defeat stress (Krishnan *et al*, 2007). However, social avoidance-susceptible mice shown no difference in BDNF mRNA in the NAc relative to controls or resilient mice; and further, virally-mediated knockdown of the *Bdnf* gene from the NAc using a Cre/lox system fails to alter avoidance behavior. However, a virally mediated knockdown of the *Bdnf* gene from the VTA attenuates the ability of repeated social defeat to increase BDNF levels in the NAc. It also increases the proportion of stress-resilient mice in the social avoidance assay. This suggests that release of BDNF by mesolimbic projection neurons plays a key role in mediating the development of social avoidance. Further, as mentioned in the introduction, VTA dopamine neurons in susceptible mice show a significant increase in their basal firing rate relative to control and resilient mice which seems to be mediated by a selective increase in their burst firing (Anstrom *et al*, 2009; Cao *et al*, 2009; Iñiguez *et al*, 2010; Krishnan *et al*, 2007). Stress-resilient mice show an upregulation in gene expression of several voltage-gated K+ channels relative to susceptible mice, including *Kcnf1*, *Kcnh3*, and *Kcnq3*. It has been proposed that these may be adaptations which support the development of the stress-resilient phenotype. Further, if the inward-rectifying K+ channel Kir2.1 is virally overexpressed in the VTA, this induces a stress-resilient phenotype that shows decreases in the firing of dopamine

neurons accompanied by robust reductions of BDNF levels in the NAc. Viral expression of a dominant-negative version of this channel promotes the development of a susceptible phenotype which shows enhanced stress-induced increases in BDNF levels in the NAc (Krishnan *et al*, 2007). Thus, this suggests that repeated stress could increase a hyperexcitable state in dopamine neurons which promotes increased BDNF release within the NAc, which in turn plays a key role in mediating the prodepressive effects of stress. It is therefore plausible that KOR-induced activation of p38 MAPK mediates a heterologous desensitization of GIRK channels in dopamine neurons which is partially responsible for this hyperexcitable state. This could easily be tested by determining if the increase in the basal firing of VTA dopamine neurons after repeated stress is blocked after CKO of p38α MAPK from dopamine neurons. This would need to be paired with confirmation that GIRK is desensitized in these neurons in a similar manner to that previously shown in serotonergic neurons. It has also been demonstrated that activation of the P2X4 purinoceptor in microglia induces SNARE-dependent exocytotic release of BDNF in a manner dependent on activation of p38 MAPK (Trang *et al*, 2009). Activation of p38 MAPK was also shown to be critical for P2X4-stimulated synthesis of BDNF in this assay. It has also been shown in hippocampal granule cells that blockade of voltage-gated sodium channels induces increases in BDNF expression in a manner dependent on activation of p38 MAPK. As such, it is also possible that KOR-stimulated p38 MAPK may be directly involved in BDNF signaling by VTA dopamine neurons.

Nevertheless, these experiments mark an important step forward in our understanding of how stress induces aversive and dysphoric states and how these states can mediate proaddictive changes in behavior. The work presented herein supports previous research from the lab in proposing that targeted KOR antagonists may be viable therapeutics for the treatment of drug addiction and depression. Further, it also supports the conclusion that G protein-biased ligands could induce analgesia in the absence of either KOR-induced dysphoria or subsequent proaddictive increases in drug reward.

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