Fitting the Motivational Actions of Prefrontal Cortical Mu-Opioids into an Ingestive-Behavior Network Context: Anatomical, Neuropharmacological, and Neurocircuitry-Based Studies

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ABSTRACT

FITTING THE MOTIVATIONAL ACTIONS OF PREFRONTAL CORTICAL MU-OPIOIDS INTO AN INGESTIVE-BEHAVIOR NETWORK CONTEXT: ANATOMICAL, NEUROPHARMACOLOGICAL, AND NEUROCIRCUITRY-BASED STUDIES

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An important feature of frontal cortical regions is the ability to inhibit or control motivated behavior. Dysfunction in this ability plays a prominent role in disorders with binge features, including substance abuse and eating disorders. However, the processes underlying frontal control of appetitive motivation remain poorly understood. The experiments contained within this thesis were designed to investigate the role of the μ-opioid system within the ventromedial prefrontal cortex (vmPFC) on food motivation and inhibitory control over food-directed behavior. First, we discovered that endogenous opioid release within the vmPFC of Sprague Dawley rats is necessary for the expression of impulsive, motivated behavior during prolonged food deprivation, and that stimulating μ-opioid receptors (μORs) with the mu-selective opioid agonist DAMGO is sufficient to evoke increases in food-seeking and impulsive behavior. Stimulation of prefrontal catecholamine systems with d-amphetamine failed to reproduce the profile of DAMGO effects. Second, we demonstrated that signaling at AMPA-type glutamate receptors in the nucleus accumbens shell (AcbSh), a prominent vmPFC projection target, can bidirectionally control increases in appetitive motivation evoked by μ-opioid stimulation within the vmPFC, suggesting a role for glutamatergic vmPFC to AcbSh projections in the limiting of food-directed behavior. Third, we discovered that increases in appetitive

motivation and impairments in inhibitory control elicited by intra-vmPFC μOR stimulation are blocked by co-administration of a dopamine (DA) D1 receptor antagonist into the vmPFC. This suggests that behavioral effects evoked by μ-opioid receptors in the vmPFC are dependent upon functional neurotransmission through dopamine D1 receptors. Lastly, we explored differences in the sensitivity of two vmPFC subregions, the infralimbic (ILC) and prelimbic (PLC) cortices, to DAMGO-evoked behavioral effects. We found that stimulating MORs in ILC, but not PLC, was sufficient to impair inhibitory control and increase feeding, suggesting that μOR-elicited effects previously ascribed to the vmPFC as a whole can be more specifically localized to the ILC, and that projection patterns specific to the ILC may play an important role in recruiting μOR-evoked behavioral responses. Taken together, the results from this thesis show that μ-opioid transmission within the vmPFC, though likely restricted to the ILC, is both necessary and sufficient to evoke appetitive, impulsive food-directed behavior, and that μOR-elicited effects require intact transmission through D1 receptors. Furthermore, AMPA-mediated processes in the AcbSh limit appetitive goal-seeking behavior evoked by prefrontal μOR stimulation.

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CHAPTER 1

INTRODUCTION

BACKGROUND AND SIGNIFICANCE

1. ***Clinical and pathophysiological considerations***

Bingeing is a behavioral construct consisting of an increase in appetitive goal-seeking behavior concomitant with a loss of control over limiting that behavior (American Psychiatric Association, 2013). Binge-like behavior plays a prominent role in many disorders of unrestrained, excessive appetitive motivation, including eating disorders, substance abuse, and "behavioral addictions" including sex, gambling, stealing, and Internet use (Grant *et al*, 2013). These disorders are highly comorbid (Hudson *et al*, 2007), suggesting an underlying common endophenotype that predisposes an affected individual toward impulsive, pathological goal-seeking behavior (Waxman, 2009). This would result in different diagnostic labels applied to a similar underlying process, depending on the specific focus of the abnormal behavior (e.g., drugs, food, gambling, etc.). The presence of a common underlying mechanism is further supported by a high degree of comorbidity between impulse control disorders and eating disorders (Fernandez-Aranda *et al*, 2008), and the presence of impulsive tendencies in other psychiatric disorders with binge-like features, including substance abuse (Jentsch *et al*, 2014; Mitchell *et al*, 2005) and Internet addiction (Choi *et al*, 2014; Hwang *et al*, 2014). Collectively, these "disorders of motivational regulation" significantly impair health-related quality of life and exact a serious toll on public health (Agh *et al*, 2015; Gowing *et al*, 2015). Advances in understanding the neural processes involved in dysregulated appetitive behavior toward one focus may be more broadly generalizable to others, since they might share common underlying mechanisms.

Our laboratory is particularly interested in binge-type behaviors directed toward food, an interest that mirrors broader societal concerns about obesity, unhealthy eating practices, and a

widespread increase in associated pathologies such as Type II diabetes and eating disorders (Avena *et al*, 2011). Accordingly, the most recent revision of the Diagnostic and Statistics Manual of Mental Disorders (DSM-V) now recognizes binge-eating disorder (BED) as a formal diagnosis. This disorder consists of frequent, reoccurring episodes of food-directed binge-like behavior described as simultaneously engendering heightened food intake and a subjective lack of control or helplessness in limiting eating (American Psychiatric Association, 2013). In other words, bingeing can be said to arise from an enhanced motivation to consume food coupled to a decrease in inhibitory control over food-related impulses (Schag *et al*, 2013a). Indeed, individuals with disorders of food-directed bingeing behavior display increased food-seeking behavior, self-report higher levels of trait impulsivity, and perform poorly on executive tasks assaying inhibitory control and delay discounting (Engel *et al*, 2005; Galanti *et al*, 2007; Garrido and Subira, 2013; Hege *et al*, 2015; Manasse *et al*, 2015; Meule and Platte, 2015; Nasser *et al*, 2004; Schag *et al*, 2013b). Cognitive-behavioral approaches to studying binge-related behavioral disorders often further parse impulsivity into two interrelated subcomponents; an increased sensitivity to food and food-associated cues, and a predisposition toward rash-spontaneous behavior (Dawe and Loxton, 2004). This distinction is valuable, as BED is only present in a subset of overweight/obese individuals. While an attentional bias toward food-related cues is present in overweight individuals with and without BED (Bongers *et al*, 2015; Engel *et al*, 2005; Galanti *et al*, 2007; Hege *et al*, 2015; Lattimore and Mead, 2015; Manasse *et al*, 2015; Meule and Kubler, 2014; Nijs *et al*, 2010; Schag *et al*, 2013b; Tetley *et al*, 2010; Werthmann *et al*, 2011), only individuals with BED have associated impairments in response inhibition (Manasse *et al*, 2015; Schag *et al*, 2013b). Thus, further characterization of brain regions involved in inhibiting prepotent response tendencies and processing reward-relevant information can provide

valuable insight into potential neurochemical targets for the treatment of disorders featuring binge-like behavior.

Given the behavioral characteristics of BED and other eating disorders, it is interesting to examine human neuroimaging studies that may suggest candidate substrates underlying the pathological behavior observed in these disorders. A number of functional magnetic resonance imaging (fMRI) studies of individuals with these disorders have implicated abnormal function in several frontal cortical sites that process reward-related information and mediate cognitive and executive processes. Overweight and obese individuals with BED show poor performance on response inhibition tasks and an associated hypoactivity in the prefrontal cortex (PFC), inferior frontal gyrus, and insular areas (Balodis *et al*, 2013; Hege *et al*, 2015). During the presentation of a predictive auditory or visual cue, individuals with high levels of binge eating show increased activation in several reward-related frontal sites, including orbitofrontal (OFC), anterior cingulate (ACC), and anterior insular (AIC) cortices, suggesting a hypersensitivity of the reward system to relevant food-related stimuli (Batterink *et al*, 2010; Filbey *et al*, 2012; Geliebter *et al*, 2016; He *et al*, 2014; Schienle *et al*, 2009). These interacting, yet functionally distinct, regions – particularly the ventral medial PFC – broadly appear to encode the subjective value of rewards and exert cognitive and executive control over reward-seeking behavior via projections onto subcortical structures controlling motivated behavior (Bartra *et al*, 2013; Smith *et al*, 2014).

Despite the abovementioned insights regarding the anatomical localization of deficits in psychiatric disorders with binge features, the neurochemical basis of these deficits remains unclear. One promising neurochemical candidate implicated in the expression of binge-like behavior and impulsivity is the endogenous μ-opioid system. Positron emission tomography (PET) imaging has demonstrated a correlation between self-reported measures of "trait-like"

impulsivity and higher concentrations of brain μOR concentration and stressor-induced μ-opioid peptide release in a variety of regions involved in both motivated behavior and executive function, including PFC, OFC, ACC, and the nucleus accumbens (Love *et al*, 2009). Administration of amphetamine increases endogenous opioid release in these same regions (Colasanti *et al*, 2012). Interestingly, numerous PET studies have shown changes in μOR availability and endogenous opioid release in the frontal cortex of cocaine addicts, individuals with bulimia, and pathological gamblers (Bencherif *et al*, 2005; Mick *et al*, 2016; Zubieta *et al*, 1996). Impulsivity is highly associated with misuse of opioid analgesic drugs in chronic pain patients (Marino *et al*, 2013; Vest *et al*, 2016), and exaggerated attentional and subjective reactivity to opioid-related cues can accurately predict future opioid misuse (Garland and Howard, 2014). Opioid antagonists are among the only semi-effective treatments for disorders with "loss-of-control" features, including binge-eating (Alger *et al*, 1991; Cambridge *et al*, 2013; Drewnowski *et al*, 1995; Meyer, 2008; Raymond *et al*, 2002; though see McElroy *et al*, 2013), alcoholism (Anton *et al*, 1999; Monti *et al*, 1999), pathological gambling (Bosco *et al*, 2012; Grant *et al*, 2008; Kim and Grant, 2001a; Kim *et al*, 2001b; Yoon and Kim, 2013), kleptomania (Grant *et al*, 2009), and Internet pornography addiction (Bostwick and Bucci, 2008). Finally, feeding behavior in BED is markedly hedonically-driven (Davis *et al*, 2008), which indirectly implicates endogenous opioid transmission (Fullerton *et al*, 1985; Pecina and Berridge, 2000; Yeomans and Gray, 1996, 1997; Zhang *et al*, 1998). The role of endogenous opioids, especially mu-opioids, in non-homeostatic, palatable feeding has been extensively studied (Olszewski *et al*, 2011; Pecina and Smith, 2010). Rats preferentially reduce consumption of palatable foods independent of homeostatic needs when given opioid antagonists, especially those acting at the μ-opioid receptor (Barbano and Cador, 2006; Cottone *et al*, 2008; Giuliano *et al*, 2012; Levine *et*

al, 1995; Rudski *et al*, 1994). In humans, opioid antagonists and inverse agonists reduce sensory hedonic ratings and caloric intake of palatable foods (Cambridge *et al*, 2013; Drewnowski *et al*, 1995; Nathan *et al*, 2012; Yeomans *et al*, 1996, 1997; Ziauddeen *et al*, 2013). Finally, obese BED patients with a "gain of function" genetic polymorphism in the μ-opioid receptor report higher levels of hedonic eating (Davis *et al*, 2009). Together, observations from human neuroimaging and cognitive/behavioral studies suggest the possibility that endogenous opioid transmission could contribute to the pathology of disorders featuring binge-like behavior by altering executive control and motivational functions through their actions at frontal cortical sites.

Animal studies, generally speaking, support a link between impulsivity and excessive reward seeking, and some of these studies suggest the involvement of the μ-opioid system in frontally-mediated processes of inhibitory control and binge-like behavior. Naturally impulsive rats predictably escalate self-administration of cocaine (Anker *et al*, 2009), increase responding for sucrose reward (Diergaarde *et al*, 2009), and consume higher amounts of palatable food relative to their low-impulsive counterparts (Velazquez-Sanchez *et al*, 2014). Systemic morphine administration in rats increases, whereas naloxone reduces, premature responding on multiple operant paradigms that measure inhibitory control, including the five-choice serial reaction time task (5CSRTT) and response inhibition task (Mahoney *et al*, 2013; Pattij *et al*, 2009). Mice lacking a functional μ-opioid receptor show reduced premature responding on a signaled nose poke task (Olmstead *et al*, 2009). More targeted explorations of opioid function have identified the medial PFC (mPFC) as region of particular interest. Rats that escalate ethanol intake show higher mPFC mRNA expression levels of μOR and the endogenous opioid peptide enkephalin (ENK), and intra-mPFC infusion of a μOR agonist significantly increases ethanol consumption

(Morganstern *et al*, 2012). Moreover, intra-mPFC opioid antagonists selectively reduce operant responding for palatable foods in rats predisposed to binge-like eating patterns (Blasio *et al*, 2014). These findings reinforce the role of cortical opioid signaling in modulating inhibitory control processes and identify the prefrontal cortex in particular as an important site for future research on binge-like behavior.

2. ***Central sites of feeding-modulatory opioid actions***

Aside from the studies summarized above, very little is known about the behavioral actions of opioids in frontal cortical areas outside of recent work in our laboratory (Mena *et al*, 2011; Mena *et al*, 2013; Selleck *et al*, 2015). However, there is a considerable literature on feeding-related opioid actions in subcortical sites. It is interesting to briefly review this literature to place our PFC results in a functional context. In a general sense, opioid systems in subcortical areas have been the subject of decades of extensive research, leading to a prodigious literature detailing opioid involvement in pain and analgesia, reward processes, stress, social status, sexual activity, drug abuse, and many other behaviors (Bodnar, 2016; Lutz and Kieffer, 2013; Paredes, 2014; Trang *et al*, 2015; Vanderschuren *et al*, 2016). Most relevant to the body of work in this dissertation is the literature detailing the role of central opioid actions in the control of *non-homeostatic* feeding behavior – that is, consumption of excess calories relative to what is biologically required for energy balance, as dictated by affective or cognitive factors (Corwin and Hajnal, 2005; Zheng *et al*, 2009).

2.1 Intra-accumbens opioids

2.1.1 Anatomical mapping and pharmacological specificity

Perhaps the most extensively studied site of feeding-modulated opioid actions is the Acb. Mucha and Iversen (1986) first implicated the Acb in feeding processes when they infused

opioid agonists into the structure and observed increases in food intake that were attenuated by concurrent naloxone administration. Kelley and colleagues mapped this effect within the striatum and found an anatomical gradient of opioid-induced feeding, with strong hyperphagia evoked by infusion of [D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin (DAMGO), a highly μ-specific agonist, into the AcbSh and weaker responses in more dorsal, lateral, and posterior infusion sites (Bakshi and Kelley, 1993b). Delta-opioid agonists produced smaller effects, and no response was seen with kappa-specific agonists (Bakshi and Kelley, 1993a; Castro and Berridge, 2014; Majeed *et al*, 1986).

2.1.2 Preferential enhancement of palatable food consumption

Early hypotheses regarding the opioidergic modulation of feeding behavior in the Acb evolved from studies showing that opioid antagonists selectively suppress consumption of sweetened solutions and diets over normal chow (Apfelbaum and Mandenoff, 1981; Cooper, 1983; Giraudo *et al*, 1993; Levine *et al*, 1982) and prevent the formation and expression of taste preferences (Cooper, 1983; Cooper and Turkish, 1989; Evans and Vaccarino, 1990; Lynch, 1986). Levine and colleagues demonstrated that the efficacy of naloxone at reducing food intake 1) is inversely related to the level of food deprivation the animal is subjected to (Levine *et al*, 1995; Rudski *et al*, 1994; Weldon *et al*, 1996) and 2) is dependent upon individual dietary preferences (Glass *et al*, 1996). The former finding established that opioid blockade was more important for regulating non-homeostatic feeding whereas the latter finding, when combined with aforementioned naloxone effects on the consumption of palatable foods, implicated endogenous opioid function in the mediation of the hedonic or "rewarding" experience of the food. Supported by the earlier work identifying the Acb as a locus of action for opioid-induced

feeding, it was hypothesized that opioid activity (specifically μ-opioid activity) within the Acb was implicated in the mediation of hedonically-driven feeding (Evans *et al*, 1990; Kelley *et al*, 1996; Zhang and Kelley, 1997, 2002).

2.1.3 A mechanistic explanation

Much like the studies on opioid involvement in food intake, our understanding of opioid actions in the Acb in augmenting hedonic food palatability originates from early studies looking at the effects of systemic opioid manipulation through use of hedonic taste reactivity testing, which looks at automatic orofacial taste responses generated by brainstem motor pattern generators as a measure of the hedonic quality of a taste stimulus (Grill and Norgren, 1978). Systemic morphine increases and naloxone decreases the number of evoked hedonic taste responses to sweet sucrose solutions (Doyle *et al*, 1993; Parker *et al*, 1992; Pecina and Berridge, 1995; Rideout and Parker, 1996) and suppresses aversive reactions to bitter quinine solutions (Clarke and Parker, 1995; Parker *et al*, 1992). As mentioned above, opioid stimulation (μ-opioid especially) elicits increased feeding through much of the striatum, with stronger responses in the most anterior, ventral, and medial areas – the AcbSh (Bakshi *et al*, 1993b). Based on these results, Berridge and Peciña mapped opioid feeding effects and enhancement of hedonic taste reactions at sites distributed throughout the Acb. They showed that opioid-induced eating effects and associated cellular activity were largely confined to the AcbSh (Pecina *et al*, 2000), and the opioid enhancement of positive taste reactions was confined to a small, localized hedonic "hotspot" in the rostrodorsal region of the medial shell (Pecina and Berridge, 2005). Together, these results confirm the central role of the AcbSh in mediating opioid-driven feeding and, in addition, provide a mechanism through which intra-AcbSh opioids act to drive non-homeostatic feeding behavior.

2.1.4 Macronutrient preferences

Opioids in the AcbSh clearly have a role in promoting consumption of palatable foods, but are there particular macronutrient qualities that are preferred? Early studies suggested that systemic opioid administration preferentially alters fat intake (Marks-Kaufman, 1982; Marks-Kaufman and Kanarek, 1980, 1990), though opioid-induced preference shifts are highly dependent on pre-existing preferences and fade with chronic drug application (Gosnell and Krahn, 1993; Gosnell *et al*, 1990). Unfortunately, systemic opioid studies on macronutrient preference were fundamentally flawed, as different preferences can be evoked by peripheral and central opioid actions (Marks-Kaufman *et al*, 1985) and even between structures in the brain (Mena *et al*, 2011). When infused directly into the Acb, μ-opioid receptor stimulation selectively increases intake of high-fat foods, *independent* of baseline preference and with no effect on carbohydrate consumption. This selective increase is preferentially blocked by intra-Acb naltrexone administration (Will *et al*, 2006; Zhang *et al*, 1998).

2.2 Terminal fields involved in intra-accumbens opioid-driven feeding

Opioid actions in the AcbSh have been established as pivotal for affective enhancement of palatable foods; however, an understanding of how these actions are translated into food-seeking and consummatory behaviors relies upon comprehension of how the Acb is integrated into a larger neural framework for the control of feeding behavior. As such, examining which brain areas are recruited by μ-opioid activation of the Acb can provide insight into the necessary pathways for expression of opioid-enhanced eating. Kelley and colleagues quantified Fos-like immunoreactivity (a measure of neuronal activation) in reward-relevant brain structures after DAMGO infusion into the Acb “shore” - the border between the core and shell. They observed increases in Fos-like immunoreactivity in the nucleus of the solitary tract (NTS), ventral

tegmental area (VTA), and lateral hypothalamic (LH) regions among other areas (Zhang and Kelley, 2000). Inactivation of these areas with the γ-aminobutyric acid receptor A ($GABA_A$) agonist muscimol blocks intra-Acb DAMGO-induced increases in fat intake (Will *et al*, 2003), and opioid stimulation of all three regions evokes increases in food intake (Kotz *et al*, 1997; Mucha *et al*, 1986; Stanley *et al*, 1988; Woods and Leibowitz, 1985). While it is clear that these areas interact to evoke increases in feeding behavior, an understanding of how they accomplish this can be achieved by looking more directly at the functional and anatomical connections between them.

2.2.1 The lateral hypothalamus

The lateral hypothalamic area has long been implicated in feeding and motivational processes (Barbano *et al*, 2016; Hoebel and Teitelbaum, 1962; Margules and Olds, 1962; Stuber and Wise, 2016). Broadly speaking, manipulations that functionally excite or activate the LH (e.g. electrical stimulation, glutamate agonism) elicit strong feeding effects and recruit appetitive seeking behavior (Delgado and Anand, 1953; Olds and Milner, 1954; Stanley *et al*, 1993a), whereas functional inhibition or ablation of the LH (such as with lesions or GABA agonists) suppresses feeding behavior and seeking processes (Anand and Brobeck, 1951; Kelly *et al*, 1979; Stricker *et al*, 1978). The AcbSh sends a strong GABAergic projection to the LH (Heimer *et al*, 1991; Meredith *et al*, 1993), and functional inhibition of the AcbSh disinhibits LH neurons and evokes increases in feeding (Stanley *et al*, 1993b; Stratford and Kelley, 1999a). DAMGO-elicited feeding effects in the Acb can be blocked by infusion of muscimol, a $GABA_A$ agonist, into the LH (Maldonado-Irizarry *et al*, 1995; Stratford *et al*, 1999a). However, the exact neurochemical systems and anatomical projections through which LH-mediated feeding effects are recruited have yet to be delineated. LH efferents are widespread and include a number of

structures that have been implicated in feeding and reward-processing, including (among others) the central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST), VTA, ventral pallidum (VP), and NTS as well as multiple subregions of the frontal cortex, including PFC, ACC, and OFC (Berk and Finkelstein, 1982; Jin *et al*, 2016; Stuber *et al*, 2016). Projections to the VTA have been most directly linked to the recruitment of feeding behavior, as optogenetic stimulation of LH-VTA projecting fibers induces both feeding and self-stimulation (Gigante *et al*, 2016).

Within the LH is a neuronal subpopulation that serves as the sole producer of the neuropeptide orexin (also known as hypocretin; Aston-Jones *et al*, 2010; Baldo *et al*, 2003), which has been associated with the recruitment of feeding behavior (Baldo *et al*, 2004; Zheng *et al*, 2003). The well-characterized role of orexin in regulating arousal and behavioral-state (Berridge *et al*, 2010) has led to a proposed role for the peptide in the coordination of feeding behavior with appropriate arousal states (Haynes *et al*, 1999; Kotz *et al*, 2002; Parise *et al*, 2011; Williams *et al*, 2001; Zheng *et al*, 2003). Orexin neurons in the LH are heavily connected to other areas of the hypothalamus and the NTS, with moderate projections to the PFC, OFC, VP, and VTA (Baldo *et al*, 2003; Jin *et al*, 2016; Peyron *et al*, 1998). Antagonism of orexin receptors in the VTA blocks increases in fat intake evoked by intra-Acb DAMGO administration (Zheng *et al*, 2007), and orexin agonists in the VP amplify hedonic "liking" reactions to sweet taste in a manner similar to the aforementioned opioid hotspot in the rostrodorsal AcbSh (Ho and Berridge, 2013), suggesting that orexin signals from the LH may serve to amplify the hedonic impact of food reward (Castro *et al*, 2015)

2.2.2 The ventral tegmental area

The VTA sends large, dense dopaminergic (DA) projections to the Acb (Domesick, 1988; Swanson, 1982). In return, the Acb sends back strong GABA- and enkephalinergic projections to mediate VTA signaling (Kalivas *et al*, 1993; Watabe-Uchida *et al*, 2012). Within the VTA, μ-opioid receptors are located on GABAergic interneurons (Margolis *et al*, 2012; Sesack and Pickel, 1992), and likely act by limiting tonic inhibition on DA projection neurons, facilitating DA transmission in feeding-relevant brain structures (Kalivas, 1993). DA within the Acb has been well characterized in the modulation of anticipatory and instrumental processes of food seeking, serving as an integrator of limbic signaling and gating access to motor outputs (Mogenson *et al*, 1980; see Fields *et al*, 2007 and Salamone and Correa, 2012 for review).

Systemic and local activation of opioid receptors in both the VTA and the Acb increases release of DA in the Acb (Devine *et al*, 1993; Leone *et al*, 1991; Ostrowski *et al*, 1982) and evokes feeding responses (Badiani *et al*, 1995; Hamilton and Bozarth, 1988; Mucha *et al*, 1986; Noel and Wise, 1993, 1995). DA levels in the Acb are increased in rats that are food-deprived and anticipating access to a palatable meal (Church *et al*, 1987; Radhakishun *et al*, 1988; Taber *et al*, 1998; Wilson *et al*, 1995), and both VTA lesions and intra-Acb DA antagonists block sucrose consumption and prevent systemic morphine-induced increases in sucrose feeding (Shimura *et al*, 2002). Opioid communication between the structures is particularly important for feeding responses, as intra-VTA opioid-induced feeding is blocked by intra-Acb naloxone and intra-Acb opioid-induced feeding is blocked by intra-VTA naloxone (Bodnar *et al*, 2005; MacDonald *et al*, 2003). The extensive interaction between DA and opioids on interactions between the Acb and VTA reflects the importance the two neurochemical systems play in regulating ingestive behavior.

2.3 Other opioid-sensitive feeding-control sites in the brain

While opioid actions in the AcbSh, LH, and VTA have been most extensively described, a large number of subcortical structures have also been shown to exert opioid-sensitive feeding effects, including the dorsal neostriatum, VP, central nucleus of the amygdala (CeA), parabrachial nucleus (PBN) of the pons, nucleus of the solitary tract (NTS), and the paraventricular (PVN), dorsomedial, and ventromedial hypothalamic nuclei (DiFeliceantonio *et al*, 2012b; Gosnell *et al*, 1986; Smith and Berridge, 2005; Stanley *et al*, 1988; Woods *et al*, 1985). An extensive review of their involvement in opioid-mediated feeding behavior is beyond the scope of this dissertation; however, an understanding of their basic neuroanatomical and functional interconnectivity can provide valuable insight into additional dimensions of information processing during ingestive behavior.

The dorsal striatum has been long implicated in the mediation of movements, habit formation (e.g. action chunking and automation), and responses to learned cues (Graybiel, 2008; Smith and Graybiel, 2016). Infusion of DAMGO into rat anteromedial dorsal striatum evokes robust increases in palatable food intake without amplifying hedonic impact (DiFeliceantonio *et al*, 2012b; Zhang *et al*, 2000), and unexpected access to palatable chocolate candy elicits surges of endogenous ENK release the same area (DiFeliceantonio *et al*, 2012b). Neurochemical staining for μOR in the neostriatum reveals a "patchwork"-like structure, with MORs concentrated within discrete "patches" or "striosomes" scattered among a matrix of otherwise undifferentiated tissue (Herkenham and Pert, 1981). Interestingly, these striosomes receive converging inputs from limbic-associated areas of frontal cortex, including OFC, ACC, and parts of PFC (Crittenden and Graybiel, 2011). These observations seem concordant with a role for dorsal striatum in the overconsumption of palatable food (DiFeliceantonio *et al*, 2012b).

The VP is the primary anatomical output of the Acb (Groenewegen *et al*, 1999), and much like the AcbSh, intra-VP infusion of μ-opioid agonists and GABA agonists into a anatomically-defined "hotspot" increases food intake and hedonic impact of sweet solutions (Shimura *et al*, 2006; Smith *et al*, 2005; Stratford *et al*, 1999b). This hotspot is directly influenced by LH orexin neurons, as mentioned previously (Ho *et al*, 2013). Moreover, the hedonic hotspots in the AcbSh and VP functionally interact, as opioid blockade in one structure can completely block enhancements in hedonic impact evoked by DAMGO the other structure (Smith and Berridge, 2007). However, while μ-opioid-elicited enhancements in hedonic impact can be blocked by VP opioid blockade, increases in food intake persist (as would be expected, given the direct modulation of LH activity by direct AcbSh projections; Smith *et al*, 2007; Stratford *et al*, 1999a). These observations suggest that the VP and AcbSh cooperate generate hedonic impact for food, and provide an anatomical route that dissociates neural coding of the hedonic quality of food from the recruitment of mechanisms for feeding food-seeking.

The amygdala has an important role in associating environmental stimuli with the availability of rewards and assigning incentive qualities to those cues that predict future rewards (Holland and Gallagher, 2004). While the amygdala as a whole is a functionally and anatomically heterogeneous structure, the CeA in particular has been identified as an especially important structure for generating motivational salience. Infusion of DAMGO into the CeA produces a pronounced elevation in appetitive behavior directed at reward-predictive cues, turning a predictive cue into a "motivational magnet" (DiFeliceantonio and Berridge, 2012a; Mahler and Berridge, 2009). The CeA likely directs appetitive behavior via direct projections to the AcbSh as well as reciprocal connections with the AIC and descending projections onto the LH, NTS, and PBN (Hopkins and Holstege, 1978; Johnson *et al*, 1994; Saper, 1982).

The rostral NTS and PBN are brainstem relays for gustatory and visceral taste information to feeding-related areas in the thalamus, cortex, amygdala, and hypothalamus, and hence lie in an ideal position to alter signals passed on to the telencephalon (Ricardo and Koh, 1978; Saper, 1982). Infusion of DAMGO into PBN increases consumption of standard and palatable chow, but intra-PBN mu-opioid antagonists only reduce intake of standard chow (Ward and Simansky, 2006). This suggests that PBN neurons regulate food intake regardless of hedonic value, yet their role can be overridden or bypassed by higher-order systems that process hedonic quality of the food. While the role of MORs in NTS is still unclear, it is interesting to note that intra-NTS opioid antagonists block increases in feeding elicited by μOR agonists in the CeA, and feeding increases evoked by DAMGO in the NTS are blocked by intra-CeA opioid antagonists (Giraudo *et al*, 1998).

The involvement of the PVN, DMH, and VMH in opioid-evoked feeding is unsurprising, as hypothalamic nuclei in general (including the LH) have been well characterized in monitoring energy homeostasis and evoking changes in feeding drive and energy expenditure in response to nutritional and other signals (Williams *et al*, 2001). Interactions between hypothalamic regions are very complex and the intricacies of their function have yet to be delineated. However, the general function of each region in modulating the expression of feeding behavior is worth noting. The VMH has classically been considered a "satiety center" within the hypothalamus, as stimulating VMH activity inhibits feeding and lesions in the region evoke overeating and weight gain (Stellar, 1954). The DMH has an important role in the expression of circadian rhythms, and may have an important role in entraining circadian rhythms to the availability of food (Gooley *et al*, 2006). The PVN contains a large number of appetite-modifying neuropeptides in addition to opioids, and is particularly sensitive to neurochemical manipulations that evoke changes in

feeding and energy expenditure (Williams *et al*, 2001). All three regions (in addition to the LH) express leptin receptors (Schwartz *et al*, 1996), which allow them to monitor energy stores in adipose tissue and adjust feeding levels accordingly (van Swieten *et al*, 2014). Together, the hypothalamus integrates a large number of central and peripheral signals, maintaining energy homeostasis by regulating feeding and energy expenditure based on the needs of the animal.

2.4 Common themes in opioid mediation of feeding behavior

From the discussion above, it is possible to distill several points relevant to opioid control of feeding into a number of key features that are shared amongst opioid-sensitive subcortical eating sites studied to date. Firstly, changes in food intake evoked by opioid actions are critically dependent upon connections to and processing within the hypothalamus. Secondly, increased opioid activity in feeding-related structures enhances palatability-driven feeding, with a strong macronutrient preference for fats (at least from opioid-sensitive zones in the Acb). Lastly, increases in eating are most strongly evoked by selective μ-opioid stimulation with only weak, sporadic involvement of delta or kappa opioid receptors.

3. ***The medial prefrontal cortex: a novel site for telencephalic opioid modulation of food motivation and food impulsivity?***

Based on the discussion above, it is interesting to evaluate the mPFC (specifically the ventral mPFC) as a candidate feeding-modulatory structure, and to evaluate whether the little that is known about behavioral actions of PFC opioids "fits" the characteristics of opioid feeding responses elicited from subcortical sites.

3.1 Unique behavioral characteristics evoked by vmPFC μ-opioid receptor stimulation

Recent studies in our lab have more directly assessed the involvement of opioid signaling in the vmPFC in the modulation of feeding behavior. Mena *et al* (2011) showed that infusion of

DAMGO into the vmPFC markedly increased intake of standard rat chow, and that this increase could be evoked by DAMGO infusion into the entirety of prelimbic (PLC) and infralimbic (ILC) cortices as well as ventral orbitofrontal cortex. Intra-vmPFC infusion of delta- or kappa-specific opioid agonists failed to elicit changes in food intake, mirroring strong mu-specific opioid effects on feeding observed in the AcbSh (Bakshi *et al*, 1993a; Castro *et al*, 2014; Majeed *et al*, 1986). More surprisingly, when rats were given concurrent, free access to both carbohydrate- and fat-rich diets, intra-vmPFC DAMGO selectively increased intake of the carbohydrate-rich test diets. This selective augmentation of carbohydrate intake was preserved when rats were divided into carbohydrate-preferring and fat-preferring subpopulations, suggesting that this effect was not due mere hedonic enhancement (which would amplify intake of a preferred food option). Furthermore, the carbohydrate bias evoked by vmPFC μOR stimulation differs from the selective enhancement of fat intake observed after DAMGO infusion into the Acb (Will *et al*, 2006; Zhang *et al*, 1998). Lastly, glutamate blockade in the LH attenuates increases in feeding evoked by μOR stimulation of the vmPFC (Mena *et al*, 2013; also, see **Chapter 3**). As previously mentioned (see **Section 2.4**), opioid-sensitive feeding sites in subcortical structures also produce feeding responses with these three key features – 1) strong μ-opioid selectivity, 2) sensitivity to macronutrient content of the food, and 3) dependence upon hypothalamus-mediated processes. Hence, the prominent features of intra-vmPFC μOR-evoked feeding responses are consistent with those in other opioid-sensitive subcortical eating sites. However, the tendency of prefrontal μOR stimulation to elicit selective intake of carbohydrates rather than fats suggests that the cortical actions of opioids exhibit unique features that differentiate them from subcortical opioid effects. Understanding how these unique features rise requires a more in-depth discussion of the

anatomical and neurochemical structure of the vmPFC, including how its function may be modulated by opioid signaling.

3.2 Dorsal-ventral distinctions in medial prefrontal cortex

The mPFC in the rat constitutes the majority of the medial wall of the brain anterior and dorsal to the genu of the corpus callosum, and can be further subdivided into prelimbic (PLC), infralimbic (ILC), and anterior cingulate (ACC) cortices, and occasionally including parts of precentral/medial agranular (PrCm), dorsal peduncular (DP), and even medial orbitofrontal (mOFC) cortices (Gaykema *et al*, 2014; Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007; Vertes, 2004). Because it covers a substantial portion of the rat frontal lobe, the mPFC is often organized into dorsal/dorsomedial (dmPFC) and ventral/ventromedial (vmPFC) regions based on architectonics, function, and connectivity with other brain structures. Generally speaking, dorsal aspects of mPFC receive proportionately more sensory-motor input and are integrated with cortical association areas, while ventral mPFC is highly interconnected with limbic structures such as the amygdala, hippocampus, and medial basal forebrain (Heidbreder *et al*, 2003; Kesner and Churchwell, 2011). However, functionality within the PFC does not overlay cleanly onto anatomical projection patterns, and retrograde tracers in PFC projection targets often show a diffuse, graded labeling of PFC neurons that transcends structural boundaries (Gabbott *et al*, 2005). This is especially apparent when looking at corticostriatal projection patterns (see below for a more in-depth discussion). Hence, there is no definitive, agreed-upon dividing line between dorsomedial and ventromedial regions of the PFC. Studies that emphasize anatomical or structural features of the PFC commonly group PrCm and ACC into dmPFC and ILC/PLC into vmPFC, whereas studies of function often (though not always) include dorsal PL in dmPFC rather than vmPFC. This creates a “gray area” in the center of the

PFC, where dorsal PL can be considered part of either region. While this somewhat accurately reflects the gradient-like transition from sensorimotor dmPFC to viscerolimbic vmPFC, it does create difficulties when discussing studies that differentiate between the two regions.

For this dissertation, we use "vmPFC" to refer to both PLC and ILC in their entirety. We make this assertion for two reasons: 1) we originally found that increases in feeding and motor activity can evoked by intra-PFC μ-opioid stimulation throughout the entirety of PLC and ILC, suggesting that the two regions mediate this function in parallel (Mena *et al*, 2011; see **Section 3.1**), and 2) cortical projections onto the ventral striatum, which contains the Acb, are densest in these regions and almost nonexistent dorsal to the PLC/ACC boundary (Gabbott *et al*, 2005; Voorn *et al*, 2004; see discussion below). However, in acknowledgement of controversies over how to effectively parse dorsal versus ventral mPFC, we directly assess differences in function between ILC and dorsal PLC in **Chapter 5**.

3.3 Anatomical connectivity

3.3.1 Afferents

The vmPFC lies in an ideal position to monitor processed sensory information and ongoing subcortical reward processes relevant to feeding. Feeding-relevant cortical afferents to the vmPFC come from AIC, which processes direct taste and visceral sensory information received from thalamic relays (Frank *et al*, 2013; Jezzini *et al*, 2013; Kobayashi, 2011; Maffei *et al*, 2012), and OFC, which maintains a real-time representation of the reward- and incentive-value of food (Kringelbach *et al*, 2003; O'Doherty *et al*, 2001; Rolls, 1997; Rolls and Grabenhorst, 2008; Schoenbaum and Setlow, 2001). Within the vmPFC, PLC and ILC share reciprocal connections between themselves, and are also highly interconnected with structures in dmPFC (Hoover *et al*, 2007). The vmPFC also receives information from subcortical reward-

and feeding-modulatory structures via both direct and indirect inputs. Orexin neurons in the LH directly project to the entire medial wall (including ACC, PLC, and ILC) with a rostro-caudal increasing gradient, matching mPFC orexin receptor expression (Conde *et al*, 1995; Hahn and Swanson, 2010), as well as information from ongoing reward processing in the striatum and pallidum via projections from relay neurons in the mediodorsal, paratenial, and paraventricular nuclei of the thalamus (Conde *et al*, 1990). These projections provide valuable feedback onto prefrontal neurons as part of large-scale cortical-striatal-pallidal-thalamic (CSPT) feedback loops (Thompson and Swanson, 2010). In fact, the vmPFC serves as a key integrative node in a wider medial prefrontal network that takes processes sensory information and uses it to guide reward-related behavior (Ongur and Price, 2000).

Other important afferents to the vmPFC include projections from monoaminergic nuclei in the midbrain and brainstem, including the VTA, locus coeruleus, laterodorsal tegmental nucleus, and dorsal and medial raphe (Hoover *et al*, 2007). Afferents from the basolateral amygdala allow the vmPFC to monitor choice outcomes, assess risks, and facilitate adjustments in choice biases (St Onge *et al*, 2012). Lastly, inputs from the hippocampal formation and associated cortical areas provide the vmPFC with spatial and contextual information (French and Totterdell, 2002; Hoover *et al*, 2007; Vertes, 2006). These varied inputs arising from reward, autonomic, sensory, and environmental monitoring systems place the vmPFC in a central position to integrate the rewarding and motivational properties of food with an internal representation of the organism's internal state and environmental status.

3.3.2 Efferents

A significant proportion of vmPFC output projections are onto structures vitally involved in interpreting the hedonic and incentive value of food stimuli and generating the requisite motor

behaviors associated with feeding. Aside from thalamic projections, the two densest efferent targets of the PFC are the ventral striatum (which contains the AcbSh) and the LH (Gabbott *et al*, 2005; Hurley *et al*, 1991; Vertes, 2004). Given the central role both structures play in the modulation of non-homeostatic feeding behavior, it is valuable to describe their interconnection with the vmPFC in greater detail.

The prefrontal cortex as a whole sends prominent, topographically-organized glutamatergic projections to the striatal complex, with sensorimotor areas preferentially projecting to dorsolateral striatum and limbic/visceromotor areas preferentially targeting ventromedial striatum (McGeorge and Faull, 1989). Accordingly, vmPFC projects heavily to the ventral striatum with light/moderate projections to dorsal striatum whereas the dmPFC projects almost exclusively to the dorsal striatum (Gabbott *et al*, 2005). There are also interesting differences between prelimbic and infralimbic projection patterns targeting specifically the Acb. While PLC pyramidal neurons project throughout the extent of the Acb (both core and shell), ILC fibers more heavily target the medial shell (Berendse *et al*, 1992; Gabbott *et al*, 2005; McGeorge *et al*, 1989; Voorn *et al*, 2004). Interestingly, within the AcbSh, fibers arising from PLC concentrate in high density cell clusters that are immunoreactive for enkephalin, whereas fibers arising from ILC avoid enkephalin-immunoreactive areas (Berendse *et al*, 1992). Given the previously described role of opioid transmission within the AcbSh in hedonic evaluation and recruiting feeding responses (via inhibition of AcbSh→LH GABAergic projections), the relative segregation of endogenous opioid expression in proximity to PLC but not ILC projections suggests differential roles for each structure in modulating AcbSh activity (see **Chapter 5**).

Similar to the dorsal-ventral gradient formed by corticostriatal projections, corticohypothalamic projections appear to vary on a rostrocaudal axis, though it should be

mentioned that the tract-tracing literature is not in full agreement. Anterograde tracers injected into the rostral vmPFC reveal an innervation pattern consisting of the entire rostrocaudal extent of the lateral and posterior hypothalamic areas, whereas projections from the caudal vmPFC preferentially target more medial areas, including the perifornical and dorsomedial regions (Floyd *et al*, 2001; Gabbott *et al*, 2005). When smaller, more targeted infusions of anterograde tracers were used to parse apart vmPFC subregions, it was shown that ILC projects significantly throughout the dorsomedial, perifornical, and lateral hypothalamic areas, whereas PLC fibers "traverse the hypothalamus en route to the brainstem" with light terminations in posterior, supramammilary, and lateral hypothalamic regions (Hurley *et al*, 1991; Vertes, 2004). However, when Gabbott and colleagues (2005) infused the retrograde tracer wheat germ agglutinin conjugated with horseradish peroxidase (WGA-HRP) into the LH, they found strong labeling throughout the PFC, including substantial labeling within PLC. While some labeling in PLC would be expected due to its aforementioned "light" terminations in the LH, the density of PLC labeling shown by Gabbott appeared to rival or even exceed that seen in ILC. This apparently contradictory finding can be partially explained by the demonstrated ability of WGA-HRP to escape axons and axon terminals and be subsequently taken up and transported both anterogradely and retrogradely by neighboring axons (Gerfen *et al*, 1982). Thus, LH-localized WGA-HRP could invade fibers of passage originating from PLC even if they don't terminate in the region. However, this finding does not fully explain why the density of PLC projection neurons labeled by intra-LH WGA-HRP infusion is comparable to that seen in ILC neurons. While additional studies using less promiscuous retrograde tracing compounds are needed for a deeper characterization of ILC- and PLC-specific projection pathways to the LH, existing studies

clearly show strong vmPFC innervation of the LH and support an important role for vmPFC modulation of LH function.

While projections to the AcbSh and LH are likely to play a central role in vmPFC modulation of feeding-related processes, projections to other structures involved in goal-directed behavior likely contribute as well. Chief among these are direct projections from the vmPFC to the VTA, a subset of which synapse directly onto dopaminergic VTA projections to the Acb (Carr and Sesack, 2000; Gabbott *et al*, 2005). In addition, PLC and ILC both send substantial projections to the amygdalar complex, with PLC targeting the central and basolateral nuclei and ILC targeting the medial, basomedial, cortical, and central nuclei of the amygdala (Gabbott *et al*, 2005; Vertes, 2004). As mentioned previously, DAMGO infusion into the central nucleus (CeA) produces profound increases in appetitive behavior directed at predictive cues (Mahler *et al*, 2009). Moreover, an increased sensitivity to reward-associated cues is prominent feature of binge-like behavior (Dawe *et al*, 2004). Structures in the vmPFC project directly to autonomic regions in the brainstem, including the NTS and PBN, which act as relays for ascending visceral and gustatory information (Gabbott *et al*, 2005; Terreberry and Neafsey, 1987). Interestingly, microstimulation of both ILC and PLC suppresses ongoing gastric motility, including reductions in gastric tone and the amplitude of gastric contractions (Hurley-Gius and Neafsey, 1986). In summary, efferent projections from the vmPFC reach nearly every opioid-sensitive feeding site in the brain, further emphasizing that the vmPFC lies in an ideal position to both monitor and modulate food-seeking and consummatory processes.

3.4 Monoaminergic modulation of prefrontal function

Perhaps the best-known and most widely studied aspect of the prefrontal cortex is the finely-tuned modulation of its function by ascending monoamine systems. These powerful

neuromodulators, including the dopamine (DA), norepinephrine (NE), and serotonin (5-HT) systems (among others), strongly innervate the PFC as well as a large variety of subcortical structures involved in generating feeding responses (Robbins, 2005; Robbins and Arnsten, 2009). While a comprehensive review of monoamine influences over prefrontal function is beyond the scope of this thesis, understanding the neuromodulatory effects of one candidate system can provide information about functioning of prefrontal neurocircuitry, which may help to create a context for interpreting the behavioral effects of vmPFC opioids. Furthermore, given the well-characterized PFC-modulatory effects of monoamines, the question arises as to whether monoamines and opioids interact within the PFC to govern appetitive motivation and inhibitory control (see **Chapter 4**).

Of the most widely studied monoamine neurotransmitters within the PFC, the DA system is most likely to be involved in the modulation of feeding processes and to interact with μ-opioid signaling. DA levels in the vmPFC are heightened during feeding and presentation of food-related cues in both hungry and sated rats (Bassareo *et al*, 2002; Bassareo and Di Chiara, 1997; Carlson *et al*, 1987; Hernandez and Hoebel, 1990; Taber and Fibiger, 1997). Selective vmPFC DA lesions impair inhibitory control and augment hunger-induced feeding responses (Galosi *et al*, 2015; Sokolowski and Salamone, 1994). Dopamine and opioid systems have also been shown to interact in the nucleus accumbens, particularly in the modulation of appetitive behavior directed towards food-related cues. Pretreating Acb neurons with μ-specific opioid agonists increases feeding-evoked DA release (Taber *et al*, 1998) and induces sensitization of amphetamine-induced locomotor activity and responding for conditioned reward (Cunningham *et al*, 1997; Cunningham and Kelley, 1992). Hence, the DA system represents an ideal candidate

for prefrontal opioid/DA interactions, and understanding how the DA system modulates prefrontal activity may provide useful information about the actions of opioid signaling as well.

Dopaminergic modulation of prefrontal function at both cellular and circuitry levels has been the subject of several studies. D1-type DA receptors in the PFC have been particularly well-characterized, especially their role in optimizing working memory performance (Arnsten and Pliszka, 2011; Floresco and Magyar, 2006; O'Donnell, 2003). D1 receptors in the PFC are expressed on pyramidal cells and in primarily parvalbumin-containing GABAergic interneurons (Gaspar *et al*, 1995; Le Moine and Gaspar, 1998; Santana *et al*, 2009). Within the PFC, DA exhibits concentration-dependent effects (Trantham-Davidson *et al*, 2004). Moderate or "optimal" concentrations of cortical DA increase activity at D1 receptors on fast-spiking interneurons, which release GABA onto prefrontal pyramidal cells and suppress spontaneous cell firing (Gorelova *et al*, 2002; Seamans *et al*, 2001b). These same DA concentrations simultaneously activate D1 receptors on pyramidal neurons, resulting in membrane depolarization and facilitated firing of the cell-depolarizing inputs (Gorelova and Yang, 2000; Seamans *et al*, 2001a; Seong and Carter, 2012). Thus, optimal levels of D1 stimulation result in PFC pyramidal cells being held in "up states"; that is, periods of sustained membrane depolarization and suppression of spontaneous cell firing (Lewis and O'Donnell, 2000; O'Donnell, 2003). During up states, strong PFC inputs are able to drive pyramidal cell firing in a way that is resistant to interfering input and noise, resulting in stable, strongly active stimulus representations (Durstewitz *et al*, 2000; O'Donnell, 2003; Seamans *et al*, 2001a). However, at non-optimal concentrations of DA, the integrity of evoked signals is eroded, as insufficient DA levels fail to suppress spontaneous firing, producing a noisy background of PFC activity, and excessive PFC DA levels begin to suppress firing activity in general, including from strong

evoked sources. Thus, the concentration-dependent nature of D1 actions in the PFC gives rise to an inverted-U dose response function, with optimal D1 stimulation levels providing enhanced signal-to-noise ratio and maximizing performance on PFC-dependent tasks (Vijayraghavan *et al*, 2007).

3.5 Mu-opioid modulation of the prefrontal cellular network

The abovementioned mechanisms underlying intra-PFC D1 signaling reveal several loci at which μ-opioid receptors could exert functional effects. Presently, the cellular actions of cortical opioid systems are very under-studied in comparison to PFC monoamines, and also in comparison to opioid influences in *subcortical* areas – especially the striatum, amygdala, hypothalamus, and ventral tegmental area. Nevertheless, studies to date have revealed important neuromodulatory effects of opioids within the PFC and have begun to identify cellular sites of μ-opioid action. Endogenous opioid peptides (preproenkephalin, β-endorphin, and endomorphins) are found in modest amounts in prefrontal areas (Leriche and Mendez, 2010; Martin-Schild *et al*, 1999; Taki *et al*, 2000) and exert their effects via μ-opioid receptors concentrated in layers I, III, and V of the neocortex (Mansour *et al*, 1987). Though few studies have explicitly examined the cortical localization of μORs, there is consensus in their localization on GABAergic interneurons, but not on glutamatergic pyramidal cells (Ferezou *et al*, 2007; Taki *et al*, 2000). Stimulation of μ-opioid receptors attenuates intracellular sodium currents in interneurons, suppressing interneuron activity and thereby reducing inhibitory tone on excitatory PFC projection neurons (Witkowski and Szulczyk, 2006). This would lead presumably to an increase in neuronal excitability, resulting in higher levels of neural output on downstream prefrontal projection targets, including the AcbSh and LH (Gabbott *et al*, 2005). However, it should be noted that recent studies of opioid receptor expression patterns in dissociated rat mPFC cell

cultures have noted μOR expression on "triangular cell bodies with apical and basal neurites" – morphological features typically associated with pyramidal neurons (Olianas *et al*, 2012). Moreover, stimulation of MORs in freshly dispersed rat mPFC pyramidal neurons modulates high-threshold Ca^{2+} channel currents via protein kinase A (PKA)-mediated activity, and this effect is reversed by opioid antagonists (Rola *et al*, 2008). While the expression of μ-receptors on prefrontal pyramidal neurons has yet to be conclusively shown, their potential influence on cell excitability or on signaling in Ca^{2+}-mediated pathways could profoundly affect processing within the mPFC and glutamatergic output onto feeding-related vmPFC projection targets.

There is also evidence for μ-opioid receptor localization on the presynaptic terminals of thalamocortical projections to cortical layer V (Marek and Aghajanian, 1998). These receptors work in opposition to excitatory presynaptic serotonin 2A ($5\text{-}HT_{2A}$) receptors to modulate glutamate release onto pyramidal cells in the mPFC (Marek *et al*, 1998; Marek *et al*, 2001). Hence, stimulation of these μ-opioid receptors would presumably negatively modulate glutamatergic signals from the medial thalamic nucleus (by opposing local, intra-PFC $5\text{-}HT_{2A}$-mediated glutamate release), eroding feedback from cortico-striatal-pallidal-thalamic (CSPT) behavioral processing loops and possibly also relieving inhibitory interneurons from an important source of excitatory drive.

Based on these considerations, we have developed a working hypothesis stating that the net effect of μ-opioid signaling in the PFC is to (1) disrupt *local PFC network function*, and (2) *disinhibit PFC output onto subcortical structures*, abnormally activating multiple downstream nodes responsible for modulating appetitive motivation. Disruption of normal prefrontal network function would impair local mPFC processing underlying executive inhibitory control mechanisms, resulting in a motivationally-driven organism with limited ability to suppress

impulsive behaviors. Moreover, based on discussions above, we also anticipate that local μ-opioid effects on the PFC cellular network would be regulated by PFC monoamines (including dopamine acting through D1 receptors), although the precise nature of this putative interaction is difficult to predict *a priori*. Finally, based on the input-output connectivity of the vmPFC described above, we further propose that this subregion plays a particularly important role (relative to more dorsal sectors) in generating dysregulated motivated responses. The overarching goal of this thesis project is to test these premises and predictions.

4. *Summary*

Binge-eating disorder is characterized by enhanced appetitive motivation towards food coupled with a decrease of inhibitory control over food-directed behavior, and is hypothesized to be a food-directed manifestation of a broader underlying etiology also seen in substance abuse and behavioral addictions (Dawe *et al*, 2004; Grant *et al*, 2013). Accordingly, recent findings associate increases in prefrontal cortical μ-opioid receptor concentration and opioid peptide release with "trait-like" impulsivity (Love *et al*, 2009). Signaling through μ-opioid receptors in subcortical areas is vitally involved in the enhancement of hedonic pleasure and the subsequent recruitment of motivational processes in the nucleus accumbens shell that engage feeding-modulatory circuits in the lateral hypothalamus. The mPFC receives projections from a large number of cortical structures involved in processing information about gustation, somatovisceral function, and reward, and projects strongly onto both the nucleus accumbens shell and lateral hypothalamus (Gabbott *et al*, 2005), placing it in an ideal position to exert top-down control over the expression of feeding behavior. This claim is supported by our findings that μ-opioid stimulation of the vmPFC increases food intake, enhances lever pressing for a sucrose reward, and impairs inhibitory control of food-reinforced responding (see below). It is interesting to note,

however, that the macronutrient preference for carbohydrate-rich foods elicited by prefrontal mu-opioid stimulation (Mena *et al*, 2011) deviates from that seen in a large number of subcortical structures (most notably the Acb), which strongly favor consumption of fats (Will *et al*, 2003; Zhang *et al*, 1998). This suggests that opioid-responsive systems in the vmPFC exhibit some unique characteristics that are distinguishable from opioid-mediated processes in the AcbSh.

5. Thesis Outline & Aims

The overall aim of this thesis was to characterize the involvement of μ-opioid signaling in the ventromedial prefrontal cortex in the modulation of executive and motivational processes governing ingestive behavior, using direct intracranial microinfusions coupled to operant and behavioral observation procedures. The experiments aimed to measure changes in motivated behavior and the integrity of inhibitory control processes in both *ad-libitum* and food-restricted animals, with the additional goal of integrating mu-opioid-evoked responses into existing frameworks and models of PFC function. We discovered that μ-opioid receptors in the ventromedial PFC play an essential role in the recruitment of motivational food-seeking processes and erosion of inhibitory control during both prolonged and acute periods of food deprivation. To the best of our knowledge, we are the first laboratory to show a modulation of prefrontal executive function and recruitment of downstream feeding effectors by local manipulation of the μ-opioid system. Subsequent experiments were designed to elucidate functional interactions between μ-opioid and dopamine receptors within the PFC, the role of PFC projections to the nucleus accumbens shell in modulating food-seeking behavior, and the anatomical locus of DAMGO-elicited effects within the ventromedial PFC, all with the goal of broadening our understanding of the observed behavioral effects.

Chapter 2 focuses on describing the central role that opioid receptors play in the expression of impulsive, motivated behavior during periods of prolonged food deprivation, as well as the surprisingly robust impact that stimulating intra-vmPFC μORs has on inhibitory control processes and appetitive food-seeking behavior during low-deprivation states. Experiments in this chapter examine the effects of various concentrations of DAMGO, infused directly within the vmPFC, on the differential reinforcement of low-rates (DRL) and progressive ratio (PR) operant procedures during both short- and long-duration food deprivation in rats. DRL measures an animal's ability to withhold a prepotent lever press response, whereas PR assesses the appetitive strength of a reinforcer. Characterization of the behavioral profile, including deficits in inhibitory control and enhanced appetitive motivation, will be described. The pharmacological specificity of this effect in comparison with monoamine manipulations will also be described.

Experiments in Chapter 3 utilize a dual cannulation technique to determine the role of the AcbSh in modulating appetitive behavior evoked by stimulation of μOR receptors in the vmPFC. The study in this chapter examines how AMPA signaling in the shell can be manipulated to bidirectionally control the expression of motivated behavior driven by PFC opioid activation. A discussion attempts to incorporate the observed "limiting" actions of PFC→AcbSh projections into a wider framework describing the "fragmented" behavioral profile of intermingled appetitive and exploratory responses evoked by PFC μ-opioid systems.

In Chapter 4, intra-vmPFC interactions between μ-opioid signaling and DA signaling through D1 receptors (a particularly important receptor for PFC-based cognitive functions, including working memory) are explored. The study tests our broader hypothesis that DAMGO-elicited effects in the vmPFC are functionally linked to the broader milieu of PFC

neuromodulator action. In the discussion for this chapter, I integrate our discovery that D1 signaling plays a "permissive" role in enabling the expression of vmPFC μOR-elicited effects with existing models of PFC D1 receptor function.

Chapter 5 describes a high-resolution microinfusion mapping study designed to more accurately localize DAMGO-elicited changes in feeding behavior and inhibitory control within subregions of the vmPFC. Though earlier studies of μOR-mediated behavioral effects showed μ-opioid sensitivity throughout the vmPFC, anatomical placements in more recent studies have increasingly clustered in more ventral parts of the vmPFC, specifically the infralimbic cortex (ILC) as opposed to the more dorsal prelimbic cortex (PLC). The use of concentrated, lower volume drug preparations and a smaller gauge microinfusion apparatus allowed us to probe intra-vmPFC DAMGO sensitivity with higher resolution and acuity. We hypothesized that ILC would show a higher sensitivity to DAMGO-induced effects on feeding and inhibitory control compared to PLC, at least partially due to the ILC's high degree of interconnectivity with vmPFC projection targets, including the AcbSh and LH, crucial to the expression of intra-vmPFC μOR-elicited effects.

Finally, the General Discussion contained in Chapter 6 will summarize all the experimental findings in this dissertation and provide a framework by which our results fit into a larger context, including a proposed circuitry-based model of PFC function that incorporates both μ-opioid and dopamine systems. Future directions of the current work as well as clinical implications will be discussed.

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CHAPTER 2

Attenuation of hunger-induced deficits in impulsive action and appetitive motivation after opioid blockade in the ventromedial prefrontal cortex

The experiments in this chapter were completed by myself. Technical assistance was provided by Mr. Kenneth Sadeghian, Mr. Curtis Lake, Ms. Viridiana Estrada, and Mr. Justin Riederer. Dr. Matthew Andrzejewski provided assistance with computer programming and data analysis. General experimental design, data analysis, and conclusions were developed with the help of my thesis advisor, Dr. Brian A. Baldo. These results have been published in full in Neuropsychopharmacology:

Selleck RA, Lake C, Estrada V, Riederer J, Andrzejewski M, Sadeghian K, Baldo BA (2015). Endogenous opioid signaling in the medial prefrontal cortex is required for the expression of hunger-induced impulsive action. *Neuropsychopharmacology, 40,* 2464-2474.

Abstract

Opioid transmission and dysregulated prefrontal cortex (PFC) activity have both been implicated in the inhibitory-control deficits associated with addiction and binge-type eating disorders. Unknown, however, is whether endogenous opioid transmission *within* the PFC modulates inhibitory control. Here, we compared intra-PFC opioid manipulations to a monoamine manipulation (d-amphetamine) in two sucrose-reinforced tasks: progressive ratio (PR), which assays the motivational value of an incentive, and differential reinforcement of low response rates (DRL), a test of inhibitory control. Intra-PFC methylnaloxonium (M-NX, a limited-diffusion opioid antagonist) was given to rats in a 'low-drive' condition (2 h food deprivation), and also after a motivational shift to a 'high-drive' condition (18 h food deprivation). Intra-PFC D-[Ala2,N-MePhe4, Gly-ol]-enkephalin (DAMGO, a μ-opioid agonist) and d-amphetamine were also tested in both tasks, under the low-drive condition. Intra-PFC M-NX nearly eliminated impulsive action in DRL engendered by hunger, at a dose (1 μg) that significantly affected neither hunger-induced PR-enhancement nor hyperactivity. At a higher dose (3 μg), M-NX eliminated impulsive action and returned PR breakpoint to low-drive levels. Conversely, intra-PFC DAMGO engendered 'high-drive-like' effects: enhancement of PR and impairment of DRL performance. Intra-PFC d-amphetamine failed to produce effects in either task. These results establish that endogenous PFC opioid transmission is both necessary and sufficient for the expression of impulsive action in a high-arousal, high-drive appetitive state, and that PFC-based opioid systems enact functionally unique effects on food impulsivity and motivation relative to PFC-based monoamine systems. Opioid antagonists may represent effective treatments for a range of psychiatric disorders with impulsivity features.

Introduction

Deficient inhibitory control over appetitively motivated behavior occurs in multiple psychiatric disorders; prominent examples include binge-eating disorder and bulimia nervosa, drug addiction, compulsive sexual behavior, and pathological gambling (DSM-5, 2013; Frascella, Potenza, Brown, & Childress, 2010). The neural basis for unregulated appetitive motivation in these disorders is not fully understood. However, considerable evidence implicates functional abnormalities in frontal cortical sites that are engaged by reward-associated cues and that modulate impulsive reward-seeking behavior (Lock, Garrett, Beenhakker, & Reiss, 2011; Schienle, Schafer, Hermann, & Vaitl, 2009). Accordingly, human neuroimaging studies have revealed aberrant frontal activity in drug and behavioral addictions, and in eating disorders (Seo et al., 2013; Uher et al., 2004; Volkow et al., 2005). Furthermore, neuropsychological assessments in individuals with these disorders have shown deficits in frontally-mediated processes such as impulse control and decision-making (Lock et al., 2011; Robbins, Gillan, Smith, de Wit, & Ersche, 2012; Schag et al., 2013). Unclear, however, is the neuropharmacological basis for this frontal dysfunction. A possible clue derives from the fact that opioid receptor antagonists exhibit some efficacy across several disorders characterized by the loss of control over appetitively motivated behavior (Cambridge et al., 2013; Kim, Grant, Adson, & Shin, 2001; Mitchell, Tavares, Fields, D'Esposito, & Boettiger, 2007; Volpicelli, Alterman, Hayashida, & O'Brien, 1992). The role of opioid transmission (particularly in the nucleus accumbens) has been extensively studied in the context of food and drug reward (for example, see Trigo, Martin-Garcia, Berrendero, Robledo, & Maldonado, 2010; Zhang, Balmadrid, & Kelley, 2003). With regard to inhibitory control *per se*, however, opioids have received comparatively less attention, outside of a small number of systemic pharmacology and gene-knockout studies which demonstrate that μ-opioid receptor (μOR) signaling robustly promotes impulsivity (Kieres et al., 2004; Mahoney, Silveira, & Olmstead, 2013; Olmstead, Ouagazzal, & Kieffer, 2009; Pattij, Schetters, Janssen, Wiskerke, & Schoffelmeer, 2009). To date, the possibility that

endogenous opioids act *within* frontal cortex to modulate (or provoke) impulsive reward-seeking action has never been explored.

Here, we studied μOR signaling in the ventromedial prefrontal cortex (vmPFC) across two sucrose-reinforced tasks that assay complementary aspects of binge-like behavior: differential reinforcement of low response rates (DRL), which tests the ability to suppress 'impulsive-like' responses, and progressive ratio (PR), which probes the motivational value of an incentive. Our goals were: (1) to determine whether endogenous intra-vmPFC opioid signaling is *necessary* for diminished inhibitory control occurring in a high-drive state, by blocking vmPFC-localized opioid receptors after a motivational shift from 2-h to 18-h of food restriction; (2) to investigate whether intra-vmPFC μOR stimulation is *sufficient* to cause loss of inhibitory control in a 'low-drive' state. Effects on impulsive action in DRL were compared and contrasted to motivational effects in PR. Furthermore, μOR-agonist effects were compared to those of intra-vmPFC d-amphetamine (AMPH), to evaluate functional differences between PFC-based opioid vs. monoamine systems in the behavioral constructs under study.

Methods

1. Subjects

Subjects were male Sprague-Dawley rats (Harlan; Madison, WI), weighing 275-300 g upon arrival at the laboratory. Rats were housed in a light- and temperature- controlled vivarium, under a 12:12-h light-dark cycle (lights on at 7:00 AM). Food and water were available *ad libitum* except as indicated for various experiments. Animals were handled daily to reduce stress. Testing occurred between 1200-1800 h. All facilities and procedures were in accordance with National Institutes of Health guidelines, and were approved/supervised by the Institutional Animal Care and Use Committee of the University of Wisconsin.

2. Operant-Behavior Procedures

Behavioral testing was carried out in standard Plexiglas operant chambers housed in sound-attenuating cabinets. Ventilation fans in the chambers provided mild masking noise throughout the sessions. The chambers contained two retractable levers placed 6 cm apart, and a pellet receptacle positioned between the levers, and three stimulus lights plus a house light positioned above the levers. After acclimation to the housing facility, rats underwent an initial training period during which they were maintained at 90±2% of free-feeding body weight using scheduled feedings. During this initial phase, all rats were trained to lever-press on a conjoint random-time 30s/fixed ratio 1 schedule (RT-30s/FR-1) in which a sucrose pellet was delivered every 30s regardless of the rats' behavior while single lever presses also resulted in sucrose-pellet delivery. Once the rats were reliably retrieving all pellets during the session (typically within 2-3 days), the RT-30s component was removed. Hence, at this point, all subjects were responding on an FR-1 schedule.

Next, separate cohorts of rats underwent different training progressions for the progressive ratio (PR) versus differential reinforcement of low response rates (DRL) tasks. For PR training, once responding was achieved on FR-1, rats advanced to FR-3, FR-5, and finally PR-2 schedules. The PR-2 contingency consisted of a constant increase in the number of lever-presses required to obtain each successive reinforcer (+2 presses, such that one response was required for the first reinforcer, three for the second, five for the third, etc.). PR sessions lasted 120 min. After 2-3 days on the PR-2 schedule, rats were returned to *ad libitum* food access in their home cages. Thereafter, rats were food-deprived for 2 h immediately preceding each testing session. This mild level of food restriction yielded highly stable levels of baseline responding, such that either increases or decreases from baseline (as engendered by experimental manipulations) could be detected. Rats were maintained on the PR-2 schedule until

stability was achieved (i.e., <10% variability in the number of reinforcers earned in each of three sequential daily testing sessions).

For DRL training, once stability was achieved on FR-1, rats advanced to a variable-interval 15s schedule (VI-15s), then to a VI-30s and finally to a DRL-15s schedule. In DRL schedules, after a reinforcer is earned, subjects are required to withhold responding during an unsignaled, fixed time-period (in our case, 15s). Once this delay interval has elapsed, the subject can then respond to earn the next reinforcer. However, each time a 'premature' response is emitted (i.e., one that is not separated from the previous response by at least 15 s), the delay timer is reset. To achieve optimal performance, therefore, the timing of consecutive responses (interresponse times (IRTs)) must exceed the delay interval. Successful performance in DRL schedules is thought to require intact executive processes of inhibitory control.

After 2-3 days on DRL, rats were switched to *ad libitum* food access, with 2-h food restriction immediately before each testing session. Rats were then re-baselined under this new food restriction schedule. DRL sessions lasted 20 min.

3. Experimental Design

Experiment 1: Effects of intra-vmPFC methylnaloxonium (M-NX) on PR and DRL performance. After recovery from surgery, rats were re-baselined on their respective operant tasks (PR-2 or DRL-15s) under 2-h food restriction, as described above. Upon exhibiting stable baseline responding (no more than ±10% variability over three consecutive testing days), rats were acclimated to the microinfusion procedures with saline injections. Rats were re-baselined after these injections, whereupon drug testing commenced.

Food was removed from the home cages 18 h prior to each testing day, resulting in a motivational shift from their 'low-drive' baseline state, into a 'high-drive' state. On testing days, rats received intra-vmPFC infusions (0, 1, or 3 μg/0.5μl M-NX) and were placed into the operant chambers for their respective PR or DRL session. Separate groups of rats were used for the two operant tasks (N=8 for PR; N=7 for DRL). To ensure that peak drug effects would coincide with the 20 min DRL sessions, infusions were given 15 min prior to testing, with rats placed in their home cages without food for the 15-min post-injection period. PR sessions, however, were longer (120 min); thus, to minimize the chance that the sessions would outlast the duration of drug effects, rats were placed into operant chambers for PR testing immediately after infusions. Doses were counterbalanced according to Latin square designs, with two interim days of drug-free testing (under 2-h food deprivation) separating the drug-infusion days. Two days after completion of all doses, rats underwent three additional testing days on which they received intra-vmPFC M-NX infusions (0, 1, or 3 μg/0.5μl) in the baseline 2-h food deprivation state. Again, dose order was counterbalanced across subjects according to a Latin square design. One drug-free interim testing day separated each drug-infusion day.

After completion of this second M-NX dose-response assessment, rats were tested with intra-PFC saline or M-NX (1 μg), under 18-h food deprivation, in a behavior-observation procedure. The PR and DRL groups were split, with one part of each group receiving intra-PFC M-NX (1 μg; N= 8; 4 PR and 4 DRL rats), and the other part, intra-PFC saline (N=7; 3 PR and 4 DRL rats). Procedural details of the behavioral-observation test are given below.

Experiment 2: Effects of intra-vmPFC DAMGO and AMPH on PR and DRL responding. Post-surgery baselining, and preliminary sham and saline infusions, were given as described in Experiment 1. For PR, intra-vmPFC DAMGO (0, 0.25, and 2.5 μg/0.5 μl) and intra-vmPFC AMPH (0, 0.75, 1.5, and 5.0 μg/0.5μl) were tested in separate groups of rats (N= 6 for DAMGO; N= 6 for AMPH). For each drug,

doses were given according to within-subjects Latin square designs, with 2-3 interim days of drug-free baseline testing separating the drug-infusion days. For DRL, DAMGO (0, 0.25, and 2.5 μg/0.5 μl) and AMPH (0, 1.5, and 5.0 μg/μl) were tested in the same rats (N=8), in counterbalanced order (i.e., half the rats received DAMGO first, the other half, AMPH first). All testing with DAMGO and AMPH was carried out under 2-h food deprivation.

4. Behavioral Observation Procedure

Rats were habituated to clear polycarbonate cages (9.5 in. width X 17 in. length X 8 in. height), identical to the home cages except for wire grid floors. Sucrose pellets were placed in glass jars affixed to the testing cage floors; water was available in overhead water bottles. Thirty-minute habituation sessions were carried out on two sequential days. On the third day, rats were food deprived for 18 h, and Rats were injected with their respective treatments, whereupon they were placed in the testing cages and videotaped with a digital camcorder for 75-min sessions. For the first 30 min of each session, a wire covering was placed over the sucrose jars so that the sucrose could be seen and smelled, but not accessed. The wire covering was then removed, and rats were allowed free access to the sucrose (and water) for 45 min. An experimenter blind to treatment viewed the digital files. Spontaneous ambulation, rearing, drinking bouts, and grooming bouts were recorded both pre- and post-screen removal. In addition, screen approaches were recorded before screen-removal, and sucrose-eating bouts recorded after screen-removal. Behaviors were recorded using an event recorder interfaced to a PC-based laptop computer (Bakshi & Kelley, 1991).

5. Surgical Procedures

Stereotaxic surgery under Isoflurane anesthesia was carried out as described elsewhere (Perry, Baldo, Andrzejewski, & Kelley, 2009). Bilateral guide cannulae were aimed at the vmPFC (near the dorsal border of infralimbic cortex). We have found in previous studies that strong μ-opioid-driven

feeding responses can be elicited from this area (Mena, Sadeghian, & Baldo, 2011). Cannulae were placed at an acute angle (19° from vertical) to avoid damage to the medial wall of the cortex and anchored in place with dental acrylic (New Truliner, Skokie, IL) and skull screws (Plastics One, Roanoke, VA). Coordinates for cannulae placements were as follows: anteroposterior, +3.0 mm anterior to bregma; mediolateral, ±2.2 from the midline; dorsoventral, -2.7 from the skull surface (2.5 mm above the final infusion site). Wire stylets (10.1 mm long, 0.008 in. diameter) were placed in the cannulae to prevent blockage. Animals were given intramuscular injections of penicillin (0.3 mL of a 300,000 U/mL suspension; Phoenix Pharmaceuticals, St. Joseph, MO) and buprenorphine (0.35 mL of a 0.03 mg/mL solution; Hospira, Lake Forest, IL), placed in a warm recovery cage, returned to their home cages upon awakening, and given a recovery period of no less than 4 days (with daily handling and health checks) before resumption of behavioral testing.

6. Microinfusion Procedures and Drugs

Intracerebral microinfusions were carried out according to standard procedures (see Perry *et al.*, 2009). Stainless steel injectors (fashioned from 33-gauge tubing) were lowered into the brain bilaterally to extend 2.5 mm past the tips of the guide cannulae. Injectors were attached with polyethylene tubing (PE-10; Becton Dickinson, Sparks, MO) to 10 µL-capacity glass Hamilton syringes, which were mounted on a Harvard Apparatus (Cambridge, MA) microdrive pump. The infusion rate was 0.32 µL/min, and the total infusate volume was 0.5 µL for all experiments. Injectors were left in place for 1 min after the infusion to allow for diffusion of the injectate, whereupon injectors were removed and wire stylets replaced. Methylnaloxonium (M-NX, a lipophobic derivative of the opioid receptor antagonist, naloxone), DAMGO (µ-opioid agonist), and the non-specific monoamine releaser d-amphetamine (AMPH) were obtained from Sigma-Aldrich (St. Louis, MO). The intra-PFC AMPH dose-range used here is similar to that used in prior studies (Vezina, Blanc, Glowinski, & Tassin, 1991; Yates et al.,

2014), and is clearly behaviorally active when infused into the nucleus accumbens (Bakshi & Kelley, 1991; Carney & Kelley, 1991). All drugs were dissolved in sterile 0.9% saline immediately prior to infusions.

7. Statistical Analyses

Data were analyzed using within-subjects factorial ANOVAs as required by the experimental designs. Contingent upon significance in the ANOVAs, post-hoc comparisons among means were conducted with Tukey's test. Data from the behavior-observation experiment were analyzed with unpaired t-tests. The level of statistical significance was set at $P< 0.05$ for all experiments.

Results

1. Blockade of vmPFC opioid receptors with M-NX reversed the impulsivity observed in a high-drive state. At the highest dose of M-NX (3 μg), and only at this dose, 3 of the 7 rats failed to respond in the DRL sessions. These rats did not emit any lever presses during the 20-min sessions. However, inspection of their behavior in the operant chambers revealed no apparent behavioral impairment. Locomotion, rearing, sniffing, etc. inside the chambers was indistinguishable from their behavior on other test days, and was also indistinguishable from rats in their cohort that successfully responded under 3 μg M-NX. Furthermore, there was no systematic difference in injector placement for any of the rats within this M-NX experiment. Hence, to ensure the veracity of statistical inferences, the data were analyzed in two ways: first, with all doses included, omitting rats that didn't respond at 3 μg M-NX; second, with all rats included, omitting the 3-μg dose. Both analyses support the same conclusions, and both are presented here.

In DRL, shifting rats from a low-drive state (2-h food deprivation) to a high-drive state (18-h food deprivation) significantly impaired *response efficiency* [main effect of drive: $F(1, 3)= 75.54$, $P= 0.003$

with all doses; F(1,6)= 41.24, P= 0.0007 with all rats]. Efficiency ratios were calculated by dividing the number of reinforced lever presses by the total number of presses (i.e., reinforced + unreinforced) for each session, and expressing these ratios as percentages. Intra-vmPFC M-NX significantly reversed this hunger-induced efficiency decrement [dose X drive interaction: F(2,6)= 6.69, P= 0.029 with all doses; F(1,6)= 7.36, P= 0.035 with all rats, Fig. 1A]. In contrast, M-NX had no effect on nose-poking into the food hopper during the DRL sessions. Shifting rats to the high-drive state significantly elevated nose-poking [main effect of drive: F(1,3)= 18.84, P= 0.023 for all doses; F(1,6)= 8.40, P= 0.027 for all rats]. Intra-vmPFC M-NX did not, however, alter nose-poking under either the low-drive state, nor did it alter the elevated rate of nose-poking observed in the high-drive state [Fs=0.24-3.18, not significant (n.s.), Fig 1B].

Further analysis was conducted on the temporal spacing of lever presses, comparing vehicle and the 1-μg M-NX dose (i.e., the dose at which all rats responded). Responses for each session were grouped according to their inter-response times (IRTs); frequency distributions of responses by IRT bin were generated. For clarity, IRTs were collapsed into 3-s bins. *Inefficient lever presses* consisted of unreinforced, 'premature' responses that did not meet the 15-s IRT requirement. *Reinforced lever presses*, on the other hand, were spaced at least 15-s apart. Rapid, closely spaced responses in the 'ultra-short' IRT bin (0-3 s) are thought to reflect loss of inhibitory control (Doughty & Richards, 2002). Intra-vmPFC M-NX significantly reversed the hunger-induced augmentation of inefficient lever-pressing [drug X drive X IRT bin: F(5,30)= 3.13, P= 0.022; Fig. 1D]. Following this 3-way interaction, data were further analyzed by time-bin using ANOVAs, followed by Tukey's tests. For the first four 'inefficient-IRT' bins, saline-treated rats in the high-drive state emitted significantly more inefficient responses than they did in the low-drive state; in these time-bins, M-NX-treatment returned high-drive responding to low-drive levels (Fig 1D). [Fs= 5.4-10.0; Ps= 0.008-0.0004]. Importantly, intra-vmPFC

M-NX failed to decrease the number of *reinforced responses* under either drive condition. Finally, M-NX did not alter responding, inefficient or otherwise, when given in the low-drive condition.

2. Intra-vmPFC opioid receptor blockade attenuated food motivation in the progressive ratio task. Shifting rats from 2 h to 18 h food deprivation markedly increased responding in the PR task, reflected as both increased responding on the active lever and in increased 'breakpoint' (i.e., the last completed ratio, calculated by applying the formula $X + (X - 1)$, where X is the number of cumulative reinforcers for each session) [main effects of drive: F(1,7)= 32.08, P= 0.0008 for active lever-presses; F(1,7)= 49.35, P= 0.002 for breakpoint; Fig. 2 A,C]. Eighteen hours of food deprivation also increased responding on the inactive lever [F(1,7)= 8.45, P= 0.023] and augmented nose-poking [F(1,7)= 12.96, P= 0.009; Fig. 2 B,D].

Intra-vmPFC M-NX dose-dependently reduced active lever presses [main effect of drug: F(2,14)= 6.81, P= 0.0086] and breakpoint [F(2,14)= 9.98, P= 0.002]. These drug effects were due mainly to actions in the high-drive condition [drug X drive interactions [F(2,14)= 5.66, P= 0.016 for active lever presses; F(2,14)= 5.11, P= 0.022 for breakpoint; Fig. 2]. Post-hoc means comparisons indicated that the high M-NX dose (3 μg), given in the 18-h deprivation state, returned active lever presses and breakpoint from 'high-drive' to 'low-drive levels'. Nevertheless, this dose did not alter responding when given in the low-drive state. Importantly, the lower dose (1 μg) had no statistically significant effects in PR in either the high- or low-drive state. Finally, intra-vmPFC M-NX altered neither inactive-lever responding nor nose-poking into the food hopper.

3. Intra-vmPFC opioid receptor blockade did not impair spontaneous activity, sucrose-directed approach, or sucrose intake in 18-h food-deprived rats. To further assess whether the apparent 'rescue' of inhibitory control by 1 μg M-NX was the non-specific consequence of general motoric or motivational impairments, rats from the M-NX DRL and PR experiments were challenged with either

saline or 1 µg M-NX, and their spontaneous activity, feeding, and food-approach behavior was assessed in a behavior-observation procedure (see Materials and Methods for full details). Briefly, in the first 30 min of this test, behaviors were recorded and rats had free access to water, but a see-through wire screen prevented access to sucrose pellets (the same pellets as were used in the operant chambers). Next, the screen was removed, and rats were permitted access to sucrose pellets and water for 45 min. There were no significant effects of M-NX on ambulation, rearing, grooming, or drinking in either the pre-screen or post-screen phase, nor did intra-vmPFC M-NX alter screen approaches or post-screen sucrose intake (t-values= -1.3-2.1, n.s.). Activity with the screen in place, summarized as horizontal+vertical movement (i.e., ambulation counts + rearing counts), is shown in Fig. 1C. All additional measures from this study are summarized in the Supplementary Materials, Table S1. These observations indicate that blockade of vmPFC-localized opioid receptors with 1 µg M-NX does not impair spontaneous motor activity, food approach, or food intake (when food is available with low effort), arguing against the presence of gross motor or motivational impairments at this dose.

4. Intra-vmPFC DAMGO, but not d-amphetamine, augmented food motivation and impaired inhibitory control of food-seeking responses. In the PR task, intra-vmPFC DAMGO given under the low-drive state increased active lever pressing [$F(2,10)= 10.41$, $P=0.004$], breakpoint [$F(2,10)= 21.42$, $P=0.0002$] and nose-poking [$F(2,10)= 21.24$, $P= 0.0003$], but did not alter inactive lever-pressing. These effects are summarized in Fig. 3 and Supplementary Figure S1. Intra-vmPFC AMPH was, however, devoid of effects on any of these measures [Fs=0.67-2.58, n.s.; Fig. 3 B,D,F]. Two days after completion of AMPH dose-response testing, the same rats were challenged with intra-vmPFC DAMGO (2.5 µg) as a positive control. DAMGO produced a significant response, relative to saline and AMPH, on breakpoint [$F(4,20]= 6.67$, $P= 0.0014$], active lever-pressing [$F(4,20)= 5.75$, $P= 0.003$], and nose-poking [$F(4,20)= 18.50$, $P<0.0001$], but not inactive lever-pressing [$F(4,20)= 1.36$, n.s.].

Intra-vmPFC DAMGO also produced 'high-drive-like' effects in the DRL task. Because a subset of rats (4 out of 12) did not respond in DRL at the highest DAMGO dose (2.5 μg), the data were analyzed in two ways: with all doses included, omitting rats that didn't respond at 2.5 μg DAMGO; and with all rats included, omitting the 2.5-μg dose. Both analyses are presented here. Note also that, in this experiment, DAMGO and AMPH were tested in the same rats in counterbalanced order. There were no effects of drug order for any of the DRL measures [Fs= 0.06-0.32, n.s.].

DAMGO, but not AMPH, robustly diminished task efficiency [$F(4,28)= 11.12$, $P<0.0001$ with all doses; $F(3,33)= 23.88$, $P< 0.0001$ with all rats]. Post-hoc analyses indicated that, at the 0.25-μg DAMGO dose, efficiency scores were significantly lower than those seen with saline or either of the two AMPH doses, and at the 2.5-μg DMGO dose, efficiency scores were lower than for 5-μg AMPH. Furthermore, efficiency levels at both AMPH doses were virtually identical to saline (see Fig. 4A). DAMGO also significantly elevated nose-poking into the food hopper, an effect similar to that seen with 18-h food deprivation (see previous section) [$F(4,28)= 15.07$, $P< 0.0001$ with all doses; $F(3,33)= 17.31$, $P<0.0001$ with all rats; see Fig. 4B]. IRT analysis, focusing on comparisons among saline, 0.25 μg DAMGO (the dose at which all rats responded), and the highest AMPH dose (5.0 μg), indicated that DAMGO significantly altered responding relative to saline or AMPH [IRT bin X drug interaction: $F(10, 110)= 13.89$, $P< 0.0001$]. Post-hoc means comparisons revealed that, for all 'inefficient-response' bins (i.e., IRTs< 15 s), the number of lever-presses was significantly greater for DAMGO-treated relative to saline- or AMPH-treated rats (see Fig. 4C). Numbers of reinforced responses, however, did not differ across the treatment groups.

5. Analysis of intra-vmPFC injector placements. As shown in Fig. 5, placements fell mainly in the infralimbic area of medial prefrontal cortex, with some placements in the ventral prelimbic territory. For the DAMGO/AMPH DRL study, it was noted that the rostro-caudal range of placements was greater

compared to other experiments. Therefore, we analyzed efficiency, nosepokes, reinforced responses, and inefficient responses in the four rats with the most rostral placements, and the four with the most caudal placements, with 'placement' as a between-subjects factor in the ANOVA. This analysis failed to reveal drug X placement interactions for any of the aforementioned measures [Fs= 0.19-1.18, n.s.], indicating that drug effects did not differ across the rostro-caudal placements in this experiment.

Discussion

The present findings reveal a novel role of vmPFC-based opioid receptor signaling in the control of food-related motivation and impulsivity. Blockade of intra-vmPFC opioid receptors with M-NX almost completely reversed the deficit in DRL task efficiency incurred by shifting rats from a low-drive (2-h food deprivation) to a high-drive (18-h food deprivation) state. This inhibitory-control improvement did not appear to be an artifact of drug-induced motor slowing or motivational impairment, for several reasons. First, neither reinforced DRL responses, nose-poking into the food hopper, nor general exploratory activity were affected by intra-vmPFC M-NX. Second, the 1-μg M-NX dose, which strongly reduced hunger-induced inefficient responding in DRL, failed to significantly alter PR breakpoint, inactive-lever responses, or nose-poking. At a slightly higher dose (3 μg), intra-vmPFC M-NX attenuated the hunger-induced amplification of PR break-point; again, there were no effects on inactive-lever responses and nose-poking. Third, M-NX produced no behavioral effects at any dose in the baseline, low-drive state. Conversely, stimulation of vmPFC-localized μ-opioid receptors in the low-drive state recapitulated behavioral features of 18-h deprivation: notably, amplification of PR break-point and decrease in DRL efficiency. Nevertheless, DAMGO did not provoke inactive-lever pressing, suggesting that the behavioral changes were not the outcome of non-specific motoric arousal. It is important to note that M-NX is a nonspecific opioid antagonist whose utility arises from its limited tissue diffusion, allowing for better localization of action. Future studies utilizing more specific mu-

specific antagonists would establish whether the 'rescue' of impulse control in hunger is reliant specifically upon blockade of the μ-opioid receptor subtype. Nevertheless, the present findings represent the first demonstration (to our knowledge) that intra-vmPFC opioid signaling is both necessary and sufficient for the expression of inhibitory-control deficits in the context of food-seeking behavior.

Inefficient responding, including 'bursts' of closely-spaced responses (i.e., those characterized by ultra-short IRTs), is a standard feature of DRL response topography and has been argued to represent an 'impulsivity-like' failure to suppress prepotent but disadvantageous action (Doughty & Richards, 2002; Sokolowski & Salamone, 1994). Strikingly, the lower dose of intra-vmPFC M-NX 'rescued' DRL response-efficiency in the high-drive state without significantly altering the general activational properties of this state (nosepoking, hyperactivity, breakpoint-enhancement). This suggests a possible dose-dissociation between prefrontal processes governing inhibitory control mechanisms and recruiting motivational mechanisms---i.e., impulsive action at the 1-ug dose was reduced, even though 'wanting' of the goal was relatively intact. This inference is further supported by the fact that 1 μg M-NX did not alter sucrose approach or intake in the behavioral-observation test. Future studies employing more demanding PR and DRL schedules are warranted to further test this interesting possibility. The fact that M-NX was devoid of effects in the 2-h deprivation state suggests that basal vmPFC opioid tone is low, but is elevated (thereby becoming behaviorally relevant) in a state of heightened arousal/appetitive drive. Amplification of vmPFC μOR signaling with exogenous DAMGO administration both impaired DRL performance and increased PR breakpoint. Together, these results demonstrate a role for state-related opioid signaling in modulating the inter-related processes of appetitive motivation and inhibitory control over food-seeking behavior.

The present results add to a growing body of evidence that μ-opioids mediate functionally unique effects relative to other PFC-based neurochemical systems. The striking dissociation between DAMGO

and AMPH shown here agrees with our previous finding that, while intra-vmPFC DAMGO provoked hyperphagia, a wide variety of intra-vmPFC dopaminergic, noradrenergic, or serotonergic agonists or antagonists failed to do so (Mena et al., 2011). Indeed, to our knowledge, no other neurochemical manipulation of the PFC recapitulates the entire μ-opioid 'behavioral phenotype' of food intake, food-reinforced operant responding, and impulsivity. Moreover, the present results highlight important differences in the behavioral actions of AMPH in the PFC versus nucleus accumbens (Acb). The dose-range of AMPH used here engenders significant hyperactivity and robustly increases responding in PR and other operant tasks when infused into the Acb (Bakshi & Kelley, 1991; Carney & Kelley, 1991; Zhang et al., 2003), in clear contrast to the lack of effects seen in the present study. With regard to impulsive action, systemic infusion of AMPH provokes premature responding in the 5-choice serial reaction time (5-CSRT) task; this effect is blocked by intra-Acb dopamine antagonist infusions, naloxone infusions, or 6-hydroxydopamine lesions (Cole & Robbins, 1989; Pattij, Janssen, Vanderschuren, Schoffelmeer, & van Gaalen, 2007; Wiskerke et al., 2011). Furthermore, intra-Acb AMPH infusion strongly elevates inefficient DRL responding (Neill, 1976). These results contrast the lack of intra-vmPFC AMPH effects in either PR or DRL seen here, or in a prior study reporting negative effects of intra-PFC AMPH (in a similar dose range as used here) on a delay-discounting procedure (Yates et al., 2014). In fact, prior work has shown that 6-OHDA lesions of the PFC cause inefficient responding in DRL (Sokolowski & Salamone, 1994), and blockade of PFC-localized D1 and D2 dopamine receptors engenders impulsive choice in a delayed-reinforcement task (Pardey, Kumar, Goodchild, & Cornish, 2013). Conversely, intra-PFC AMPH attenuated the hyperactivity induced by intra-Acb AMPH (Vezina et al., 1991). Together, these results indicate that an optimal level of PFC-based dopamine transmission is required for intact inhibitory control. Pharmacologically elevating PFC monoamine release with AMPH does not improve inhibitory control in the baseline state (as seen here), but may become relevant when there is a challenge to the system. This pattern of results could reflect a

'stabilizing' action of monoamines on cortical networks, which is not apparent when network efficiency is already near its ceiling. Furthermore, the fact that intra-PFC opioid agonists produce the same effects on inhibitory control as do intra-PFC 6-OHDA lesions or dopamine antagonist infusions could indicate oppositional effects of PFC-based opioid and dopamine systems. It is interesting to posit that optimal levels of dopamine could 'buffer' against the disruptive effects of opioid signaling in heightened arousal/drive states. It would be interesting, for example, to assess whether intra-vmPFC AMPH improves DRL performance in a high-drive state.

Presently, the mechanisms by which opioids modulate the PFC cellular network are unclear. The few studies that have been done, however, indicate that μOR signaling profoundly modulates cortical activity. Endogenous opioid peptides (present in the PFC as enkephalin (ENK), beta-endorphin, and endomorphins (Ferezou et al., 2007; Martin-Schild, Gerall, Kastin, & Zadina, 1999)) act upon μORs at key points within the PFC cellular network. PFC μORs are localized on GABA interneurons (not pyramidal cells) (Ferezou et al., 2007; Taki, Kaneko, & Mizuno, 2000), and endogenous ENK acts at these receptors to suppress interneuron activity and to reduce inhibitory currents onto pyramidal cells (Ferezou et al., 2007; Witkowski & Szulczyk, 2006). This action removes an inhibitory component from cellular network function, presumably disinhibiting the network in a manner similar to μ-opioid actions in the hippocampus (McQuiston & Saggau, 2003). Mu-opioid receptors also appear to function as heteroreceptors on thalamocortical nerve terminals, interacting with serotonin 2A receptors to modulate glutamate release (Marek & Aghajanian, 1998; Marek, Wright, Gewirtz, & Schoepp, 2001). These multiple actions have the potential to strongly shape patterns of activation in the PFC, altering ongoing discharge patterns, changing input/output mappings, and enacting other processes that govern PFC engagement of subcortical systems. Our recent work, for example, suggests that intra-vmPFC μOR stimulation engenders heightened glutamate signaling in multiple terminal fields, including the Acb and hypothalamus (Mena, Selleck, & Baldo, 2013; also see **Chapter 3**). Considering evidence of PFC-Acb

functional connections in modulating drug reinstatement, attentional performance, and other processes that tax inhibitory control (Bossert et al., 2012; Christakou, Robbins, & Everitt, 2004; Peters, LaLumiere, & Kalivas, 2008), this pathway may be particularly relevant for PFC-opioid-induced impulsive action seen here.

Human-imaging studies suggest that exaggerated activity within select frontal sites, including ventromedial aspects of PFC and anterior cingulate cortex (ACC) (areas roughly homologous to the site studied here), contributes to inhibitory-control deficits in a variety of psychiatric disorders characterized by dysregulated appetitive motivation (Karhunen et al., 2000; Schienle et al., 2009; Seo et al., 2013; Uher et al., 2004) . The present results join a growing number of studies indicating that these cortical sites represent crucial loci of clinically relevant opioid action. In humans, ligand-PET studies have demonstrated frontal cortical μ-opioid peptide release in association with sweetened-alcohol drinking (Mitchell et al., 2012) and μOR upregulation in frontal sites including the PFC and ACC robustly predicts the severity of craving and rapidity of relapse in cocaine users (Gorelick et al., 2008; Zubieta et al., 1996). Mu-opioid receptors are upregulated in the PFC (along with the Acb and amygdala) in individuals with trait impulsivity, and these individuals display exaggerated stressor-induced PFC opioid release (Love, Stohler, & Zubieta, 2009). In animal studies, PFC-localized μ-opioid peptides are elevated after exposure to 'binge-like' palatable-feeding or cocaine self-administration schedules, and in rats predisposed to excessive ethanol intake (Blasio, Steardo, Sabino, & Cottone, 2013; Morganstern, Liang, Ye, Karatayev, & Leibowitz, 2012). Finally, intra-PFC naloxone infusion reduced food-reinforced PR responding in rats that had experienced a 'binge'-inducing schedule of sugar access (Blasio et al., 2013). Together with the present results, these studies raise the possibility that supernormal opioid transmission could underlie the frontal cortical dysregulation observed in fMRI studies across multiple binge-type disorders. The PFC may therefore represent a crucial site at which naltrexone and similar drugs act to ameliorate a bingeing endophenotype.

An important future goal is to determine whether endogenous PFC μ-opioid signaling plays a role in mediating executive deficits in other types of high-arousal states, beyond food-motivated states. If so, opioid blocking drugs may have clinical utility beyond current use in binge-type eating disorders and alcoholism; for example, these drugs may also improve performance in a wider range of psychiatric conditions in which extreme arousal impedes executive function. The present findings provide a mechanistic justification for pursuing such possibilities.

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Figure 1. Treatment with intra-ventromedial prefrontal cortex (vmPFC) methylnaloxonium (M-NX) reversed impulsive responding in the differential reinforcement of low-rates (DRL) task after rats were shifted from 2-hour to 18-hour food deprivation states. Rats (n = 7) shifted from a low-drive (2 h) to high-drive (18 hr) state showed impaired response efficiency (i.e., the ratio of reinforced lever presses to total lever presses (**A**) and increased nose-poking behavior (**B**). Intra-vmPFC M-NX attenuated deficits in response efficiency but had no effect on nose-poking behavior. In (**A**): *P< 0.05, main effect of drive; #P< 0.05, different from all low-drive means and from high-drive+3-µg M-NX. In (**B**): *P< 0.05, main effect of drive. Spontaneous locomotor activity ('movements', the sum of horizontal movement (locomotion-'loco') and vertical movement (rearing-'rear') was not effected by M-NX, (**C**). Analysis of inter-response times (IRT; timing of consecutive responses, (**D**) revealed that the motivational shift to a high-drive state resulted in an increase in responding across the four shortest 'inefficient-response' IRT bins; inhibitory control was significantly rescued by intra-vmPFC M-NX (1 ug). In (**D**): *P < 0.05, different from all 'low-drive' means; #P< 0.05, different from 'high-drive'+saline, in each respective time-bin. Error bars depict one S.E.M.

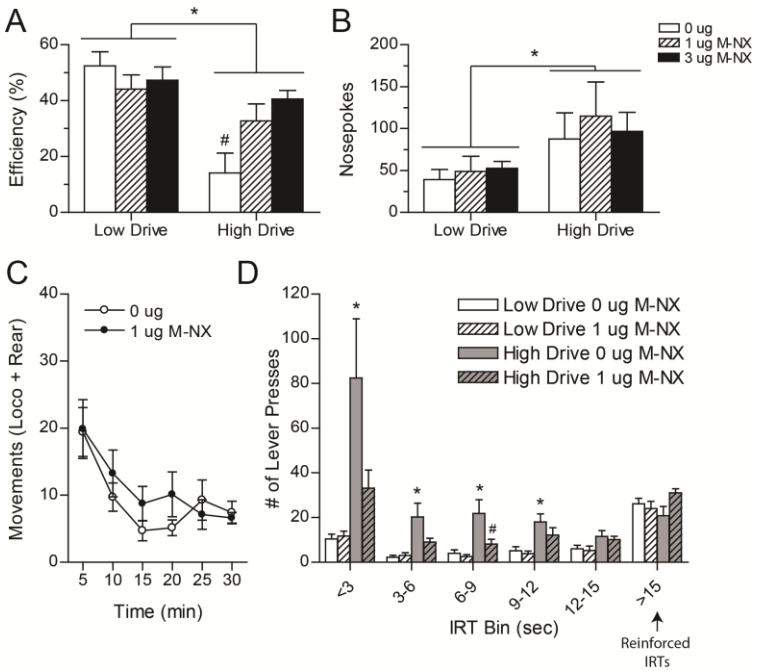


Figure 2. Treatment with intra-ventromedial prefrontal cortex (vmPFC) methylnaloxonium (M-NX) attenuated the hunger-induced amplification of food motivation in the progressive ratio task. Rats (*n* = 7) shifted from a low-drive (2 h) to high-drive (18 h) state showed increases in responding on the active (**A**) and inactive (**B**) levers. The motivational shift also resulted in a higher 'breakpoint' (i.e., the last completed ratio, **C**). Intra-vmPFC infusions of M-NX (3 ug) restored active lever presses and breakpoint to 'low-drive' levels. Increases in nose-poking into the food hopper (**D**) were not altered by M-NX administration. Progressive ratio sessions were 2 h long. *P< 0.05; main effect of drive; #P< 0.05, different from high drive + saline; †P< 0.05, different from 'high-drive + 1 μg M-NX'. Error bars depict one S.E.M.

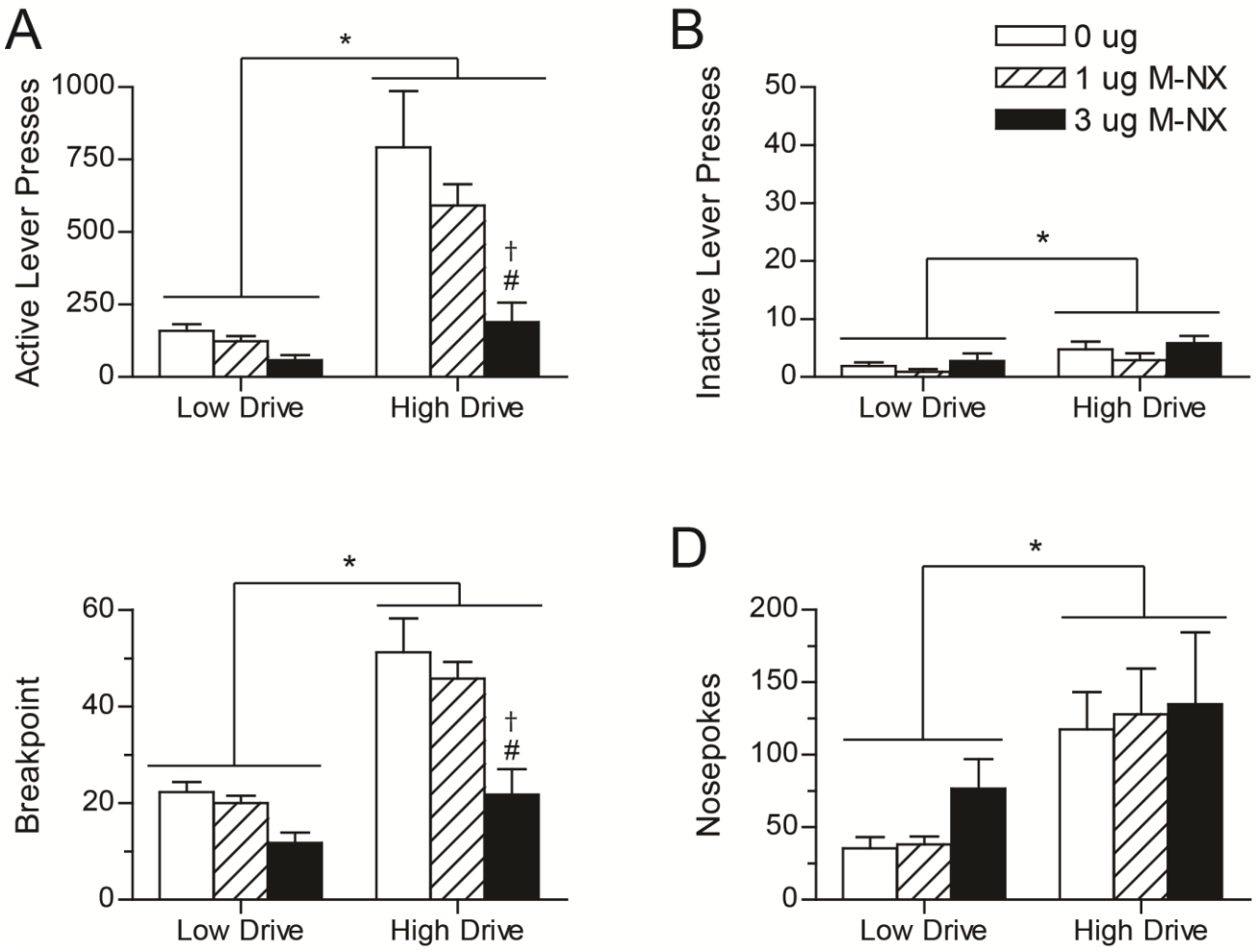


Figure 3. Rats (*n*=6) treated with intra- vmPFC infusions of the μ-opioid agonist DAMGO during the low-drive state showed increases in food motivation, whereas rats (*n*=6) treated with intra-vmPFC infusions of d-amphetamine (AMPH) did not exhibit any change in pressing for sucrose reward in the progressive ratio task. Intra-vmPFC DAMGO increased responding on the active lever (**A**), resulting in an increased breakpoint (i.e., the last completed ratio, **C**). Nose-poking was also increased (**E**). Intra-vmPFC AMPH infusions had no effect on active lever pressing (**B**), breakpoint (**D**), or nose-poke behavior (**F**). The same rats, when challenged with DAMGO, showed significant increases in all three measures. In (**A**), (**C**), (**E**): *P < 0.05, different from saline; # P < 0.05, difference between the two DAMGO doses. In (**B**), (**D**), (**F**): *P < 0.05, different from all within-subject saline and AMPH treatments. Error bars depict one S.E.M.

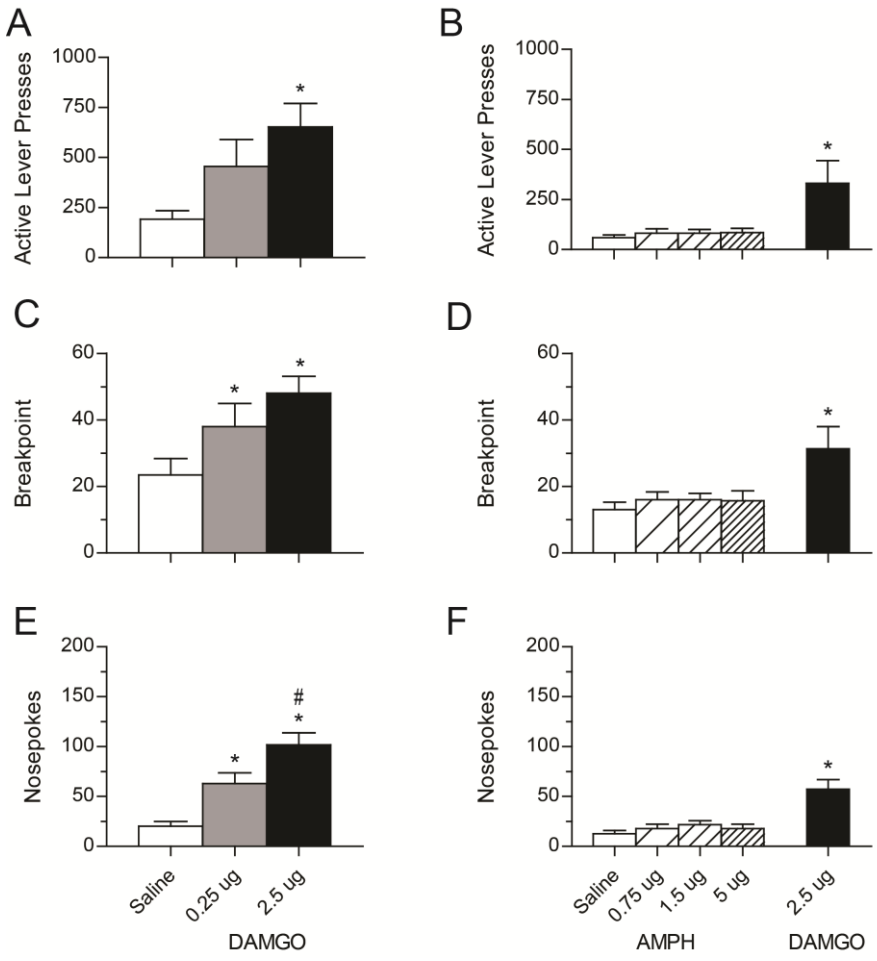


Fig. 4: Rats (*n*=8) treated with intra- vmPFC infusions of DAMGO displayed increased impulsivity in the DRL task. In the same rats, AMPH failed to alter DRL responding. Intra-vmPFC DAMGO infusions impaired response efficiency (**A**) and increased nose-poking behavior (**B**). IRT analysis revealed that DAMGO (0.25 µg) increased responding for all 'inefficient-response' IRT bins (**C**). The number of reinforced responses (i.e., the >15s bin) did not differ between groups. In (**A**): *P < 0.05, different from saline; #P< 0.05, different from both AMPH doses; †P< 0.05, different from AMPH-5µg. In (**B**): *P< 0.05, different from saline; #P< 0.05, different from both AMPH doses. In (**C**): *P< 0.05, different from saline; #P< 0.05, different from AMPH in each respective time-bin. Error bars depict one S.E.M.

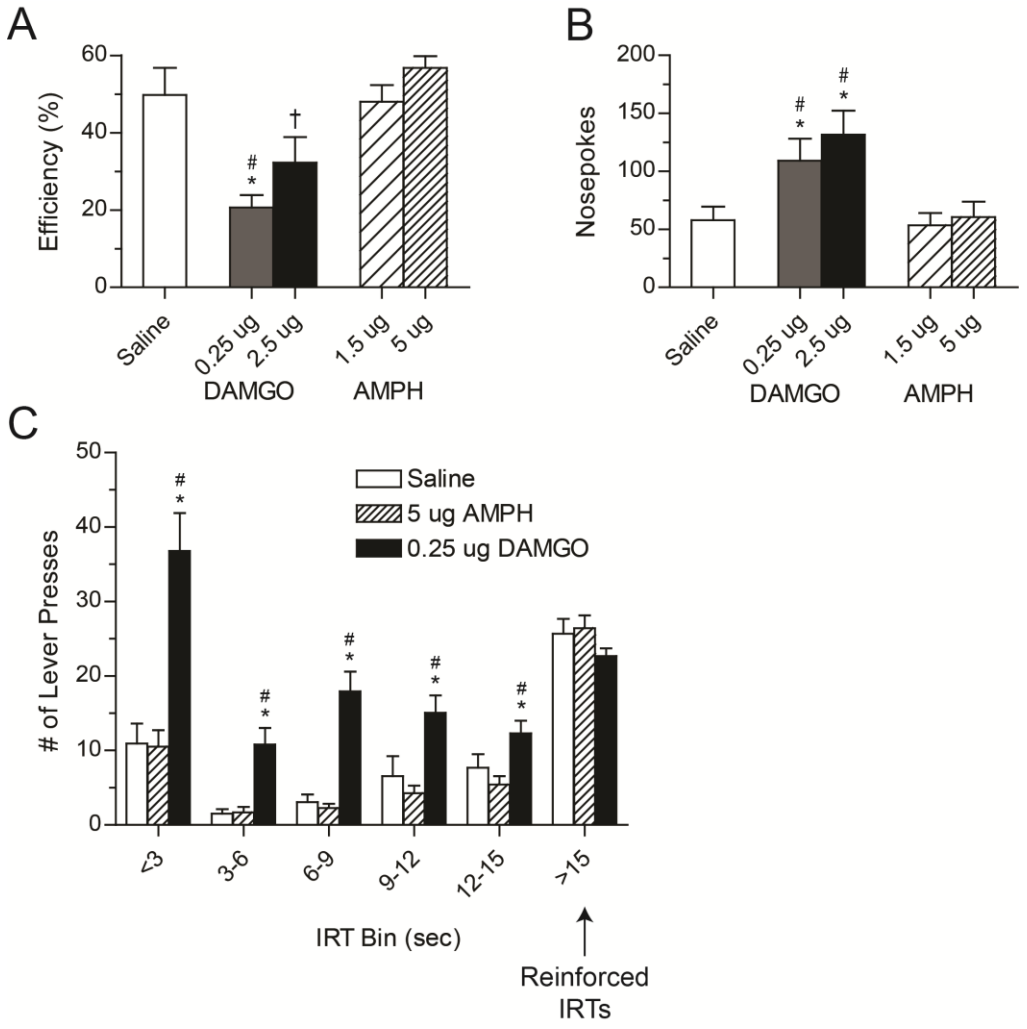
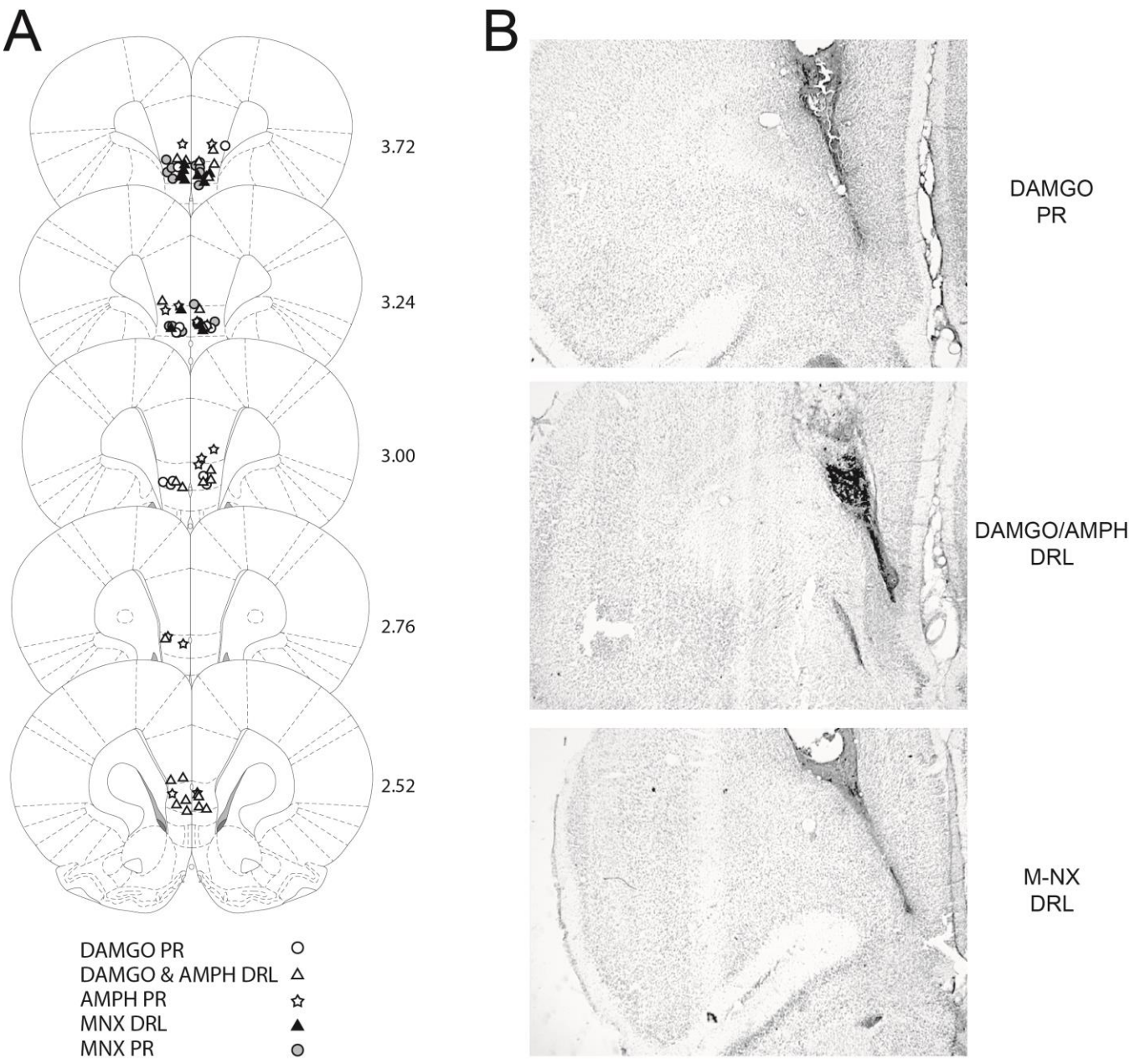


Figure 5. Chartings for injector placements in all experiments (**A**). Different shapes (for different experiments) depict the placement of injector tips. Photomicrographs illustrate representative examples for the DAMGO PR (**B**), DAMGO & AMPH DRL (**C**), and M-NX DRL (**D**) experiments.



CHAPTER 3

Glutamate signaling in the nucleus accumbens shell opposes increases in appetitive motivation elicited by μ-opioid receptor stimulation in the ventromedial prefrontal cortex

The experiments in this chapter were completed by myself. Technical assistance was provided by Dr. Jesus Mena and Mr. Kenneth Sadeghian. Dr. Matthew Andrzejewski provided assistance with programming for behavioral testing equipment. General experimental design, data analysis, and conclusions were developed with the help of my thesis advisor, Dr. Brian A. Baldo. These results have been published in The Journal of Neuroscience:

Mena JD*, Selleck RA*, Baldo BA (2013). Mu-Opioid Stimulation in Rat Prefrontal Cortex Engages Hypothalamic Orexin/Hypocretin-Containing Neurons, and Reveals Dissociable Roles of Nucleus Accumbens and Hypothalamus in Cortically Driven Feeding. *J Neurosci*, *33*(47), 18540-18552.

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ABSTRACT

Mu-opioid receptor (μOR) stimulation within the ventromedial prefrontal cortex (vmPFC) modulates feeding behavior through the recruitment of feeding-related vmPFC projection targets in the nucleus accumbens shell (AcbSh) and the lateral-perifornical hypothalamic areas (LH-PeF). We previously found that glutamate blockade in these areas differentially alters intra-vmPFC μOR-evoked feeding behavior in rats. Intra-LH-PeF glutamate antagonism blocks, whereas intra-AcbSh glutamate antagonism augments, hyperphagia evoked by intra-vmPFC μOR stimulation. Increases in food consumption, however, could be a reflection of a variety of underlying behavioral processes. The goal of this study was to more specifically test whether increases in intra-vmPFC μOR-elicited food motivation are modulated by glutamate signaling in the AcbSh using a sucrose-reinforced progressive ratio (PR) operant task, which assays the motivational value of an incentive by requiring progressively higher amounts of effort to obtain the goal. Blockade of AMPA-type glutamate receptors in the AcbSh augmented increases in PR breakpoint elicited by vmPFC μOR stimulation, whereas intra-AcbSh AMPA stimulation reduced breakpoint and increased nonspecific responding on an inactive lever. These findings suggest that glutamatergic vmPFC-AcbSh projections have the capacity to suppress specific appetitive behaviors via AMPA-mediated activation of a specific "appetitive response-gating" neuron population. Dysfunction within this projection could result in inappropriate limiting of appetitive drive, suggesting treatment strategies that combine μOR blockade with manipulation of substrates in subcortical PFC projection targets in the treatment of eating disorders with binge features.

INTRODUCTION

Central μ-opioid receptors (μORs) have been strongly implicated in natural- and drug-reward, and in addiction and binge-type eating disorders (Berner, Bocarsly, Hoebel, & Avena, 2011; Bodnar, 2004; Gosnell & Levine, 2009; Haile, Kosten, & Kosten, 2008; Kelley & Berridge, 2002). Recent evidence suggests that prefrontal cortex (PFC) may be an important site of μ-opioid peptide action. For example, human neuroimaging studies have demonstrated μOR signaling in PFC in association with drug intake (Colasanti et al., 2012; Gorelick et al., 2005; Mitchell et al., 2012). Nevertheless, at present, knowledge of the subcortical pathways through which intra-PFC μOR signaling influences appetitively motivated behavior (important for placing imaging results in a network context) is limited.

We previously discovered that μOR stimulation, alone among a variety of other opioid and monoamine receptor manipulations within rat ventral medial PFC (vmPFC), elicits appetitive behavior together with intense hyperactivity (an "eat-and-run" pattern) (Mena, Sadeghian, & Baldo, 2011). This response is particularly notable because it is characterized by significantly shortened feeding bouts that are often terminated by abrupt shifts into other behaviors such as rearing or grooming (Mena et al., 2011). The vmPFC is highly interconnected with structures that mediate diverse aspects of appetitively motivated behavior (Gabbott, Warner, Jays, Salway, & Busby, 2005; Vertes, 2004). Prominent among these are the nucleus accumbens shell (AcbSh) and lateral/perifornical hypothalamic areas (LH-PeF), which represent amongst the densest of all vmPFC terminal fields (Gabbott et al., 2005; Hahn & Swanson, 2010; Vertes, 2004), and are the subcortical sites most frequently represented in bifurcating vmPFC projection neurons (Gabbott et al., 2005). The LH-PeF and AcbSh both strongly modulate appetitive behavior, but through dissociable mechanisms. Activation of the LH-PeF with glutamate

agonists or electrical stimulation engenders feeding (Berthoud & Munzberg, 2011; Li, Deurveilher, & Semba, 2011; Stanley, Willett, Donias, Ha, & Spears, 1993; Wise, 1974). In the AcbSh, however, *blockade* of AMPA-type glutamate receptors, or muscimol-induced inactivation, elicits overeating (via LH-PeF activation) (Baldo et al., 2004; Kelley & Swanson, 1997; Maldonado-Irizarry, Swanson, & Kelley, 1995; Stratford & Kelley, 1999), while electrical stimulation or AMPA-receptor stimulation *arrests* feeding and promotes general motoric activation (Ikeda et al., 2003; Krause, German, Taha, & Fields, 2012; Stratford, Swanson, & Kelley, 1998). Hence, glutamate-mediated activation in LH-PeF vs. AcbSh elicits dissociable behavioral processes; simultaneous recruitment of these conjointly innervated vmPFC projection targets could therefore contribute distinct (and potentially opposing) elements to the overall behavioral profile elicited by PFC μ-opioid stimulation.

More recent work from the lab provides direct evidence for functional interactions between the vmPFC and its downstream projection targets, the LH-PeF and AcbSh, in the mediation of appetitive feeding behavior. Intra-LH-PeF NMDA antagonism blocks, whereas intra-AcbSh AMPA antagonism augments, hyperphagia evoked by intra-vmPFC μOR stimulation (Mena, Selleck, & Baldo, 2013), which suggests that previously detailed interactions between AcbSh and LH are relevant for top-down behavioral effects evoked by μOR actions in the vmPFC. It is important to note, however, that increases in food consumption are not necessarily indicative of the recruitment of appetitive food-*seeking* processes. For example, functional inhibition of the AcbSh using GABA agonists or glutamate receptor antagonists dramatically increases free-feeding in ad libitum-maintained rats (Maldonado-Irizarry et al., 1995; Reynolds & Berridge, 2001; Stratford & Kelley, 1997, 1999; Stratford et al., 1998), but does not increase instrumental responding for food or a Pavlovian conditioned stimulus for food

(Burns, Everitt, Kelley, & Robbins, 1994; Zhang, Balmadrid, & Kelley, 2003). Conversely, intra-AcbSh administration of the indirect DA agonist d-amphetamine increases instrumental responding for food and food-associated conditioned reinforcers (Cunningham & Kelley, 1992; Kelley & Delfs, 1991; Phillips, Robbins, & Everitt, 1994; Zhang et al., 2003) but decreases intake of freely-available food pellets (Bakshi & Kelley, 1991). Hence, the relationship between food consumption and instrumental responding for food is complex, and one cannot necessarily assume that a targeted neurochemical manipulation that increases one measure will also increase the other. Our previous work has established that intra-vmPFC μOR stimulation increases effort-based responding (Selleck et al., 2015; see Chapter 2), it is unknown whether this increase is mediated by GABA- and glutamate-dependent substrates in the AcbSh.

Here, we studied the role of AMPA signaling in the AcbSh in the mediation of appetitive, goal-seeking behavior elicited by μ-opioid signaling in the vmPFC using the sucrose-reinforced progressive ratio (PR) operant task. We performed a dual-site microinfusion study, with simultaneous infusions of DAMGO into the vmPFC and an AMPA receptor antagonist in the AcbSh, to evaluate the ability of AMPA blockade in the ventral striatum to alter increases in appetitive motivation evoked by stimulation of μORs in the vmPFC. Based on findings (detailed above) showing that AMPA antagonists in the AcbSh augment feeding evoked by intra-vmPFC DAMGO stimulation, we predicted that AMPA blockade in the AcbSh will augment increases in appetitive motivation elicited by μOR stimulation of the vmPFC.

METHODS

Subjects: Subjects were male Sprague-Dawley rats (Harlan; Indianapolis, IN), weighing 275-300 g upon arrival in the laboratory. Rats were pair-housed in clear polycarbonate cages (9.5-in. width X 17-in. length X 8-in. height), with cob bedding, in a light- and temperature-controlled

vivarium. Animals were maintained under a 12:12 h light/dark cycle (lights on at 0700 h). Food and water were available *ad libitum*, except as indicated for various experiments. Animals were handled gently daily to reduce stress. Testing occurred between 1100 and 1600 h, during the light phase of the animals' dark/light cycle. All procedures were evaluated and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison, and are in accordance with the guidelines promulgated in *the NIH Guide for the Care and Use of Laboratory Animals*. Facilities have been approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

Operant-Behavior Procedures: Behavioral testing was carried out in standard Plexiglas operant chambers housed in sound-attenuating cabinets. Ventilation fans in the chambers provided mild masking noise throughout the sessions. The chambers contained two retractable levers placed 6 cm apart, and a pellet receptacle positioned between the levers, and three stimulus lights plus a house light positioned above the levers. Upon arrival in the laboratory, rats were food restricted and maintained at 90±2% of free-feeding body weight for initial operant training. Rats were trained to lever-press for 45-mg sucrose pellets (BioServe; Frenchtown, NJ). First, rats were exposed to a conjoint Random Time 30s/Fixed Ratio 1 (RT-30/FR-1), with both levers extended. Only one lever was active; manipulating the inactive lever had no programmed consequences (but these presses were recorded). Depressing the active lever was followed by a pellet delivery into the food receptacle, a small light within the receptacle flashing briefly, and the house light turning off and the red stimulus light turning on for 5 s. After his 5s period the chamber returned to its "active" state (house light on, red light off).

Once rats were reliably retrieving sucrose pellets, the RT-30 component was removed. Hence, at this point, all subjects were responding on an FR-1 schedule. Rats were then trained

progressively through FR-3 and FR-5 schedules; once high rates of responding on FR-5 were achieved (at least 300 active lever-presses/session), rats were switched to a Progressive Ratio-2 (PR-2) schedule. Here, the number of responses required for each successive reinforcer was increased by two (i.e., one response was required for the first pellet, three for the second, five for the third, etc.). After two days on the PR-2 schedule, rats were returned to *ad libitum* feeding, and henceforth received daily PR-2 sessions after acute, short-term food deprivation (2 h) immediately before the session. We found that this low level of food deprivation yielded highly stable responding, within a range that could be either increased or decreased by drug treatments. Rats were trained on PR-2 to stability (± 15% reinforcers earned on three consecutive days), whereupon they underwent cannula implantation surgery.

Surgical Procedures:

Rats (weighing 300-330 g at the time of surgery) were anesthetized with Isofluorane gas and secured in a Kopf stereotaxic frame. The toothbar was set at –4.0 mm below the interaural line. Bilateral stainless steel cannulas (10 mm long, 23 gauge) were implanted targeting vmPFC and AcbSh according to standard stereotaxic procedures. Coordinates of the injection site were (vmPFC) AP: +3.0 mm from bregma, ML: ±2.1 mm from midline, DV: -5.2 mm from skull surface with cannulas angled at 19 degrees from vertical to avoid damage to the medial wall of the PFC and (AcbSh) AP: +2.0 mm from bregma, ML: ±1.0 mm from midline, DV: -7.2 mm from skull surface. Angled placements were not used for the AcbSh. Cannulas were fixed in place 2.5 mm above the target sites with dental acrylic (New Truliner, Skokie, IL) and anchoring skull screws (Plastics One, Roanoke, VA). Wire stylets (10 mm long, 30 gauge) were placed in the cannulas to prevent blockage. Rats were given an intramuscular injection of Penicillin (0.3 ml of a 300,000 unit/µl suspension; Phoenix Pharmaceuticals. St. Joseph, MO), placed in heated

recovery cages, returned to their home cages upon awakening, and given a recovery period of no less than five days (with daily health checks) before being returned to a schedule of daily PR-2 testing. Once rats again achieved stable baselines, drug infusions commenced.

Microinfusion Procedures and Drugs

For intracerebral microinfusions, rats were held gently, and stylets removed from the guide cannula. Stainless steel injectors, connected via polyethylene tubing (PE-10, Becton Dickinson, Sparks, MD) to 10-µl capacity Hamilton syringes (Hamilton, Reno, NV) on a Harvard microdrive pump, were lowered to the site of infusion. The flow rate for infusions was 0.32ul/min. The total infusate volume for the bilateral infusions was 0.5ul/side. For the double cannulation studies, infusions into vmPFC preceded infusion into AcbSh by approximately 5 min. DAMGO, CNQX disodium salt hydrate, and AMPA hydrobromide were all obtained from Sigma Aldrich (St. Louis, MO) and dissolved in sterile saline. For CNQX, the saline was warmed slightly during mixing to aid solubility. After infusions, injectors were left in place for an additional minute to allow for diffusion of the injectate into the tissue. Injectors were then removed, and wire stylets replaced.

Experimental design

Rats (n=5) received intra-vmPFC infusions of saline or DAMGO (0.25 µg), and intra-AcbSh infusions of saline or CNQX (100 ng) according to a completely within-subjects design, with the order of drug treatments counterbalanced across subjects. After these treatments were completed, all rats received a challenge with saline in the vmPFC and AMPA (50 ng) in the AcbSh (vmPFC infusions were given first, followed 10 min later by intra-AcbSh infusions, whereupon rats were immediately placed into the operant chambers for the start of the session).

Each drug-infusion day was separated from the next by at least two interim days, on which rats were tested on the PR-2 task with no intracerebral infusions.

Verification of placements:

At the end of the experiment, rats were deeply anesthetized with Isofluorane and perfused transcardially with a 0.9% saline solution followed by 10% formalin in phosphate buffer. Brains were collected and stored in 10% formalin. Coronal sections (60 μm) were cut through the infusion sites on a cryostat microtome, collected on slides, stained with cresyl violet, and subsequently reviewed to verify correct placement of the intracerebral injections. Images of representative sections from each experiment were captured using Scion Image software on a computer interfaced with a microscope-mounted Hitachi HV-C20 CCD camera.

Statistical analyses

Experiments were analyzed with two-factor ANOVAs. Fisher's PLSD test or Bonferroni-corrected t-tests were used for post-hoc comparisons among means. The level of statistical significance was set at $P< 0.05$.

RESULTS

Intra-vmPFC DAMGO augments sucrose-reinforced progressive ratio (PR) responding; intra-AcbSh infusion of CNQX potentiates this effect while intra-AcbSh AMPA reduces breakpoint and engenders non-specific lever-pressing.

As shown in Fig. 1, Intra-vmPFC DAMGO robustly elevated PR responding on the active lever. The DAMGO-induced potentiation of PR was markedly potentiated by the 100-ng intra-AcbSh CNQX dose (main effect of DAMGO: $F(1, 4)= 41.1$; $P< 0.004$; CNQX: $F(1, 4)= 6.4$; $P< 0.07$; DAMGO X CNQX: $F(1, 4)= 11.0$; $P< 0.03$; Figure 1A). Indeed, total lever presses for the

DAMGO+CNQX combination often exceeded 1,000 presses per session. Responding on the inactive lever was low for all groups (in the range of 1-7 presses), but elevated slightly by CNQX and the DAMGO+CNQX combination (main effect for DAMGO: $F(1, 4)= 3.2$, n.s.; CNQX: $F(1, 4)= 12.2$; $P< 0.03$; DAMGO X CNQX: $F(1, 4)= 5.8$; $P< 0.08$).

All rats were then challenged with AMPA in the AcbSh (and saline in the vmPFC); AMPA effects were compared to all other treatments using one-way ANOVAs followed by Fisher's PLSD post-hoc tests. Intra-AcbSh AMPA significantly reduced PR responding on the active lever ($F(4, 16)= 24.8$; $P< 0.0001$) and substantially increased responding on the inactive lever ($F(4, 16)= 4.5$; $P< 0.02$). Post-hoc comparisons (summarized in Fig. 1) indicated that these AMPA effects differed significantly from the effects of saline into both sites, CNQX into the AcbSh, or DAMGO into the vmPFC. To further explore the possibility that intra-AcbSh AMPA degraded stimulus control in the PR task, we calculated a lever-discrimination index (active lever presses/total lever presses) in which indiscriminate responding is indicated by a value of 0.5. AMPA significantly reduced lever discrimination relative to all other groups ($F(4, 16)= 3.5$; $P< 0.04$). Together, these findings with the PR task further indicate that AMPA signaling in the AcbSh "opposes" specific goal-directed appetitive behaviors associated with intra-vmPFC μ-opioid signaling by provoking a competing behavioral set of generalized, indiscriminate motor activation.

Histological verification of intra-tissue injection placements

Representative micrographs of injector placements into vmPFC and AcbSh (Figure 1, E and F) of dual-cannulated animals reveal that cannula and injector tracks are clearly visible with no unusual damage to the targeted areas.

DISCUSSION

As previously described (Selleck et al., 2015; see Chapter 2), stimulation of intra-vmPFC μORs evoked an increase in lever pressing for a sucrose reward, with an associated increase in PR breakpoint. When intra-vmPFC μOR blockade was paired with the blockade of AMPA-type glutamate receptors in the AcbSh, evoked increases in PR responding were markedly potentiated. However, stimulation of AcbSh-localized AMPA receptors reduced lever pressing and PR breakpoint while simultaneously increasing responding on the inactive lever, suggestive of a degradation of stimulus control. These findings provide evidence that glutamatergic activity in the AcbSh opposes food motivation driven by μ-opioid activation of the vmPFC.

Previous work has shown that AMPA signaling within the AcbSh has an important role in limiting or suppressing food consumption and food-directed appetitive processes. Stimulation of AcbSh-localized AMPA-type glutamate receptors suppresses feeding (Stratford et al., 1998), and electrical stimulation of the nucleus accumbens immediately arrests consummatory sucrose licking (Krause et al., 2012). Conversely, AMPA (but not NMDA) blockade or chemical inactivation of the AcbSh provokes feeding; importantly, this feeding effect is thought to be mediated by LH activity (Kelley & Swanson, 1997; Maldonado-Irizarry et al., 1995; Stratford & Kelley, 1999). Electrophysiological studies revealed a subpopulation of Acb neurons that show inhibitions that begin immediately before the onset of consummatory sucrose licking and persist during consumption, but do not occur during locomotion unrelated to consumption (Krause et al., 2012; Taha & Fields, 2005, 2006). In a conceptually related finding, a subpopulation of AcbSh units showed phasic inhibitions during instrumental actions and sucrose-receptacle entries; basal activity of these units was suppressed by vmPFC lesions, suggesting that these neurons are under excitatory control by vmPFC projections (Ghazizadeh, Ambroggi, Odean, & Fields, 2012).

Together, these findings suggest that the glutamatergic projection from the vmPFC to the AcbSh has the capacity to suppress specific goal-directed behaviors via AMPA-mediated activation of a specific "appetitive response-gating" neuron population. In line with this, we observed that altering glutamate signaling in the AcbSh was able to bidirectionally modify changes in appetitive responding evoked by vmPFC μOR stimulation. This finding is particularly noteworthy in light of prior studies showing that functional inactivation of the AcbSh alone has no effect on appetitive goal-seeking behavior (as opposed to simple feeding; Burns et al., 1994; Zhang et al., 2003), further illustrating differences between neural control of goal-seeking versus consummatory behavior (Baldo & Kelley, 2007). Finally, along with inhibiting feeding, intra-AcbSh AMPA infusion also engenders considerable exploratory-like behavior (ambulation) (Stratford et al., 1998), and unilateral AMPA but not NMDA stimulation in the AcbSh elicits intense motor activation manifested as contraversive rotational behavior (Ikeda et al., 2003). These results indicate that AMPA signaling in the AcbSh recruits motor repertoires that are incompatible with feeding. In concert with this, we found that AMPA blockade in the AcbSh augmented increases in sucrose-reinforced PR responding elicited by μOR stimulation in the vmPFC, whereas infusion of AMPA alone into the AcbSh reduced PR breakpoint while engendering non-specific responding on the inactive lever.

These findings are consistent with the hypothesis that μ-opioid activation of the vmPFC produces supernormal vmPFC activation and glutamatergic outflow, perhaps through μ-OR-mediated suppression of a local inhibitory network, onto downstream targets of vmPFC projections – most notably for our purposes, the AcbSh (Taki et al., 2000; Witkowski and Sulczyk 2006; Ferezou et al., 2007). With regard to circuit connections, a recent study found a closed-loop anatomical relationship among infralimbic PFC, AcbSh (similar to the zone targeted

here), ventral pallidum, and a tightly circumscribed anterior LH region, via a thalamic relay (Thompson and Swanson, 2010). AMPA signaling in the closed-loop vmPFC-AcbSh circuit could engage a behavioral set that competes with or "interrupts" specific goal-directed appetitive behaviors (see discussion in Baldo & Kelley, 2007), whereas while glutamate transmission in broader hypothalamic areas simultaneously engages appetitive drive. Such a mechanism could underlie the "fragmented" locomotor/feeding profile typically observed after μ-opioid stimulation of the vmPFC, and possibly dysregulated feeding responses and breakdown in executive control seen in binge-type eating disorders.

The present results may have important clinical implications. Recent evidence suggests that the PFC is a crucial site of opioid action in several "disorders of appetitive motivation," including addiction (Chang et al., 2010; Colasanti et al., 2012; Gorelick et al., 2005; Mitchell et al., 2012); however, to date, the subcortical network through which intra-PFC μOR signaling modifies behavior and cognition has not been characterized. The present work suggests that the AcbSh has an important role as a limiter of cortically-derived appetitive drive. This insight may suggest treatment strategies that combine μOR blockade (which shows some efficacy against food bingeing and drug craving (Berner et al., 2011; O'Brien, 2005)) with rationally selected manipulation of substrates within subcortical PFC projection targets.

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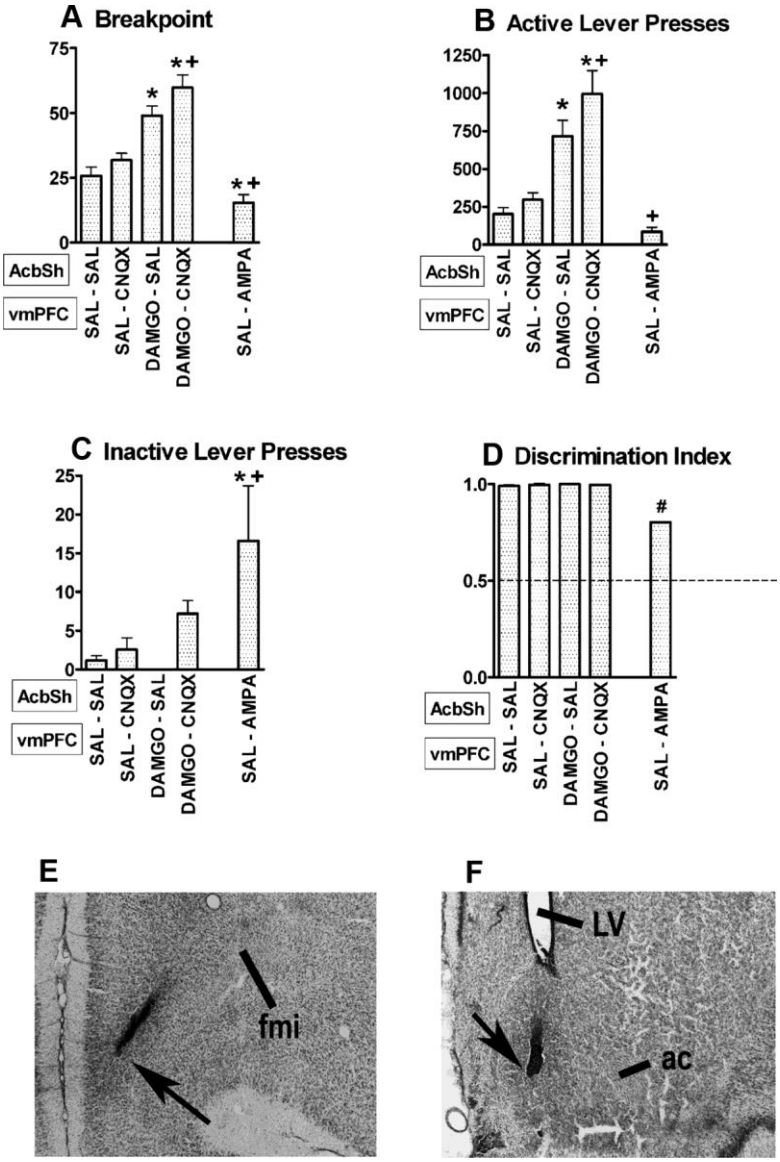
Figure 1. Effects of intra-Acb shell AMPA manipulations and intra-vmPFC DAMGO on PR responding. **A**, Shows breakpoint (the last successfully completed ratio requirement). **B**, **C**, Show responding on the active and inactive levers, respectively. **D**, Shows discrimination index, the ratio of correct responses to total responses (1.00 reflects perfect discrimination, 0.5 reflects chance responding or loss of discrimination). **A-D**, **p*<0.05, different from Sal-Sal and Sal-CNQX, +*p*<0.05, different from DAMGO-Sal. Error bars indicate 1 SEM. Photomicrographs illustrate typical placements in the vmPFC (**E**) and AcbSh (**F**). Arrows point to injector tips. Fmi, forceps of corpus callosum; ac, anterior commissure; LV, lateral ventricle.



CHAPTER 4

Effects of intra-ventromedial prefrontal mu-opioid receptor stimulation on inhibitory control and appetitive motivation are dependent on intact signaling through D1 dopamine receptors

The experiments in this chapter were completed by myself. Technical assistance was provided by Mr. Kenneth Sadeghian, Mr. Brandon Buchholtz, and Mr. Curtis Lake. Dr. Matthew Andrzejewski provided assistance with programming for behavioral testing equipment. General experimental design, data analysis, and conclusions were developed with the help of my thesis advisor, Dr. Brian A. Baldo. These results have been submitted in full for publication in the journal Neuropsychopharmacology:

Selleck RA, Buchholtz BD, Lake C, Sadeghian K, Baldo BA. (Under review) Prefrontal cortical mu-opioid modulation of inhibitory control and appetitive motivation is dependent upon intact dopamine D1 receptor signaling.

ABSTRACT

Opioid transmission within the prefrontal cortex (PFC) has been implicated in psychiatric disorders featuring increased impaired inhibitory control over appetitive motivation, such as addiction and binge-eating disorder. We have previously shown that infusions of DAMGO (D-[Ala2,N-MePhe4, Gly-ol]-enkephalin, a μ-opioid agonist) into the ventromedial PFC (vmPFC) increased responding on two sucrose reinforced tasks - differential reinforcement of low response rates (DRL), a test of impulsive action, and progressive ratio (PR), an assay of the appetitive strength of an incentive. These effects are not reproduced by intra-PFC infusion of a variety of DA agonists and antagonists, suggesting that manipulation of DA systems alone is not sufficient to reproduce μOR-like effects on impulsive action or feeding behavior. Nevertheless, this does not rule out *interactions* between PFC DA and μ-opioid systems in mediating effects on processes of inhibitory control over motivated behavior. Here we used intra-vmPFC drug cocktails containing DAMGO and SCH-23390 (a D1-specific DA antagonist) to determine whether increases in impulsive, motivated responding elicited by stimulation of intra-vmPFC μ-opioid receptors (μORs) require intact signaling through intra-vmPFC D1 DA receptors. Simultaneous blockade of D1 receptors with SCH-23390 attenuated both impairments in inhibitory control and enhancements in PR breakpoint elicited by intra-vmPFC μOR stimulation. Furthermore, D1 blockade reversed the increase in general exploratory-like activity engendered by intra-vmPFC μOR stimulation. These results establish that intra-vmPFC D1 signaling is required for the expression of behavioral effects evoked by opioid transmission within the PFC, and further suggest that D1 signaling tone plays an enabling or permissive role in allowing μOR-elicited effects to be expressed. Simultaneous targeting of both μ-opioid and D1 systems in the

PFC may represent a more efficacious treatment strategy for psychiatric disorders with impulsivity features.

INTRODUCTION

Multiple psychiatric disorders are characterized by insufficient inhibitory control over appetitive motivation, including binge eating disorder, substance use disorders, and pathological gambling (American Psychiatric Association, 2013). Neuroimaging studies of individuals with these disorders show abnormal function in frontal cortical areas that are responsive to reward-associated cues and that modulate reward-seeking behavior (Donnelly *et al*, 2014; Feja and Koch, 2015; Schienle *et al*, 2009). Nevertheless, the neurochemical basis for frontal dysfunction in these disorders is still unclear. A previous study from our lab suggested a role for μ-opioid signaling in the ventromedial prefrontal cortex (vmPFC) in the modulation of impulsive reward-seeking behavioral states (Selleck *et al*, 2015). Stimulation of vmPFC μ-opioid receptors (μOR) using the μ-selective agonist DAMGO increased effort expended in lever pressing for sucrose reward on a progressive ratio (PR) schedule, and also impaired the animal's ability to temporarily withhold a disadvantageous prepotent lever-press response in a differential reinforcement of low-rates (DRL) paradigm (Selleck *et al*, 2015). When shifted into a naturally motivated, impulsive behavioral state (prolonged food deprivation), blockade of vmPFC opioid receptors returned animals to near-normal patterns of responding on PR and DRL tasks (Selleck *et al*, 2015). Hence, μ-opioid signaling in the vmPFC is necessary for impairments in frontal inhibitory control over food-directed motivated behavior, and increased intra-vmPFC μOR signaling evokes increases in motivated, impulsive behavior.

While the abovementioned results show that μ-opioid activity modulates the expression of cortically-driven motivated ingestive behavior, the role of other neurochemical systems within the PFC in modulating μOR activity is unknown. The dopamine (DA) system seems a particularly promising candidate. DA levels in the vmPFC are heightened during feeding after

food deprivation (Carlson *et al*, 1987; Hernandez and Hoebel, 1990; Taber and Fibiger, 1997) and after presentation of palatable foods and food-related cues to sated rats (Bassareo *et al*, 2002; Bassareo and Di Chiara, 1997). Selective vmPFC DA lesions evoke deficits in DRL performance and augment hunger-induced feeding responses (Galosi *et al*, 2015; Sokolowski and Salamone, 1994). However, we have previously shown that stimulation of intra-vmPFC DA transmission using indirect agonists has no effect on DRL performance or PR breakpoint; nor did intra-vmPFC administration of D1 or D2-selective DA antagonists alter food intake (Mena *et al*, 2011; Selleck *et al*, 2015). Hence, manipulating intra-vmPFC DA receptors is not sufficient to evoke changes in food-directed motivated behavior. The possibility remains, however, that opioid and dopaminergic systems within the vmPFC interact to mediate cortical influences over feeding behavior. General support for DA/opioid interactions is provided by previous work in the nucleus accumbens, a prominent vmPFC projection target (Baldo and Kelley, 2007; Gabbott *et al*, 2005). For example, pretreatment of nucleus accumbens neurons with μ-specific opioid agonists increases feeding-evoked DA release (Taber *et al*, 1998) and induces sensitization of amphetamine-induced locomotor activity and responding for conditioned reward (Cunningham *et al*, 1997; Cunningham and Kelley, 1992).

The goal of this study was to explore the role of D1 signaling in modulating μ-opioid responses within the vmPFC as an initial step toward the broader objective of investigating the interaction of vmPFC opioids and monoamines. The D1 receptor was selected here because it has a well-established role in modulating PFC function, including working memory (Berridge and Arnsten, 2013; Kesner and Churchwell, 2011; Vijayraghavan *et al*, 2007; Zahrt *et al*, 1997). Blockade of D1 receptors in the vmPFC prevents enhancements in control over impulsive action evoked by the antidepressant milnacipran (Tsutsui-Kimura *et al*, 2013). Stimulation of vmPFC

projection neurons that express D1 receptors increases food intake (Land *et al*, 2014). In the present study, we explored whether intra-vmPFC D1 receptor signaling modulates μOR-evoked behaviors using μ-opioid agonist and D1 antagonist drug cocktails infused directly into the vmPFC coupled with three discrete behavioral paradigms. These three paradigms were progressive ratio (PR), an operant task assaying the motivational strength of a food reinforcer, differential reinforcement of low-rates (DRL), a task that measures the ability to withhold a disadvantageous prepotent lever press response, and a behavioral observation paradigm, which measures unconditioned food intake and spontaneous motor activity. Based on established findings showing that prefrontal DA signaling modulates inhibitory control over motivated behavior, as well as the abovementioned literature showing known interactions between subcortical opioid and dopamine systems on ingestive behavior (Cunningham *et al*, 1997; Cunningham *et al*, 1992; Sokolowski *et al*, 1994; Taber *et al*, 1998), we hypothesized that blockade of D1 DA receptors in the vmPFC would alter the behavioral effects of μ-opioid stimulation. The use of parallel DRL, PR, and unconditioned behavior tasks enabled the detection of possible dissociations in opioid/DA interactions across the interacting, yet distinct, processes of appetitive motivation and inhibitory control of motivated behavior.

METHODS

Subjects

Subjects were male Sprague-Dawley rats (Harlan, Madison, WI) weighing 275-300 g upon arrival at the laboratory. Rats were housed in a light- and temperature-controlled vivarium, under a 12:12 hr light-dark cycle (lights on at 0700 hr). Food and water were available *ad libitum*, except as indicated for various experiments. Animals were handled daily to reduce

stress. Testing occurred between 1100 and 1500 h. All facilities and procedures were in accordance with National Institutes of Health guidelines and were approved/supervised by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

Operant-Behavior Procedures

Operant training and testing procedures were conducted in standard operant chambers made of sheet aluminum and Plexiglass, enclosed within ventilated, sound-attenuated chests. Ventilation fans in the chambers provided masking noise continuously throughout each session. One wall of the chamber contained two retractable levers spaced 6 cm apart. Space equally between the levers was a pellet receptacle into which 45 mg sucrose pellets (BioServ; Frenchtown, NJ) could be delivered from an automatic pellet dispenser. Above the receptacle was a row of three stimulus lights (red, yellow, and green) and a 28 V house light. Experiments were controlled, and behavioral input recorded, on a PC-based computer running MedPC IV for Windows (Med Associates, St. Alban, VT).

After acclimation to the housing facility, rats underwent an initial training period during which they were maintained at 90 ± 2% of free-feeding body weight using scheduled feedings. During this initial phase, rats were trained to lever press on a conjoint random-time 30 s/fixed ratio 1 schedule (RT-30 s/FR-1), in which a sucrose pellet was delivered on average every 30 s regardless of the rats' behavior while single lever presses also resulted in a sucrose pellet delivery. Once the rats were reliably retrieving all pellets during the session (typically within 2-3 days), the RT-30 s component was removed. Hence, at this point all rats were responding on an FR-1 schedule.

Next, separate cohorts of rats underwent different training progressions for the PR *vs* DRL tasks. For PR training, once responding was achieved on FR-1, rats advance to FR-3, FR-5, and finally PR-2 schedules. The PR-2 schedule consisted of a constant increase in the number of lever presses required to obtain each successive reinforcer (+2 presses, so that one response was required for the first reinforcer, three for the second, five for the third, and so on). PR sessions lasted 120 minutes. After 2-3 days at PR-2, rats were returned to *ad libitum* food access in their home cages. Thereafter, rats were food deprived for 2 h immediately preceding each testing session, a mild level of food restriction that yielded highly stable levels of baseline responding. This allowed for the detection of both increases and decreases from baseline (as engendered by experimental manipulations). Rats were maintained on the PR-2 schedule until stability was achieved (<10% variability in the number of reinforcers earned in each of three sequential daily testing sessions).

For DRL training, once consistent responding was achieved on FR-1, rats advanced to a variable-interval 15 s schedule (VI-15 s), then to a VI-30 schedule, and finally to a DRL-15 s schedule. In DRL schedules, after a reinforcer is earned rats are required to withhold responding during an unsignaled, fixed time period (15 s for a DRL-15 schedule). Once this delay interval has elapsed, the subject can then respond to earn the next reinforcer. However, each time a 'premature' response is emitted (i.e. one that is not separated from the previous response by at least 15 s), the delay timer is reset. To achieve optimal performance, therefore, the timing of consecutive responses (interresponse intervals (IRTs)) must exceed the delay interval. Successful performance in DRL schedules is thought to require intact executive processes of inhibitory control (Andrzejewski *et al*, 2011; Doughty and Richards, 2002; Sokolowski *et al*, 1994). After 2-3 days on DRL, rats were returned to *ad libitum* feeding, with 2 h food restriction immediately

before each testing session. Rats were then rebaselined under this new food restriction schedule. DRL sessions lasted 20 min and were initiated by the subject's first response.

Surgical Procedures

Surgeries were conducted with the toothbar set to 4.0 mm below interaural zero. Stainless steel guide cannulae (10 mm long; 25 gauge) were aimed at the vmPFC. Mu-opioid-driven increases in PR responding and impairments in DRL performance can be elicited from this area (Selleck *et al*, 2015). Cannulae were placed at an acute angle (19° from vertical) to avoid damage to the medial wall of the cortex and anchored in place with dental acrylic (New Truliner, Skokie, IL) and skull screws (Plastics One, Roanoke, VA). Coordinates for cannulae placements were as follows: anteroposterior, +3.0 mm anterior to bregma; mediolateral, ±2.2 mm from the midline; dorsoventral, -2.7 mm from skull surface (2.5 mm above the final infusion site). Wire stylets (10.1 mm long, 0.008 in. diameter) were placed in the cannulae to prevent blockage. Animals were given intramuscular injections of penicillin (0.3 mL of a 300,000 U/mL suspension; Phoenix Pharmaceuticals, St. Joseph, MO) and buprenorphine (0.35 mL of a 0.03 mg/mL solution; Hospira, Lake Forest, IL), placed in a warm recovery cage, returned to their home cages upon awakening, and given a recovery period of no less than 4 days (with daily handling and health checks) before resumption of behavioral testing.

Microinfusion Procedures and Drugs

For intracranial microinfusions, rats were held gently and stylets removed from the guide cannulae. Stainless steel injectors (fashioned from 33-gauge tubing) were lowered into the brain bilaterally to extend 2.5 mm past the tips of the guide cannulae. Injectors were attached with polyethylene tubing (PE-10; Becton Dickinson, Sparks, MO) to 10 µL-capacity glass Hamilton

syringes, which were mounted on a Harvard Apparatus (Cambridge, MA) microdrive pump. The infusion rate was 0.32 µL/min, and the total infusate volume was 0.5 µL for all experiments. Injectors were left in place for 1 min after the infusion to allow for diffusion of the injectate, whereupon injectors were removed and wire stylets replaced. DAMGO ([D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin), a µ-opioid agonist, was obtained from Bachem, and SCH-23390 (7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol; a D1 specific antagonist) was obtained from Sigma Aldrich. All drugs were dissolved in sterile 0.9% saline immediately before infusions.

Experimental Design

After recovery from surgery, rats were rebaselined on their respective operant tasks (PR-2 or DRL-15 s) under 2-h food restriction, as described above. Upon exhibiting stable baseline responding (no more than ±15% variability over three consecutive testing days), rats were acclimated to the microinjection procedure with sham infusions (injectors lowered into the cannula without any infusate) and intra-vmPFC saline injections on consecutive days. Rats were rebaselined after these injections, whereupon drug testing commenced.

Testing began with all rats receiving an intra-vmPFC infusion of the µ-opioid agonist DAMGO (Pre-SCH-23390; 0.25 µg/0.5 µL), followed by three testing days with intra-vmPFC SCH-23390 infusions (0, 1, or 2 µg/0.5 µL). Rats then underwent three additional testing days where they received intra-vmPFC infusions of a drug cocktail (0.5 µL) containing both DAMGO (0.25 µg) and SCH-23390 (0, 1, or 2 µg). Finally, rats received one last intra-vmPFC DAMGO infusion (Post-SCH-23390; 0.25 µg). The inclusion of three DAMGO-alone testing days (one before any drug cocktails were given, one counter-balanced into cocktail infusions (0 µg SCH +

0.25 µg DAMGO), and one after the completion of cocktails) allowed for the detection of possible shifts in µ-opioid sensitivity in the vmPFC throughout the experiment. For DRL testing, rats were placed into the operant chamber 15 min after infusions in order to ensure that peak drug effects with coincide with the 20 min DRL sessions. For PR testing (which lasted 120 min), rats were placed into the operant chamber immediately after infusion in order to minimize the chance that testing sessions would outlast the duration of drugs. Doses were counterbalanced according to Latin square designs, with two interim days of drug-free testing (under 2-h food deprivation) separating drug infusion days.

Behavioral-Observation Procedure

After recovery from surgery, rats were habituated to clear polycarbonate cages (9.5 in width x 17 in length x 8 in height), identical to the home cages except for wire grid floors. Pre-measured chow pellets were placed on the cage floors, and water was available in overhead water bottles. One hour long habituation sessions were carried out on two sequential days. Drug treatments commenced on the third day, and were identical to those detailed in the Experimental Design section. After infusion, rats were placed in the testing cages and videotaped with a digital camcorder for 60-min sessions. An experimenter blind to treatment viewed the digital files and recorded spontaneous ambulation, rearing, drinking bouts, eating bouts, and grooming bouts using an event recorder interfaced to a PC-based desktop computer.

Histology

At the end of each experiment, rats were deeply anesthetized with isoflurane and perfused transcardially with 0.9% saline solution followed by 10% formalin in phosphate buffer. Brains were collected and stored in 10% formalin. Coronal sections (60 µm) were cut through the

infusion site on a cryostat microtome, collected on slides, stained with cresyl violet, and subsequently reviewed to verify correct placement of the intracortical injections.

Statistical Analyses

Data were analyzed using two-factor repeated measures ANOVAs. Contingent upon significance in the ANOVAs, *post hoc* comparisons among means were conducted using the Student-Newman-Keuls test. The level of statistical significance was set at $P < 0.05$ for all experiments.

RESULTS

Repeated Intra-vmPFC Mu-opioid Agonist Infusions Revealed No Evidence of Shifts in Mu-opioid Receptor Sensitivity Across the Duration of the Study

To assess whether the repeated intra-vmPFC DAMGO treatments of the within-subjects design led to either tolerance or sensitization, we compared DAMGO responses from multiple time points (pre-SCH-23390, 0 µg SCH-23390 + 0.25 µg DAMGO, and post-SCH-23390; see Experimental Design) for each individual experiment (DRL, PR, and behavioral feeding microstructure). There were no detectable changes on DAMGO's effects on DRL measures (efficiency, nosepokes; Fs = 0.84 - 2.06, NS; not shown) or PR measures (active lever presses, inactive lever presses, breakpoint, nosepokes; Fs = 0.18 - 1.13, NS; not shown) over the course of the experiment. There were also no changes in any behaviors measured in the observational paradigm (food intake, eating bouts, ambulation, rears; Fs = 0.25 - 1.48, NS; not shown). Hence, values from the three DAMGO treatments for each animal were averaged for subsequent analysis and presented in figures.

Increases in Progressive Ratio Performance Evoked by Intra-vmPFC Mu-opioid Receptor Stimulation were Blocked by Concurrent Antagonism of Intra-vmPFC D1 DA Receptors.

Overall, DAMGO produced an increase in responding that was reversed dose-dependently by SCH-23390, as evidenced by main effects of DAMGO ($F(1,5) = 19.80$, $P = 0.0067$ for active lever presses; $F(1,5) = 19.43$, $P = 0.007$ for breakpoint), SCH-23390 ($F(2,5) = 8.00$, $P = 0.0084$ for active lever presses; $F(2,5) = 6.445$, $P = 0.0159$ for breakpoint), and DAMGO x SCH-23390 interactions ($F(2,10) = 5.88$, $P = 0.0205$ for active lever presses; $F(2,10) = 5.90$, $P = 0.0203$ for breakpoint; Figures 1a and d). A similar pattern of results was observed for nosepokes (DAMGO: $F(1,5) = 87.94$, $P = 0.0002$; SCH-23390: $F(2,5) = 8.92$, $P = 0.006$; DAMGO x SCH-23390 interaction: $F(2,10) = 17.17$, $P = 0.0006$; Figures 1c). However, inactive lever presses were unaffected (Fs = 0.093-0.527, NS; Figure 1b). *Post hoc* means comparisons indicated that increases in active lever presses, breakpoint, and nosepokes evoked by 0.25 µg DAMGO were dose-dependently attenuated by blocking D1 DA receptors with SCH-23390 (for a summary of specific means comparisons, see Figure 1 legend). Importantly, SCH-23390 had no statistically significant effects in PR in the absence of DAMGO.

Blockade of vmPFC D1 DA Receptors with SCH-23390 Attenuated the Impulsivity Evoked by Intra-vmPFC Mu-opioid Receptor Stimulation

Intra-vmPFC DAMGO infusions produced increases in lever pressing and reduced response efficiency (main effects of DAMGO, lever pressing: $F(1,7) = 17.38$, $P = 0.0042$; efficiency: $F(1,7) = 16.22$, $P = 0.005$; Figures 2a and b). Analyses were conducted on the temporal spacing of lever presses, comparing DAMGO and SCH-23390 doses alone and in all drug cocktail combinations. Responses for each session were grouped according to their interresponse times

(IRT); frequency distributions of responses, expressed for each bin as proportions of total responses, were generated (Andrzejewski *et al*, 2011; Doughty *et al*, 2002). For clarity, IRTs were collapsed into 3-s bins and classified as either *inefficient* or *reinforced* lever presses. Inefficient lever presses consisted of unreinforced, 'premature' responses that did not meet the 15-s IRT requirement, whereas reinforced lever presses were spaced at least 15-s apart. Both DAMGO and SCH-23390 modulated DRL responding in a bin-dependent manner (bin x DAMGO interaction; $F(5,35) = 14.71$, $P < .0001$; bin x SCH-23390 interaction; $F(10,70) = 2.392$, $P = 0.0167$; Figure 2c). Following these multiple interactions with the time-bin factor, data were further analyzed by time bin using ANOVAs followed by Student-Newman-Keuls *post hoc* tests. Significant effects were seen in the 'reinforced response' bin. Rats given 0.25 µg DAMGO showed significantly fewer reinforced responses relative to all other treatment conditions. This reflects a DAMGO-induced reduction in response efficiency (i.e. a smaller proportion of total responses were reinforced), indicating an impulsivity-like effect (Andrzejewski *et al*, 2011; Doughty *et al*, 2002; Sokolowski *et al*, 1994). Most notably, 2 µg SCH-23390 significantly reversed the effect of DAMGO. The 1 µg SCH-23390 dose showed a trend towards reversing the DAMGO-induced deficit, although this did not achieve statistical significance.

Intra-vmPFC D1 DA Receptor Blockade Attenuated Motor Hyperactivity Evoked by Intra-vmPFC Mu-opioid Stimulation

In the behavioral observation paradigm, infusion of 0.25 µg DAMGO into the vmPFC significantly increased ambulation and rearing, which was reversed by concomitant administration of SCH-23390, as evidenced by significant main effects of DAMGO on locomotion, and a strong trend for rearing ($F(1,5) = 15.06$, $P = 0.0116$ for ambulation; $F(1,5) =$

5.71, $P = 0.0624$ for rearing), significant main effects of SCH-23390 ($F(2,10) = 8.74$, $P = 0.0064$ for ambulation; $F(2,10) = 25.15$, $P = 0.0001$ for rearing), and significant DAMGO x SCH-23390 interactions ($F(2,10) = 7.54$, $P = 0.0101$ for ambulation; $F(2,10) = 7.055$, $P = 0.0123$ for rearing; Figures 3a and b). *Post hoc* means comparisons indicated that co-infusion of SCH-23390 reversed the increases in ambulation and rearing evoked by 0.25 μg DAMGO, however, SCH produced no effects on cage-crossing or rearing when given alone. A summary of means comparisons is provided in Figure 3. These observations indicate that increases in spontaneous motor activity evoked by μ-opioid receptor stimulation in the vmPFC require functional vmPFC D1 DA receptor signaling, and that blocking D1 receptors alone (at the SCH-23390 doses used here) does not impair baseline spontaneous motor activity.

Somewhat surprisingly, there were no statistically significant effects in behavioral measures of eating despite multiple previous reports showing robust responses in these measures to intra-vmPFC μ-opioid receptor stimulation (Mena *et al*, 2011; Selleck *et al*, 2015). Upon closer review of the data it was noted that while all animals showed increased motor activity after intra-vmPFC DAMGO infusion, a subset of rats (hereafter referred to as "feeding-responders") appeared to concurrently increase food intake (approximately two-fold) as opposed to the remaining rats (referred to as "non-feeding-responders"). Mean food intake for the feeding-responders showed an evident increase relative to saline controls (saline = 1.3 ± 0.35 g versus DAMGO = 2.7 ± 0.22g). On an exploratory basis, we conducted one-tailed paired t-test comparing saline and 0.25 ug DAMGO responses. DAMGO significantly increased both food intake ($t(3) = -6.25$, $P = 0.004$) and the number of feeding bouts ($t(3) = -7.53$, $P = 0.0024$). These results, along with the abovementioned strong effects in the PR paradigm, generally uphold that

food-directed behavior is increased after intra-vmPFC DAMGO infusion, and that concurrent blockade of prefrontal D1 DA receptors blocks these effects.

Histological Analyses

Representative micrographs of injector placements into vmPFC reveal that placements fell mainly in the infralimbic area of ventromedial PFC (Figure 4). In the behavioral observation experiment, there was no systematic difference in injector placement for any of the rats in feeding-responder versus non-feeding-responder populations. Cannula and injector tracks were clearly visible with no unusual damage to the targeted area.

DISCUSSION

The present findings reveal a novel role for vmPFC DA signaling via D1 receptors in modulating μOR-evoked changes in food-directed motivation and impulsivity. Blockade of intra-vmPFC D1 receptors with SCH-23390 dose-dependently reversed the increases in appetitive food motivation and "impulsivity-like" impairments in DRL response efficiency evoked by vmPFC μOR stimulation with DAMGO. D1 blockade also dose-dependently reduced DAMGO-evoked increases in motor hyperactivity. On its own (absent simultaneous μOR stimulation), D1 blockade did not evoke changes in food motivation, impulsive behavior, or motor activity. Hence, D1 signaling appears to mediate a "permissive" or "enabling" function that allows μ-opioid-elicited effects to be expressed. To our knowledge, this represents the first demonstration of functionally relevant D1/μOR interactions at the level of the PFC, and, moreover, that intra-vmPFC D1 DA receptor signaling is necessary for the expression of intra-vmPFC μ-opioid-elicited functional effects within the cortical system.

The effects of vmPFC D1 antagonism on μ-opioid-elicited behavior do not appear to be an artifact of SCH-23390-induced motor impairments for a number of reasons. First (and most clearly), SCH-23390 did not reduce levels of spontaneous motor behavior (rearing and ambulation measures) below baseline, drug-free levels when given on its own or in conjunction with DAMGO. Furthermore, neither dose of SCH-23390 reduced DAMGO-evoked changes on any operant measure below drug-free baseline levels. This is especially striking for the DRL task, where the highest dose of SCH-23390 returned μOR-evoked decreases in the proportion of reinforced IRTs to baseline levels without changing the number of total lever presses (indicating that animals were more capable of withholding disadvantageous responses). Relatedly, there were no changes across SCH-23390 treatments in inactive lever presses – an indirect measure of general motor activity – on the PR task. These findings suggest that SCH-23390 blocked or attenuated behavioral effects elicited by intra-vmPFC μOR stimulation without affecting normal motor performance on the behavioral measures in the present study. Finally, changes in DAMGO-evoked behavior produced by SCH-23390 were not an artifact of any tolerance or altered sensitivity to repeated injections of DAMGO across the study, because statistical analyses of DAMGO-alone challenges given before, during, and after the series of DAMGO/SCH-23390 infusions showed that the behavioral response to DAMGO did not change across the duration of the experiment.

Several studies have begun to elucidate the mechanisms of PFC D1 function in rats at the cellular and circuit levels, especially in the context of working memory performance (Arnsten and Pliszka, 2011; Floresco and Magyar, 2006; O'Donnell, 2003). The PFC receives DA innervation via a mesocortical projection that arises from the ventral tegmental area (VTA) of the midbrain (Lindvall et al., 1974). Intra-vmPFC DA acts on D1 receptors expressed on pyramidal

cells and in primarily parvalbumin-containing GABAergic interneurons (Gaspar *et al*, 1995; Le Moine and Gaspar, 1998; Santana *et al*, 2009), where it exhibits concentration-dependent effects (Trantham-Davidson *et al*, 2004). At "optimal" levels of cortical DA activity, stimulation of D1 receptors increases firing of fast-spiking interneurons, which in turn suppress spontaneous pyramidal cell activity in the vmPFC (Gorelova *et al*, 2002; Seamans *et al*, 2001b). These same DA concentrations simultaneously activate D1 receptors on pyramidal neurons, resulting in depolarization of the membrane potential and facilitation of cell firing to depolarizing inputs (Gorelova and Yang, 2000; Seamans *et al*, 2001a; Seong and Carter, 2012). This conflux of D1 actions results in pyramidal cells being held in a "up state", a pattern of activity characterized by a sustained membrane depolarization coupled with a suppression of spontaneous cell firing (Lewis and O'Donnell, 2000; O'Donnell, 2003). During these up states, strong inputs are able to overcome heightened tonic inhibition and evoke D1-mediated increases in pyramidal cell firing, which manifest as stable, strongly active stimulus representations that are resistant to interfering inputs and noise (Durstewitz *et al*, 2000; O'Donnell, 2003; Seamans *et al*, 2001a). The DA concentration-dependent nature of D1 actions in the PFC gives rise to an inverted-U dose response function, with optimal D1 stimulation levels providing enhanced signal-to-noise ratio and maximizing performance on PFC-dependent tasks (Vijayraghavan *et al*, 2007).

The cellular and circuitry-based actions of intra-vmPFC μORs are, in comparison to DA D1 receptors, less well understood. Evidence suggests that μ-opioid signaling acts at several points within the cellular network to modulate mPFC response to excitatory input (Baldo, 2016). Endogenous opioid peptides (enkephalins, β-endorphin, and endomorphins) are all found within in the PFC (Ferezou *et al*, 2007; Martin-Schild *et al*, 1999; Taki *et al*, 2000). Immunohistochemistry and single cell PCR studies have shown that μORs are expressed on

GABAergic interneurons primarily expressing vasoactive intestinal peptide (VIP), with little to no expression on pyramidal neurons or upon parvalbumin-containing interneurons on which D1 receptors are found (Ferezou *et al*, 2007; Taki *et al*, 2000). Stimulation of intra-vmPFC μOR inhibits sodium currents in interneurons, decreasing spiking levels and reducing GABAergic miniature inhibitory postsynaptic currents (mIPSC) onto pyramidal cells (Ferezou *et al*, 2007; Qu *et al*, 2015; Witkowski and Szulczyk, 2006), possibly activating pyramidal cells via disinhibition (Tanaka and North, 1994). Application of μOR agonists to slices of PFC tissue also suppresses increases in pyramidal neuron excitatory postsynaptic currents (EPSCs) evoked by 5-HT_{2A} agonists, an effect which may be due to interactions between μOR and 5-HT_{2A} receptors on presynaptic thalamocortical glutamate terminals (Marek and Aghajanian, 1998). Regarding this function, μORs may act to possibly reshape patterns of activation by de-emphasizing thalamic input relative to other incoming signals, including those of limbic origin (Baldo, 2016). More recently, a number of studies have challenged that idea of μOR localization exclusively on interneurons, suggesting that at least some pyramidal cells may also express MORs (Juhasz *et al*, 2008; Olianas *et al*, 2012; Schmidt *et al*, 2003; Schmidt *et al*, 2001). For example, Rola and colleagues (2008) found that application of DAMGO in freshly dispersed mPFC pyramidal neurons modulated high-threshold Ca^{2+} channel currents via protein kinase A (PKA)-mediated activity, an effect that was reversed by naloxone (Rola *et al*, 2008). Presumably, changes in intracellular Ca^{2+} signaling evoked by μOR stimulation could act to alter cell excitability or modulate Ca^{2+}-dependent intracellular signaling pathways, resulting in a reduced threshold for activation either from stronger glutamatergic signaling or from disinhibition after interneuron suppression (Carafoli, 2002). Together, these μOR-mediated effects have the potential to

markedly alter processing within the PFC and increase glutamate release in multiple downstream PFC projection targets responsible for recruiting appetitive behavior (see Baldo, 2016).

The cellular sites of DA D1 receptor and μOR action detailed above suggest possible mechanisms underlying interactions between these two receptor systems in the vmPFC, which in turn could explain the behavioral results we observed in this study. D1/μOR interactions could arise from actions within individual neurons, or between different neurons in a cortical microcircuit. As mentioned above, μORs and D1 receptors are expressed on cortical inhibitory interneurons, though the preponderance of evidence suggests localization of μORs and D1 receptors on non-overlapping interneuron populations (VIP-expressing and fast-spiking parvalbumin-expressing subpopulations, respectively) (Ferezou *et al*, 2007; Le Moine *et al*, 1998; Rudy *et al*, 2011; Taki *et al*, 2000). Hence, given the current state of literature, it does not seem likely that D1-μOR interactions arise through intracellular actions on individual parvalbumin-expressing interneurons. While D1 activity on parvalbumin-containing interneurons would remain unchanged by μOR influences, pyramidal cell firing could be enhanced due to the combined effects of D1-elicited hyperexcitability and a loss of inhibitory tone arising from μOR-mediated suppression of VIP-expressing interneurons (Ferezou *et al*, 2007). In other words, active D1 tone could be required to maintain pyramidal neurons in a state through which μOR-elicited signals could be transmitted.

Another possible mechanism underlying the D1/μOR interaction seen in the current study is direct cross-talk between the two receptors on individual pyramidal cells. Olianas and colleagues (2012) showed that approximately half of D1 receptor-positive cells in the mPFC showed immunoreactivity for μOR. Many of these cells had morphological features of pyramidal neurons, e.g. “triangular cell bodies with apical and basal neurites.” In transfected HEK 293

cells, μ-opioid and D1 receptors form a functionally-active hetero-oligomer complex (Juhasz *et al*, 2008) and in cultured mouse mPFC neurons, stimulation of μORs with DAMGO potentiated the increases in cyclic AMP formation evoked by application of D1-specific agonists (Olianas *et al*, 2012). This action was shown to be dependent on adenylyl cyclase (AC) and PKA. Interestingly, the same study found that stimulation of these μORs in the absence of DA or D1 agonists had no effect, showing that functional D1 signaling was required for μOR-elicited effects to be expressed. Finally, μOR-potentiated D1 signaling increased phosphorylation of AMPA and NMDA receptors in mouse mPFC suggesting that MORs may strengthen DA D1 receptor modulation of glutamatergic neurotransmission in cortical neurons, an essential mechanism for the expression of intra-mPFC synaptic plasticity and appetitive instrumental training (Baldwin *et al*, 2002; Gurden *et al*, 2000; Olianas *et al*, 2012). The evidence detailed above suggests that, in addition to possible microcircuitry interactions among interneurons, interactions between μOR and D1 receptors signaling could also arise through converging regulatory inputs on AC-mediated cyclic AMP formation within individual pyramidal neurons. Clearly, additional work is needed to more thoroughly determine the localization of μ-opioid and D1 receptors in the vmPFC and the bases of their functional interactions. Nevertheless, regardless of the precise mechanism, the present results clearly demonstrate that MORs and D1 receptors interact to modulate vmPFC function, and that D1 receptor signaling appears to play an enabling or permissive role that allows μOR-elicited effects to be expressed.

A growing body of literature suggests that aberrant activity in select frontal areas, including the anterior cingulate cortex (ACC) and vmPFC, contributes to deficits in impulse control in a number of psychiatric disorders characterized by excessive appetitive motivation (Dong *et al*, 2016; Karhunen *et al*, 2000; Schienle *et al*, 2009; Seo *et al*, 2013; Uher *et al*, 2004).

There is some evidence that these deficits could result from supernormal opioid transmission (Blasio *et al*, 2014; Gorelick *et al*, 2008; Love *et al*, 2009; Mitchell *et al*, 2012; Morganstern *et al*, 2012; Selleck *et al*, 2015; Zubieta *et al*, 1996). Accordingly, opioid antagonists have some degree of clinical efficacy across several disorders characterized by loss of control over appetitively motivated behavior (Cambridge *et al*, 2013; Kim *et al*, 2001; Mitchell *et al*, 2007; Volpicelli *et al*, 1992). However, their use in treating binge eating has shown mixed or limited success (McElroy *et al*, 2013; Ziauddeen *et al*, 2013), suggesting that we do not fully understand the complexities of the clinical efficacy of opioid antagonists. Improved knowledge of modulatory influences upon the opioid system may suggest poly-drug strategies to enhance the clinical efficacy of opioid antagonists. For example, the work shown here suggests that simultaneously blocking D1 receptors could amplify the ability of opioid blockers to quell the functional effects of excess opioid transmission. This could lead to improved clinical outcomes in treating orders of impulsive reward-seeking. Further studies are needed to enhance our understanding of opioid- and dopamine-mediation of PFC-based networks in order to develop pharmacological treatments for disorders of inhibitory control over motivated behavior.

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Figure 1

Rats (*n* = 6) treated with intra-ventromedial prefrontal cortex (vmPFC)infusions of the selective D1 antagonist SCH-23390 showed attenuated responses to increases in food motivation elicited by co-infusion of the μ-opioid agonist DAMGO (ᴅ-[Ala2,N-MePhe4, Gly-ol]-enkephalin) in the progressive ratio (PR) task. Intra-vmPFC DAMGO increased responding on the active lever (a), resulting in increased breakpoint (i.e. the last completed ratio, d). Nose-poking was also increased (c). Co-administration of SCH-23390 attenuated increases in active lever pressing (a), breakpoint (d), and nose-poking (c) evoked by treatment with DAMGO alone. There were no effects of any treatment on responding on the inactive lever (b). **P* < 0.05, different from all other treatments; $^{\#}P$ < 0.05, different from all 0.25 μg DAMGO cocktails. Error bars depict 1 SEM.

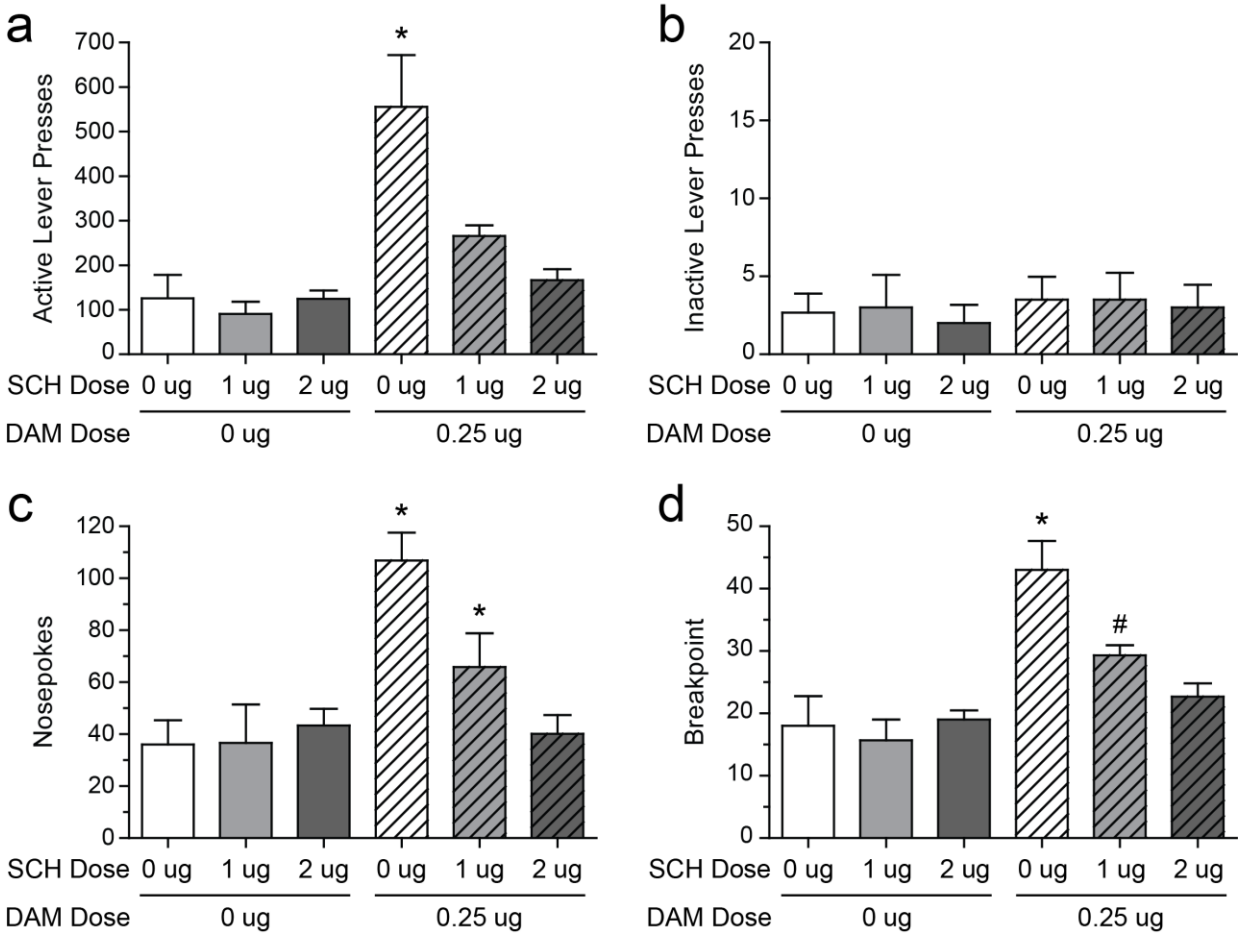


Figure 2

Co-infusion of SCH-23390 into the ventromedial prefrontal cortex (vmPFC) attenuated deficits in inhibitory control evoked by intra-vmPFC DAMGO (D-[Ala2,N-MePhe4, Gly-ol]-enkephalin) infusions in the differential reinforcement of low response rate (DRL) task. Intra-vmPFC DAMGO infusions increased the total number of lever presses emitted (A) and decreased overall response efficiency (B), and these effects were both blocked by SCH-23390. (C) Inter-response times (IRT) analysis revealed that rats (N = 8) treated with intra-vmPFC DAMGO showed a lower proportion of reinforced responses (ie, the 15+ bin) to total responses, and this effect was attenuated by coincident intra-vmPFC infusion of SCH-23390. $^{\$}P < 0.05$, main effect of DAMGO. $^{*}P < 0.05$, different from saline, SCH-23390 alone, and 2 µg SCH-23390/0.25 µg DAMGO cocktail. $^{\#}P < 0.05$, different from both SCH-23390 alone treatments. Error bars depict SEM.

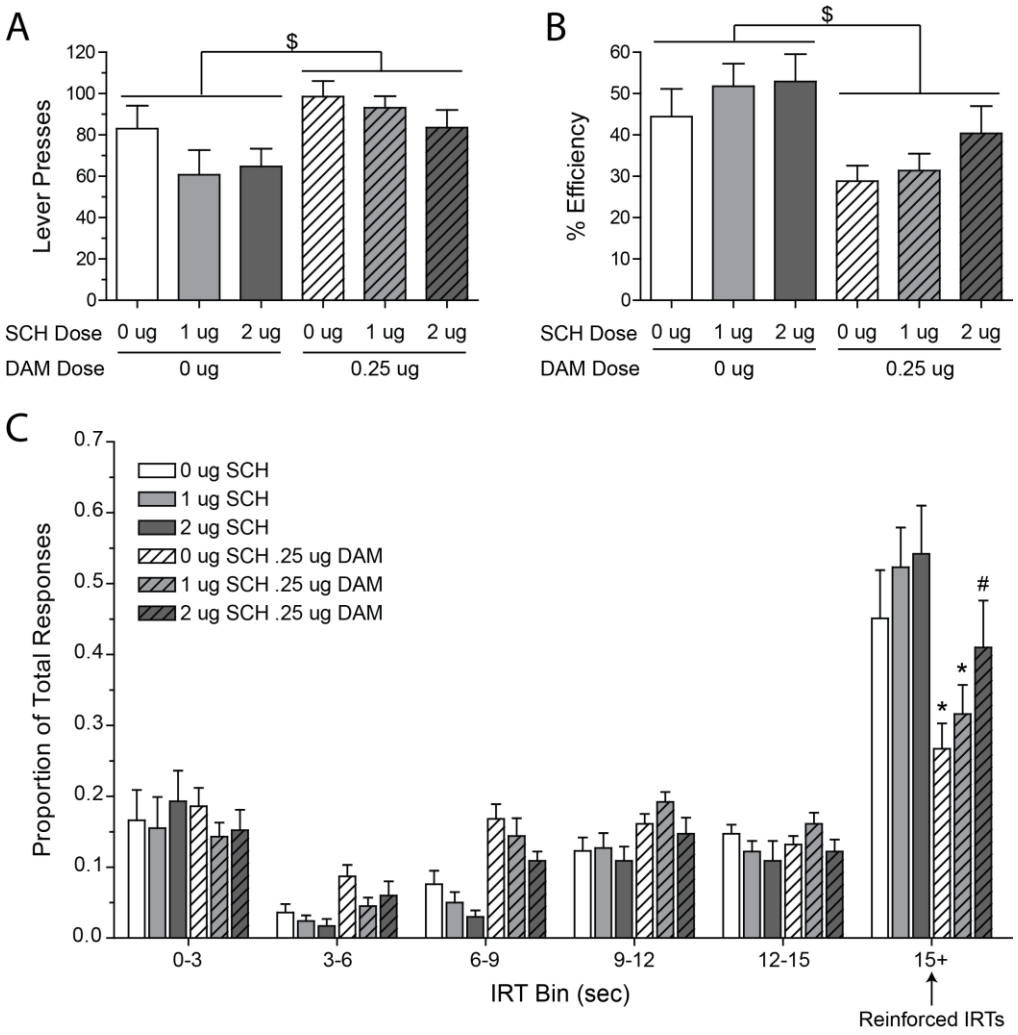


Figure 3

Treatment with intra-ventromedial prefrontal cortex (vmPFC) SCH-23390 blocked increases in spontaneous locomotor activity evoked by coincident infusion of DAMGO (D-[Ala2,N-MePhe4, Gly-ol]-enkephalin). Rats (*N* = 6) receiving intra-vmPFC DAMGO infusions increased horizontal movement (ambulation, a) and vertical movement (rearing, b), which was attenuated by coincident infusion of SCH-23390. **P* < 0.05, different from all other treatments. Error bars depict SEM.

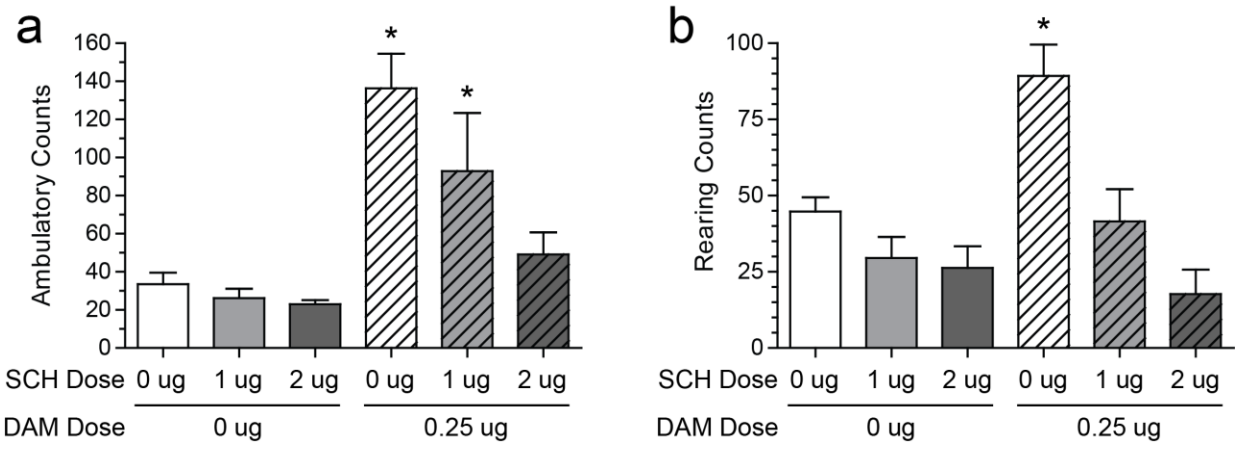
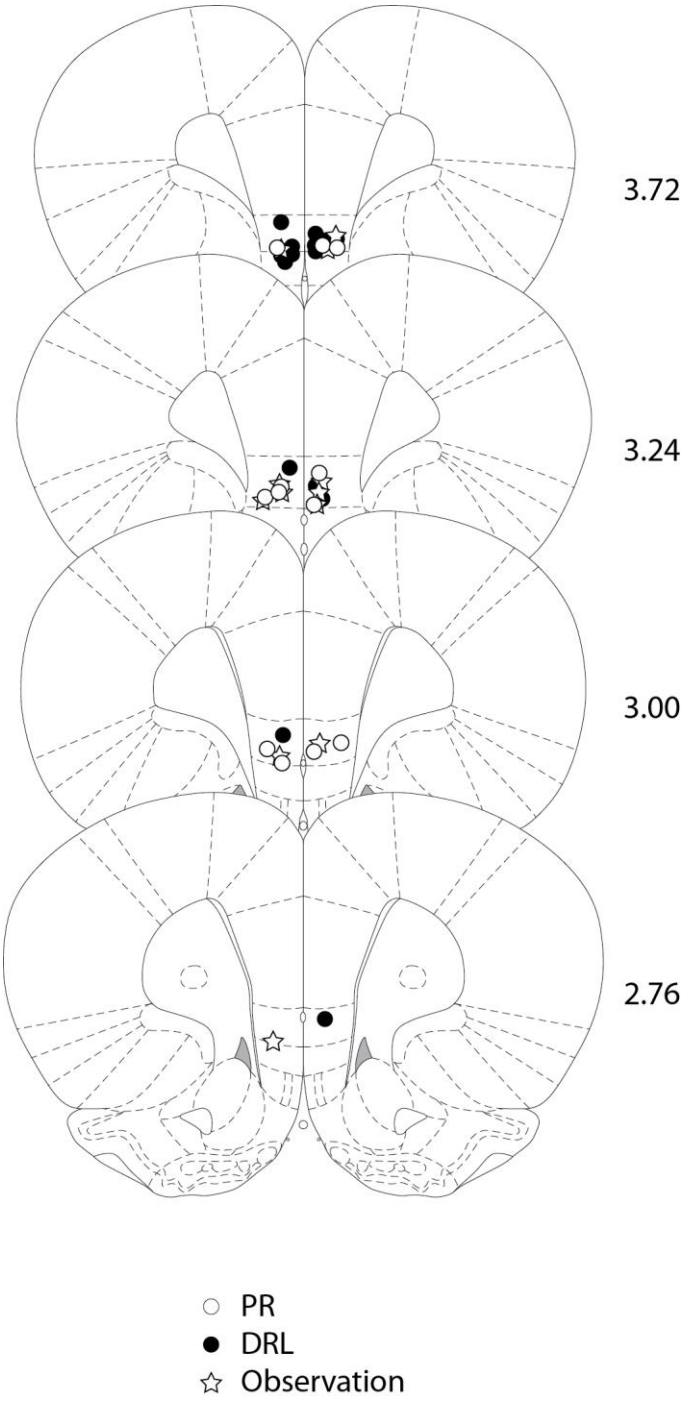


Figure 4

Chartings for injector placements in all experiments. Different shapes (for different experiments) depict the placement of injector tips.



CHAPTER 5

Deficits in inhibitory control over motivated behavior are evoked by μ-opioid stimulation in infralimbic but not prelimbic cortex

The experiments in this chapter were completed in collaboration with Ms. Juliana Giacomini. Technical assistance was provided by Mr. Brandon Buchholtz and Mr. Kenneth Sadeghian. General experimental design, data analysis, and conclusions were developed with the help of my thesis advisor, Dr. Brian A. Baldo. These findings are part of a larger, ongoing neuroanatomical mapping study with expected publication in 2017.

ABSTRACT

The frontal cortex encompasses a number of interrelated regions that interact to monitor and direct behavioral output. We have previously implicated signaling through μ-opioid receptors (μORs) in the ventromedial prefrontal cortex (vmPFC) in the recruitment of feeding processes and the loss of inhibitory control over goal-seeking behavior. However, the vmPFC is a functionally and anatomically dissociable region that comprises both prelimbic (PLC) and infralimbic (ILC) cortices, each with a unique pattern of connectivity and distinct role in modulating executive function. Here we conducted a high-resolution microinfusion mapping study, utilizing smaller gauge microinjectors and reduced infusion volumes, to compare the effects of μOR stimulation in PLC and ILC across two behavioral assays: the differential reinforcement of low rates (DRL) operant paradigm, which assays an animal's ability to suppress disadvantageous prepotent lever response tendencies (a measure of impulsive action), and a behavioral observation paradigm, which measures food approach, unconditioned feeding, and spontaneous motor activity. Stimulating μORs in ILC evoked impairments on DRL performance, whereas PLC μOR stimulation was without effect. Similarly, μOR stimulation in ILC, but not PLC, increased intake of freely available sucrose pellets in the behavioral observation paradigm. These results establish that μOR-elicited effects previously ascribed to the vmPFC as a whole can be more specifically localized to the ILC, and emphasize the importance of ILC-specific projection pathways in recruiting μOR-evoked behavioral responses.

INTRODUCTION

Converging evidence from a number of behavioral and cognitive studies show that individuals diagnosed with binge-type eating disorders show impairments in inhibitory control, which compromises their ability to limit excessive food-directed motivated behavior (Hege *et al*, 2015; Meule and Platte, 2015; Schag *et al*, 2013a; Schag *et al*, 2013b). Studies utilizing human neuroimaging techniques, such as functional magnetic resonance imaging (fMRI) or positron emission tomography (PET), have shown an association between food-directed impulsive tendencies and aberrant activity in frontal cortical areas (Filbey *et al*, 2012; Lock *et al*, 2011; Schienle *et al*, 2009). The frontal areas involved encompass a number of distinct yet interacting regions, including anterior insular (AIC), orbitofrontal (OFC), anterior cingulate (ACC) and medial prefrontal cortices (PFC). One of the primary collective functions of these regions is to provide a mechanism to monitor reward-related inputs, associate them with relevant stimuli to produce representations of expected reward value, and use these representations to guide emotional/reward-related behavioral output (Grabenhorst and Rolls, 2011; Rolls and Grabenhorst, 2008). Our lab has identified μ-opioid signaling in the ventromedial PFC (vmPFC) as a particularly important modulator of impulsive reward-seeking behavioral states. Stimulation of vmPFC μ-opioid receptors increases premature, inefficient responding in a sucrose-reinforced differential reinforcement of low-rates (DRL) task, which requires the animal to temporarily withhold a disadvantageous prepotent lever response (Selleck *et al*, 2015, see Chapter 2). During a naturally impulsive behavioral state (prolonged food deprivation), opioid antagonists in the vmPFC restore patterns of responding on the DRL task to near normal levels (Selleck *et al*, 2015; see Chapter 2). Hence, μ-opioid signaling in the vmPFC is both necessary and sufficient to evoke impulsive, motivated behavior directed towards food.

Though we previously localized our findings to the vmPFC as opposed to more dorsal sites, such as ACC, it is worth noting that the brain regions that constitute the vmPFC are not universally agreed upon, and those typically included within the region are anatomically and functionally dissociable. Two structures commonly included within the vmPFC, the prelimbic (PLC) and infralimbic (ILC) cortices, have unique sets of afferent and efferent projections. While the PLC is connected reciprocally with a wide variety of cortical structures, including ILC, AIC, and medial OFC in addition to entorhinal cortex and the hippocampal formation (HF), cortical connections with ILC are much more limited, arising primarily from PLC and to a lesser extent dorsal AIC and the HF (Conde *et al*, 1995; Hoover and Vertes, 2007; Vertes, 2004). PLC and ILC also show very different projection patterns to structures previously shown to be important in modulating behavioral output evoked by μOR stimulation of the vmPFC – including feeding-modulatory sites such as the nucleus accumbens (Acb) and lateral hypothalamus (LH) (Mena *et al*, 2013). Fibers originating from PLC distribute throughout the extent of the Acb, with a preference toward core rather than shell terminations, whereas ILC projections primarily target the medial Acb shell (AcbSh; Berendse *et al*, 1992; Gabbott *et al*, 2005; Hurley *et al*, 1991; McGeorge and Faull, 1989; Vertes, 2004; Voorn *et al*, 2004). In contrast, ILC fibers densely innervate the LH, whereas PLC fibers primarily traverse the region en route to the brainstem, with only light terminations in LH (Gabbott *et al*, 2005; Vertes, 2004). Based on these projection patterns, PLC and ILC are often differentiated through their involvement in cognitive/limbic versus affective/visceromotor functions, respectively (Hoover *et al*, 2007; Vertes, 2004).

In accordance with their different projection patterns, PLC and ILC have been shown to play distinct and sometimes opposing roles in modulating executive control; for example, in studies examining the cognitive control over conditioned fear and reward-seeking behaviors

(Gourley and Taylor, 2016; Moorman *et al*, 2015; Peters *et al*, 2009). Moreover, the psychostimulant methylphenidate displays efficacy in altering working memory performance in dorsal PLC, but not ILC (Spencer *et al*, 2012), whereas blockade of NMDA receptors in ILC, but not medial PLC, increases premature responding on the 5-choice serial reaction time task (5CSRTT; Benn and Robinson, 2014) Given these previously-established between-site differences, a more targeted exploration of μOR stimulation specifically in PLC and ILC could provide valuable information about what behavioral processes are mediated by each subregion. Furthermore, an overlay of subregion-specific anatomical projections onto cortical sites displaying μOR-evoked changes in feeding behavior could provide valuable insight into the projection pathways through which cortical μ-opioid systems exert their effects.

A meta-review of our prior vmPFC μOR studies shows that our anatomical injector placements have clustered within ILC, though they have ranged from dorsal PLC to dorsal pedunculary cortex (Mena *et al*, 2011; Mena *et al*, 2013; Selleck *et al*, 2015; see Chapters 2-4). Interestingly, initial microinfusion studies detailing μOR effects on feeding and motor hyperactivity had a larger anatomical spread (dorsal PLC to ILC) than more recent DRL studies (tightly clustered in ILC). However, direct comparisons between studies are complicated by the former use of a relatively large infusion volume (0.5 μL), which likely results in a larger functional area of drug activation (Stukel *et al*, 2008). Furthermore, our recent transition to smaller gauge cannula and microinjector tubing has resulted in reduced tissue damage during microinfusions relative to earlier studies focusing primarily on eating effects (Mena *et al*, 2011; Mena *et al*, 2013). Our recent use of these more refined techniques has resulted in a smaller area of compromised tissue integrity and presumably a more limited area of drug-evoked action in the brain. Our earlier studies have shown robust μOR involvement in the recruitment of feeding and

motivational processes. The small but significant methodological changes to be used in the present study make it easier to precisely determine the range of vmPFC sites that display μOR stimulation-evoked behavioral effects and to establish whether μOR-elicited changes in feeding and impulsive action are evoked by overlapping cortical sites.

Hence, to address whether opioid modulation of feeding and inhibitory control processes are differentially regulated by PLC and ILC, we undertook a high-resolution microinfusion mapping study in PLC and ILC cortices using a specific μOR agonist, DAMGO, across two behavioral assays: DRL and a behavioral observation paradigm, which measures food approach, unconditioned feeding, and spontaneous motor activity. We were particularly interested in comparing and contrasting μOR-sensitive areas of vmPFC that increase feeding and motor activity with those that evoke impairments in inhibitory control over motivated behavior. While the more dorsally-located placements our earlier studies suggest that μOR-induced increases in feeding behavior may be elicited throughout both PLC and ILC, the heavily ILC-localized placements in our DRL studies, paired with previously described findings showing ILC-specific effects of premature responding after NMDA antagonist administration (Benn *et al*, 2014), we predict that μOR stimulation will preferentially impair DRL performance when infused specifically into ILC, but that feeding will be more anatomically widespread.

METHODS

Subjects: Subjects were male Sprague-Dawley rats (Envigo; Madison, WI), weighing 275-300 g upon arrival in the laboratory. Rats were pair-housed in clear polycarbonate cages (9.5-in. width X 17-in. length X 8-in. height), with cob bedding, in a light- and temperature-controlled vivarium. Animals were maintained under a 12:12 h light/dark cycle (lights on at 0700 h). Food and water were available *ad libitum*, except as indicated for various experiments. Animals were

handled gently daily to reduce stress. Testing occurred between 1100 and 1600 h, during the light phase of the animals' dark/light cycle. All procedures were evaluated and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison, and are in accordance with the guidelines promulgated in *the NIH Guide for the Care and Use of Laboratory Animals*. Facilities have been approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

Operant-Behavior Procedures: Operant training and testing procedures were conducted in standard operant chambers made of sheet aluminum and Plexiglass, enclosed within ventilated, sound-attenuated chests. Ventilation fans in the chambers provided masking noise continuously throughout each session. One wall of the chamber contained two retractable levers spaced 6 cm apart. Space equally between the levers was a pellet receptacle into which 45 mg sucrose pellets (BioServ; Frenchtown, NJ) could be delivered from an automatic pellet dispenser. Above the receptacle was a row of three stimulus lights (red, yellow, and green) and a 28 V house light. Experiments were controlled, and behavioral input recorded, on a PC-based computer running MedPC IV for Windows (Med Associates, St. Alban, VT).

After acclimation to the housing facility, rats underwent an initial training period during which they were maintained at 90 ± 2% of free-feeding body weight using scheduled feedings. During this initial phase, rats were trained to lever press on a conjoint random-time 30 s/fixed ratio 1 schedule (RT-30 s/FR-1), in which a sucrose pellet was delivered on average every 30 s regardless of the rats' behavior while single lever presses also resulted in a sucrose pellet delivery. Once the rats were reliably retrieving all pellets during the session (typically within 2-3 days), the RT-30 s component was removed. Hence, at this point all rats were responding on an FR-1 schedule.

Once consistent responding was achieved on FR-1, rats advanced to a variable-interval 15 s schedule (VI-15 s), then to a VI-30 schedule, and finally to a DRL-15 s schedule. In DRL schedules, after a reinforcer is earned rats are required to withhold responding during an unsignaled, fixed time period (15 s for a DRL-15 schedule). Once this delay interval has elapsed, the subject can then respond to earn the next reinforcer. However, each time a 'premature' response is emitted (i.e. one that is not separated from the previous response by at least 15 s), the delay timer is reset. To achieve optimal performance, therefore, the timing of consecutive responses (interresponse intervals (IRTs)) must exceed the delay interval. Successful performance in DRL schedules is thought to require intact executive processes of inhibitory control (Andrzejewski *et al*, 2011; Doughty and Richards, 2002; Sokolowski and Salamone, 1994). After 2-3 days on DRL, rats were returned to *ad libitum* feeding, with 2 h food restriction immediately before each testing session. Rats were then rebaselined under this new food restriction schedule. DRL sessions lasted 20 min and were initiated by the subject's first response.

Surgical Procedures: Rats (weighing 300-330 g at the time of surgery) were anesthetized with Isofluorane gas and secured in a Kopf stereotaxic frame. The toothbar was set at –4.0 mm below the interaural line. Bilateral stainless steel cannulas (10 mm long, 23 gauge) were implanted targeting ILC or PLC according to standard stereotaxic procedures. Coordinates of the injection site were (ILC) AP: +3.0 mm from bregma, ML: ±2.0 mm from midline, DV: -5.2 mm from skull surface and (PLC) AP: +3.0 mm from bregma, ML: ±2.3 mm from midline, DV: -3.7 mm from skull surface. ILC cannulas were angled at 19 degrees and PLC cannulas at 23 degrees from vertical to avoid damage to structures along the medial wall of the PFC (specifically the anterior cingulate cortex). Cannulas were fixed in place 2.5 mm or 1.0 mm above the target sites

(ILC and PLC, respectively) with dental acrylic (New Truliner, Skokie, IL) and anchoring skull screws (Plastics One, Roanoke, VA). Wire stylets (10.1 mm long, 30 gauge) were placed in the cannulas to prevent blockage. Rats were given an intramuscular injection of penicillin (0.3 ml of a 300,000 unit/µl suspension; Phoenix Pharmaceuticals. St. Joseph, MO) and subcutaneous infusion of the non-steroidal anti-inflammatory drug meloxicam (2 mg/kg; Boehringer Ingelheim Vetmedica, St. Joseph, MO) before being placed in heated recovery cages. Upon awakening rats were returned to their home cages and given a recovery period of no less than five days (with daily health checks), with supplementary subcutaneous infusions of meloxicam (2 mg/kg) the day after surgery. Once normal recovery was confirmed, rats were returned to a schedule of daily PR-2 testing. Upon achieving stable baselines, drug infusions commenced.

Microinfusion Procedures and Drugs: For intracerebral microinfusions, rats were held gently, and stylets removed from the guide cannula. Stainless steel injectors, 12.5 mm (ILC) or 11.0 mm (PLC) long, were connected via polyethylene tubing (PE-10, Becton Dickinson, Sparks, MD) to 10-µl capacity Hamilton syringes (Hamilton, Reno, NV) on a Harvard microdrive pump. All injectors extended beyond the 10.0 mm guide cannula to reach final infusion site. The flow rate for infusions was 0.32ul/min. The total infusate volume for the bilateral infusions was 0.25ul/side. DAMGO ([D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin), a µ-opioid agonist, was obtained from Bachem. After infusions, injectors were left in place for an additional minute to allow for diffusion of the injectate into the tissue. Injectors were then removed, and wire stylets replaced.

Experimental Design: After recovery from surgery, rats were rebaselined on DRL-15 under 2-h food restriction, as described above. Upon exhibiting stable baseline responding (no more than ±15% variability over three consecutive testing days), rats were acclimated to the microinjection

procedure with sham infusions (injectors lowered into the cannula without any infusate) and intra-ILC or intra-PLC saline injections on consecutive days. Rats were rebaselined after these injections, whereupon drug testing commenced.

During the DRL testing phase, rats received four intra-ILC or intra-PLC infusions of DAMGO (0, 0.1, 0.3, or 1.0 µg/0.25 µL). Rats were placed into the operant chamber 15 min after infusions in order to ensure that peak drug effects coincided with the 20 min DRL sessions. Doses were counterbalanced according to Latin square designs, with two interim days of drug-free testing (under 2-h food deprivation) separating drug infusion days.

Behavioral-Observation Procedure: Upon completion of the DRL testing phase, rats were habituated to clear polycarbonate cages (9.5 in width x 17 in length x 8 in height), identical to the home cages except for wire grid floors. Jars containing pre-measured sucrose pellets (45 mg; identical to those used in the DRL procedure) were fastened to the cage floors, and water was available in overhead water bottles. One hour long habituation sessions were carried out on two sequential days. Drug treatments commenced on the third day, and were identical to those detailed in the Experimental Design section. After infusion, rats were placed in the testing cages and videotaped with a digital camcorder for 60-min sessions. An experimenter blind to treatment viewed the digital files and recorded spontaneous ambulation, rearing, drinking bouts, eating bouts, and grooming bouts using an event recorder interfaced to a PC-based desktop computer.

Verification of placements: At the end of the experiment, rats were deeply anesthetized with Isofluorane and perfused transcardially with a 0.9% saline solution followed by 10% formalin in phosphate buffer. Brains were collected and stored in 10% formalin. Coronal sections (60 µm) were cut through the infusion sites on a cryostat microtome, collected on slides, stained with

cresyl violet, and subsequently reviewed to verify correct placement of the intracerebral injections.

Statistical analyses: Data were analyzed using two-factor repeated measures ANOVAs. Contingent upon significance in the ANOVAs, *post hoc* comparisons among means were conducted using the Student-Newman-Keuls test. The level of statistical significance was set at $P < 0.05$ for all experiments.

RESULTS

Stimulation of Mu-opioid Receptors in Infralimbic Cortex, but not Prelimbic Cortex, Elicited Increases in Impulsive Action

Prior to analysis, we calculated response efficiency ratios by dividing the number of reinforced lever presses by the total number of presses (ie. reinforced + unreinforced) for each session, and expressed them as percentages in resulting figures. Overall, DAMGO induced a site-specific reduction in response efficiency, as evidenced by a main effect of DAMGO ($F(3,24) = 3.72$, $P = 0.025$) and a DAMGO x SITE interaction ($F(3,24) = 3.94$, $P = 0.0203$), as well as a site-specific effect on total lever pressing (DAMGO x SITE interaction ($F(3,24) = 3.04$, $P = 0.0486$). Following these two-way interactions, data were further analyzed using separate ANOVAs by site.

For ILC, DAMGO significantly reduced response efficiency ($F(3,9) = 4.22$, $P = 0.0404$) and produced notable trends toward significance on total lever presses ($F(3,9) = 3.121$, $P = 0.0807$) and premature responses ($F(3,9) = 3.721$, $P = 0.0546$). There were no effects on successful responses or nose-poking into the magazine (Fs = 1.3-1.9, not significant (NS)). SNK post hoc tests showed that, at $0.05 < \alpha < 0.1$, 1.0 µg DAMGO significantly impaired response

efficiency relative to saline and 0.1 ug DAMGO (Fig. 1). For PLC, there were no significant results or notable trends toward significance on any measure (Fs = 0.2-2.5, NS, Fig 1).

Mu-opioid Receptor Stimulation in Infralimbic Cortex, but not Prelimbic Cortex, Increases Sucrose Intake

DAMGO had a site-specific effect on sucrose intake in the behavioral observation experiment, as evidenced by a robust DAMGO x SITE interaction ($F(3,24) = 4.82$, $P = 0.0091$). There were no main effects of SITE or DAMGO (Fs = 0.4-1.22, NS). As stated above, data were further analyzed by site using individual ANOVAs followed by SNK post hoc tests. DAMGO increased sucrose intake in ILC ($F(3,9) = 5.52$, $P = 0.0199$; Fig. 2A). SNK tests showed that both the 0.3 µg and 1.0 µg DAMGO doses increased sucrose consumption relative to saline. In contrast, DAMGO had no effect on sucrose intake when injected into PLC ($F(3,15) = 0.28$, NS; Fig 2B).

Analysis of Injector Placements in Infralimbic and Prelimbic Cortices

As shown in Figure 3, Placements for both ILC and PLC fell completely within the anatomical boundaries of their respective regions. Cannula and injector tracks were clearly visible with no unusual damage to the targeted area.

DISCUSSION

The present findings demonstrate that increases in impulsive action and food consumption previously shown to be elicited by stimulation of vmPFC µ-opioid systems is mediated by µORs in ILC, but not PLC. Stimulating µORs in ILC produced significant impairments in DRL response efficiency, with strong trends toward increases in total and premature responding – a

pattern of responding that strongly resembles those seen in earlier explorations of vmPFC μOR-mediated effects on DRL performance (Selleck *et al*, 2015; also see Chapter 4). In contrast to ILC-localized results, stimulation of PLC-localized μOR receptors had no effect on DRL responding. ILC μOR stimulation robustly increased sugar consumption, whereas, again, PLC μOR stimulation had no effect. To our knowledge, this is the first demonstration that increases in impulsive action and feeding behavior previously ascribed to vmPFC μ-opioid stimulation can actually be further restricted to μOR-mediated activity solely in ILC.

Though these results are consistent with our prior findings exploring μOR-mediated effects in vmPFC, caution is warranted in their interpretation. Most notably, the sample size for the ILC placement group is small and underpowered (n = 4). A large number of subjects from the ILC group were dropped from the study because of infections, clogged cannulae, and a number of midline breaches (i.e. an off-center placement caused the injector from one side to cross the medial wall). Nevertheless, stimulation of ILC-localized μORs still elicited a DRL performance deficit and increase in sucrose intake sufficiently robust to achieve statistical significance. Planned additions to the dataset will improve statistical power and enable full detection of these effects.

It should be noted that the baseline (0 μg DAMGO) level of eating for the PLC placement group was unusually high (mean = 3.55 g consumed compared to ILC mean = 1.28 g). While the cause of this abnormally high level of baseline eating is unknown, it is unlikely to result in ceiling effects in feeding, as we have previously found that μ-opioid stimulation in the Acb shell elicits much higher levels of food consumption (>10 g) in a similar time period (Zhang and Kelley, 2000). While the conclusions we draw from this study can be strengthened by ensuring that it is properly powered, the consistency between our results and existing findings in

the literature give us confidence that these effects will be strengthened as additional subjects are added to the data set.

Our finding that μOR stimulation in ILC, but not PLC, disrupts inhibitory control over motivated behavior is in agreement with previous literature showing that ILC plays an important role in the suppression of extinguished, unrewarded, or prepotent response tendencies, whereas PLC is involved in expressing goal-directed response strategies (Gourley *et al*, 2016). ILC activity is required for the inhibition of unreinforced actions on a cued appetitive task, likely through excitatory connections to AcbSh neurons that tonically inhibit or limit reward seeking by suppressing activity in the LH (Ghazizadeh *et al*, 2012; Stratford and Kelley, 1999). Perturbation of normal ILC activity, via lesions, NMDA blockade, or infusion of GABAergic agonists, increases premature responding on the 5CSRTT, possibly though the disruption of ILC input to these "limiter" AcbSh neurons (Chudasama *et al*, 2003; Ghazizadeh *et al*, 2012; Murphy *et al*, 2012). Moreover, we have previously shown that glutamatergic processes in the AcbSh also oppose increases in appetitive motivation evoked by vmPFC (likely ILC) μOR stimulation (Mena *et al*, 2013; see Chapter 3). The "limiting" actions of ILC-AcbSh projections play an important role in providing negative feedback onto subcortical circuitry consisting of feeding-related structures (including AcbSh and LH) and their projections onto brainstem behavioral pattern generators and neuromodulator systems (Thompson and Swanson, 2010).

Regarding food intake, our initial studies showed that μOR stimulation in vmPFC subregions ranging from dorsal PLC to dorsal ILC can evoke increases in feeding (Mena *et al*, 2011); however, the current study, utilizing a more refined, high-resolution microinfusion mapping technique, shows that this effect is in fact completely restricted to the ILC. This suggests that previously described μOR-induced feeding evoked by PLC microinfusions is likely

due to drug diffusion into μOR-active feeding sites in ILC. This more anatomically-restricted range of feeding-related μOR-sensitivity, when overlaid upon known patterns of ILC efferent projections, may suggest anatomical insights into the ILC modulation of feeding and food impulsivity. As previously discussed, ILC projects more specifically to AcbSh and LH compared to PLC. We have previously shown that increases in feeding evoked by intra-vmPFC DAMGO stimulation are blocked by glutamate receptor blockade in the LH (Mena *et al*, 2013). Unknown, however, are the precise pathways through which this dual site interaction occurs. μOR signals, arising from ILC, could reach the LH through a number of different projection pathways. As previously mentioned, the vmPFC sends a substantial projection, arising primarily from ILC, directly to the LH (Gabbott *et al*, 2005; Reppucci and Petrovich, 2016; Vertes, 2004), and could therefore directly increase LH glutamate levels and recruit feeding- and arousal-related peptide systems, such as the hypocretin/orexin system (Estabrooke *et al*, 2001; Mena *et al*, 2013; Tsujino and Sakurai, 2009). However, ILC-elicited feeding responses could also be recruited via strong, D1 dopamine (DA) receptor-containing projections to the basolateral amygdala (BLA) (Land *et al*, 2014; McDonald, 1998; McDonald *et al*, 1996; Vertes, 2004). Optogenetic stimulation of D1-containing BLA projection neurons (located in both PLC and ILC) drives food intake, presumably via a two-stage circuit terminating in the LH (Land *et al*, 2014; McDonald *et al*, 1996; Reppucci *et al*, 2016). Moreover, we have previously shown that intra-vmPFC μOR-evoked increases in food-seeking behavior require intact transmission through vmPFC D1 receptors (see Chapter 4), providing indirect support for the BLA route of control. There is, however, some evidence of D1 receptors on ILC-LH projection neurons as well, though they are not as prominent as in BLA projections (Land *et al*, 2014). It should be noted that the LHA receives converging input from both BLA and ILC, which are themselves strongly

interconnected (Hoover *et al*, 2007; Hurley *et al*, 1991; Reppucci *et al*, 2016; Vertes, 2004). Regardless of the precise pathway, the present findings showing that increases in feeding can be elicited by μOR stimulation in ILC but not PLC, coupled with a high degree of overlap with ILC-LH projection neurons (Gabbott *et al*, 2005), provide indirect support for an ILC-LH functional interaction, in a general sense. Future studies utilizing chemogenetic methodology (such as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)) can more directly assess the individual contributions of ILC-LH and ILC-BLA projection pathways to the ILC μ-opioid behavioral feeding profile.

The present results demonstrate the importance of high resolution microinfusion mapping studies, as a more precise anatomical localization of behavioral and neurochemical effects can help determine which macro- and microcircuits are involved in their generation, as discussed above. The substantial differences in μOR-modulation of feeding and inhibitory control shown here between dorsal (PLC) and ventral (ILC) aspects of vmPFC suggest that other frontal areas, such as OFC and AIC, may also play distinct functional roles in modulating feeding- and reward-related processes. Indeed, we have previously shown that μOR stimulation in ventral OFC (vOFC) also elicits feeding behavior (Mena *et al*, 2011); however, it is still unknown whether intra-vOFC μOR-elicited feeding increases are accompanied by impairments in inhibitory control. Future studies directly addressing this question in this and other frontal sites can greatly expand our understanding of frontal executive processes governing reward-related behaviors.

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Figure 1. Rats treated with infusions of DAMGO into infralimbic cortex (**A**), but not prelimbic cortex (**B**), showed increased impulsivity in the DRL task. Intra-ILC DAMGO infusions showed a significant trend toward impaired response efficiency, while intra-PLC DAMGO infusions had no effect. There were no significant effects of lever pressing or premature responding for either brain site. #0.05<P< 0.1, trending towards significance compared to saline (0 ug) and 0.1 ug DAMGO. Error bars depict one S.E.M.

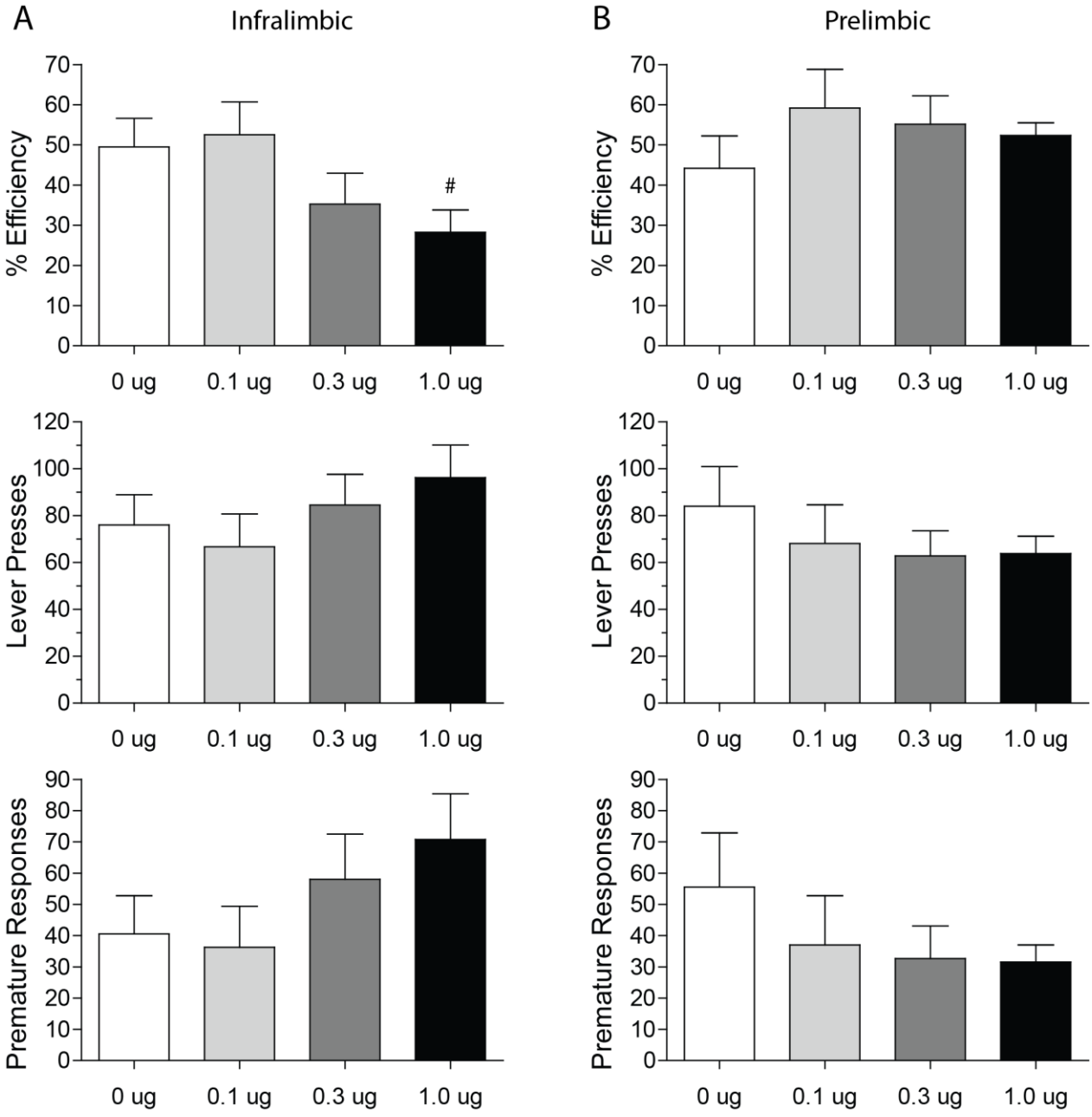


Figure 2. Rats treated with infusions of DAMGO into infralimbic cortex (**A**), but not prelimbic cortex (**B**), increased sucrose consumption during free feeding. *P<0.05, different from saline (0 ug). Error bars depict one S.E.M.

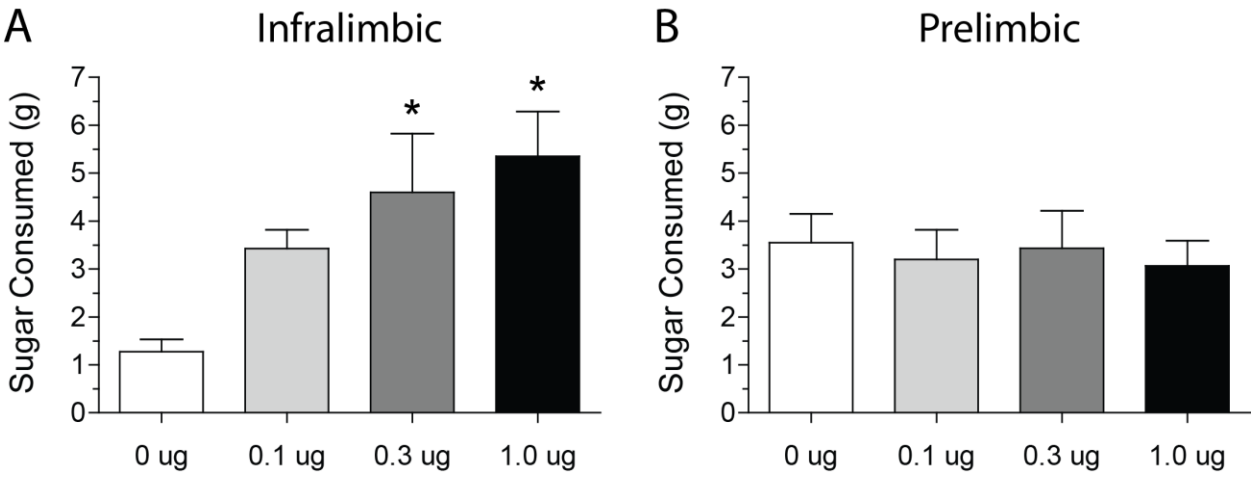
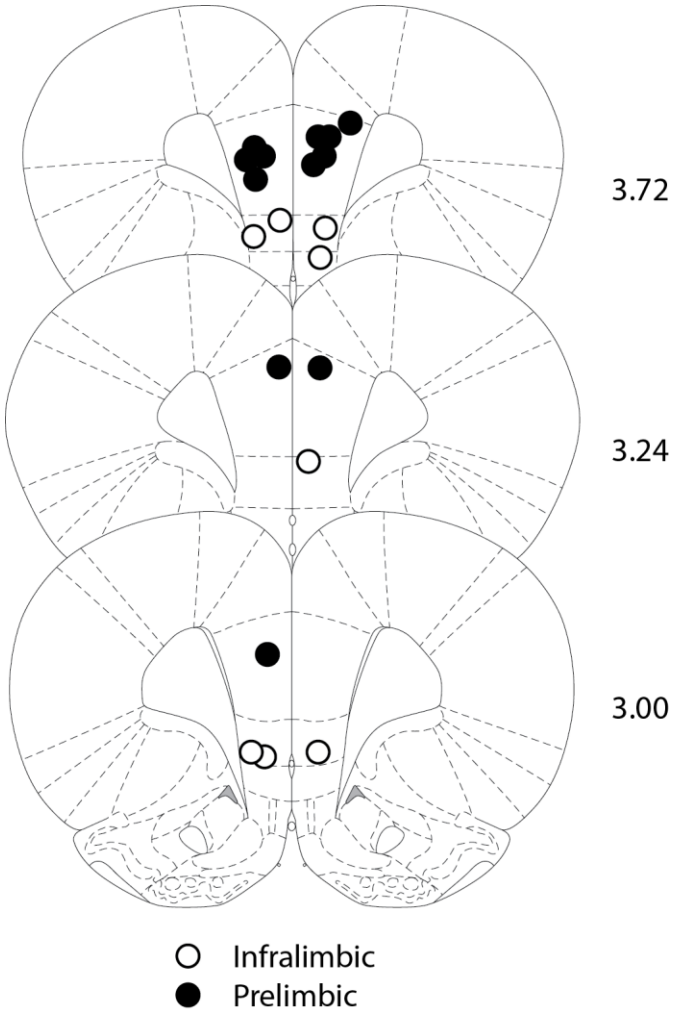


Figure 3. Chartings for injector placements in all experiments. Filled circles represent placements in prelimbic cortex, whereas empty circles represent placements in infralimbic cortex.



CHAPTER 6

GENERAL DISCUSSION

1. SUMMARY OF RESULTS

The findings presented in this thesis establish a role for μ-opioid receptors in the ventromedial prefrontal cortex in mediating inhibitory control over feeding behavior. Stimulation of PFC-localized μ-opioid receptors simultaneously increases appetitive behavior directed toward the acquisition of food and impairs the ability of an animal to inhibit food-directed motivation. These effects are not reproduced by increasing monoamine levels in the PFC (in the absence of pharmacological μ-opioid stimulation), though the expression of μ-opioid-mediated effects requires intact signaling through at least one monoamine receptor system. Collectively, these findings demonstrate a functionally unique role of μ-opioid receptor signaling in the ventromedial prefrontal cortex in modulating frontal control over motivated behavior and represent an initial step in integrating prefrontal opioid signaling into existing models of neuromodulatory influences upon frontal executive function.

In Chapter 2, we described the role of opioid transmission in the vmPFC in modulating both appetitive motivation and inhibitory control over food-directed behavior, and how this behavioral profile is both pharmacologically specific to the μ-opioid system (compared to the monoamines) and physiologically relevant during periods of prolonged food deprivation. Hungry animals become impulsive and highly motivated to obtain available food; we showed that both of these behaviors are dependent upon functional opioid signaling in the vmPFC. Moreover, when μ-opioid receptors were stimulated in low-hunger rats, they showed similar impairments in impulse control and increases in food-seeking behavior to severely food deprived rats. Finally, increasing monoamine levels in the vmPFC failed to recreate this behavioral profile. These results suggest a specialized role of μ-opioid receptors within the region on mediating food-directed, impulsive behavior.

In Chapter 3, we showed that increases in appetitive motivation induced by DAMGO infusion directly into the vmPFC are opposed by glutamatergic signaling through AMPA-type receptors in the AcbSh. Intra-vmPFC DAMGO-evoked increases in responding for sucrose reward were potentiated by AMPA-specific antagonists in the AcbSh. Moreover, stimulation of AMPA-type glutamate receptors in the AcbSh decreased appetitive motivation and degraded stimulus discrimination. The ability of glutamate signaling through AMPA receptors within the Acb shell to modulate changes in appetitive behavior generated by 'upstream' brain structures is particularly interesting, as AMPA signaling in the structure has previously been shown to inversely impact feeding behavior without concomitant changes in food-seeking behavior (Zhang *et al*, 2003). These findings implicate projections from the vmPFC to the AcbSh in the limiting of food-directed behavior, a process which possibly involves inhibitory connections to the lateral hypothalamus.

In Chapter 4, we demonstrated that increases in impulsive action, appetitive motivation and spontaneous motor activity evoked by μ-opioid receptor stimulation in the vmPFC are dependent upon intact signaling through dopamine D1-type receptors. Simultaneous blockade of vmPFC D1 receptors resulted in a dose-dependent reversal in DAMGO-induced measures of binge-like behavior and motor activity, though there were no detectable changes in operant responding or motor activity when D1 antagonists were infused alone. These results show that D1 signaling in the vmPFC is required for the expression of behavioral effects evoked by vmPFC opioid signaling, and that prefrontal D1 receptors play an enabling or permissive role in allowing μ-opioid receptor-evoked effects to be expressed. Moreover, the demonstration of functional interactions between μ-opioid and D1 receptors provides an initial route for integrating μ-opioid signaling into models of monoamine neuromodulation of the PFC.

In Chapter 5, we showed that a more anatomically-restricted range of μ-opioid sensitivity within the vmPFC – specifically within the infralimbic cortex, relative to the prelimbic area – is responsible for DAMGO-induced changes in inhibitory control and feeding behavior. DAMGO infused specifically into the prelimbic cortex had no effect on impulsive action or feeding behavior. These results begin to provide a more detailed map of μ-opioid-sensitive zones in the frontal cortex. Specifically, results from this study suggest that μ-opioid-elicited effects on inhibitory control and hyperphagia previously attributed to the vmPFC can be more accurately localized to the infralimbic cortex, and emphasize the importance of ILC-specific projection pathways in the recruitment of prefrontal μ-opioid-sensitive behavioral effects.

Collectively, this work expands our understanding of cortical control over motivated behavior, refines our understanding of the localization of μ-opioid-sensitive sites for the control of feeding, and provides an initial step toward integrating μOR signaling into existing models for the neurochemical modulation of executive control processes by the PFC.

2. MECHANISMS UNDERLYING vmPFC-μOR REGULATION OF MOTIVATIONAL FUNCTION: CELLULAR AND SYSTEMS PERSPECTIVES

A. Cellular localization of μORs within cortical microcircuits

Studies of μORs and electrophysiological recordings of μ-opioid effects on elements of the prefrontal network suggest that μ-opioids could modulate vmPFC signaling through several different routes, including 1) inhibitory effects through μORs located on GABAergic interneurons (Ferezou *et al*, 2007; Taki *et al*, 2000), 2) modulation of thalamic input to the vmPFC by μORs located on the presynaptic thalamocortical nerve terminals (Giacchino and Henriksen, 1998; Marek and Aghajanian, 1998), and possibly 3) potentiation of excitatory D1

receptor-mediated currents by μORs located on vmPFC projection neurons (Olianas *et al*, 2012; Rola *et al*, 2008). Opioid actions acting through these different μ-opioid signaling routes would act to disrupt local PFC network function and disinhibit excitatory PFC output onto subcortical structures, including the LH and AcbSh. This simultaneous activation of projections from ILC to AcbSh that interrupt or limit feeding behavior and projections from ILC to LH (either direct or indirect) that evoke feeding behavior would result in a motivationally-driven organism with limited ability to suppress impulsive behaviors. Interestingly, a more in-depth discussion of each potential route-of-action for μOR-elicited effects suggests additional features of μ-opioid modulation of PFC function.

As described in Chapter 1 and in the Discussion for Chapter 4, the most thoroughly characterized route of μOR action in the cortical mantle is through GABAergic interneurons. Taki *et al* (2000) conducted a series of double immunofluorescence studies, finding that nearly 97% of μOR-immunoreactive (ir) neurons neocortical regions of interest showed immunoreactivity for GABA as well. Moreover, μOR-ir neurons showed a high degree of overlap with vasoactive intestinal peptide (VIP), corticosterone-releasing factor (CRF), choline acetyltransferace (ChAT), calretinin, and cholecystokinin (CCK). This finding is important, as the extensive degree of overlap between VIP and μORs allows us to more narrowly identify μ-opioid-sensitive interneuronal subpopulations as well as their innervation patterns, and their role in inhibiting cortical function (see below). In addition, high levels of overlap in immunoreactivity for μORs and other transmitters such as CRF and CCK suggest that these co-expressed peptides may recreate elements of the DAMGO behavioral profile or potentially oppose μ-opioid-elicited effects. Moreover, identification of μORs on cortical interneurons inspired a number of investigators to characterize their influence within the cortical inhibitory

network. It was soon found that μOR stimulation slowed interneuronal spiking via inhibition of sodium currents, thus reducing GABAergic miniature inhibitory postsynaptic currents (mIPSC) onto pyramidal cells (Ferezou *et al*, 2007; Qu *et al*, 2015; Witkowski and Szulczyk, 2006), and activating them via disinhibition (Tanaka and North, 1994).

As mentioned above, μORs are located on a neurochemically distinct subset of cortical interneurons that express VIP (Ferezou *et al*, 2007; Taki *et al*, 2000). Moreover, uORs are concentrated in layers I, III, and V of the neocortex (Mansour *et al*, 1987). More careful analysis reveals that this laminar distribution overlaps with a subset of VIP-ergic interneurons classified as double bouquet cells, which have small, bipolar, vertically-elongated cell bodies and narrow axonal arbors that extend vertically, mostly targeting the dendrites of pyramidal cells in different layers of the cortex within a narrow column (Karube *et al*, 2004; Kawaguchi and Kubota, 1996; Kubota *et al*, 2016; Raghanti *et al*, 2010). GABAergic interneurons with these characteristic features contribute significantly to the morphology and distribution of cortical 'minicolumns', which are suggested to play a vital role in sensorimotor integration as well as the binding of perceptual and executive control information within cortical areas to guide goal-driven behavior (Opris and Casanova, 2014; Opris *et al*, 2013; Raghanti *et al*, 2010). Stimulation of μ-opioid receptors attenuates intracellular sodium currents in interneurons, suppressing interneuron activity and thereby reducing inhibitory tone on excitatory PFC projection neurons (Witkowski *et al*, 2006). Thus, invoking an interneuron-based explanation for μ-opioid-mediated effects, activating μORs on VIP-ergic interneurons could reshape inhibition patterns and signal processing within μOR-sensitive minicolumns, leading to a disinhibition of pyramidal output onto cortical projection targets.

Signaling through μ-opioid receptors could also alter processing in prefrontal networks by dampening thalamic input into the region. Prefrontal afferents from mediodorsal thalamus relay information from other brain regions involved in complex cognitive processes (Mitchell, 2015). Application of μOR agonists to slices of PFC tissue suppresses increases in pyramidal neuron excitatory postsynaptic currents (EPSCs) evoked by 5-HT_{2A} agonists, an effect which may be due to interactions between μOR and 5-HT_{2A} receptors on presynaptic thalamocortical glutamate terminals (Marek *et al*, 1998). As discussed briefly in Chapter 4, μORs may act to possibly reshape patterns of activation by de-emphasizing thalamic input relative to other incoming signals, including those of limbic origin (Baldo, 2016). One behavioral consequence of this putative mechanism would be a prefrontal hyper-responsiveness to incoming limbic signals from the amygdala and reward-responsive areas of frontal cortex, which would be able to disproportionately drive PFC output onto downstream targets.

A small number of recent studies have begun to challenge the idea of exclusive localization of μORs on interneurons. Some of these studies characterized μOR immunoreactivity on mPFC neurons that display morphological features commonly attributed to pyramidal neurons, including "triangular cell bodies with apical and basal neurites" (Juhasz *et al*, 2008; Olianas *et al*, 2012; Rola *et al*, 2008; Schmidt *et al*, 2003; Schmidt *et al*, 2001). These studies have been conducted in mice (Olianas *et al*, 2012), rats (Juhasz *et al*, 2008; Rola *et al*, 2008), and humans (Schmidt *et al*, 2003; Schmidt *et al*, 2001), though a functional role has only been described for μORs in mice and rats in two studies: Olianas *et al* (2012) and Rola *et al* (2008). Working in mouse mPFC cultures, Olianas et al (2012) reported that AC activity induced by stimulation of DA D1-like receptors was potentiated by concomitant activation of μORs, though stimulation of μORs alone had no effect. Using whole-cell patch clamp

recordings, Rola and colleagues (2008) reported that application of DAMGO decreased the amplitude of voltage-dependent Ca^{2+} currents in dispersed mPFC pyramidal cells. These results suggest that μORs located on pyramidal cells may require a change in membrane potential (likely a depolarization) in order to become functional.

It is worth noting, however, that though the pharmacological and electrophysiological descriptions reported by the Olianas and Rola papers provide strong evidence of μOR-mediated actions on excitatory PFC pyramidal neurons (especially when accompanied with the numerous immunohistochemistry studies described above), we should remain skeptical of this possibility for several reasons. Firstly, Taki *et al* (2000) and Ferezou *et al* (2007) showed via double immunofluorescence and single cell reverse transcription polymerase chain reaction (scPCR), respectively, that MORs are almost exclusively co-localized with a marker for GABAergic neurons. Taki et al (2000) found that nearly 97% of μOR-immunoreactive neurons also showed GABA immunoreactivity, whereas the Ferezou et al (2007) paper found that all μOR-positive neurons express the GABA synthesizing enzyme glutamic acid decarboxylase (GAD). This extremely high (nearly exclusive) expression rate led both authors to conclude that μORs are exclusively expressed on GABAergic interneurons (though see below). Secondly, every paper that has described μOR expression on pyramidal cells in mPFC has identified the cell type solely based on ***morphological features*** (e.g. triangular cell body with apical and basal dendrites), whereas numerous papers describing μORs on interneurons (Ferezou *et al*, 2007; Taki *et al*, 2000) have confirmed the cell type by showing ***co-expression of markers*** for GABA or GABA-synthesizing ability. It should be noted that cortical interneurons are a heterogenous, morphologically diverse class of cells, some of which show pyramidal-like features – most prominently, μOR-expressing double bouquet cells, which are vertically-elongated with

ascending axonal arbours (Kubota *et al*, 2016). Finally, to the best of our knowledge, there are no other studies (apart from those listed above) showing evidence of μOR expression on pyramidal cells in the cortex, though some studies have described μORs on excitatory projection neurons in the basolateral amygdala (Zhang *et al*, 2015) and hippocampus (Caudle and Chavkin, 1990). However, it is important to note that in hippocampus, where μOR actions have been studied more extensively, it has been demonstrated clearly that μORs are localized on interneurons that are very similar to VIP-containing interneurons in neocortex, and, moreover, that μOR stimulation disinhibits hippocampal output via inhibition of theses interneurons (Capogna *et al*, 1993; Krook-Magnuson *et al*, 2011; Zieglgansberger *et al*, 1979). Hence, taking these various lines of evidence into consideration, the evidence seems much stronger for μOR localization on interneurons within cortex.

B. Terminal fields mediating vmPFC-μOR-induced motivational effects: focus on the putative vmPFC→AcbSh 'limiter circuit'

The ability of the AcbSh to inhibit and interrupt consummatory behavior has been well characterized, as discussed in Chapter 1 and the Discussion for Chapter 3. Briefly, GABAergic medium spiny neurons (MSNs) in the AcbSh strongly project to the LH (Heimer *et al*, 1991; Meredith *et al*, 1993), and stimulation of AcbSh-localized AMPA-type glutamate receptors suppresses feeding (Stratford *et al*, 1998). Pauses in AcbSh MSN firing are required to initiate and maintain bouts of consumption, and electrical stimulation of the AcbSh immediately arrests consummatory sucrose licking (Krause *et al*, 2012). These pauses do not occur during bouts of locomotion unrelated to consumption (Taha and Fields, 2005, 2006), suggesting that they are

specifically directed towards motor repertoires involved in the eating act itself. Moreover, pauses in AcbSh firing are correlated with orofacial taste reactivity responses, such that hedonic tastes evoke inhibitory responses while aversive tastes elicit increases in firing rate (Roitman *et al*, 2005). These taste-responsive units in the AcbSh change their response when the hedonic value of a preferred taste is changed; establishment of a conditioned taste aversion (CTA) to sucrose changed sucrose-associated inhibitions in AcbSh firing into excitations (Roitman *et al*, 2010). Finally, we showed in Chapter 3 that blocking glutamate transmission through AMPA receptors potentiated increases in motivated behavior evoked by μOR stimulation in the vmPFC, suggesting that the AcbSh is able to suppress or limit appetitive behaviors as well as those directly involved in consumption (Mena *et al*, 2013).

The PFC is ideally positioned to exert executive control over Acb-mediated reward processing via excitatory projections that synapse directly onto MSNs in the AcbSh (French and Totterdell, 2002). Ghazizadeh *et al* (2012) described a subpopulation of AcbSh units that were inhibited during goal-seeking behavior and subsequent retrieval of a sucrose reward. The basal activity of these units was suppressed by lesions of the vmPFC, suggesting that these neurons are normally under excitatory control by vmPFC projections. Another study by LaLumiere *et al* (2012) showed that activation of ILC suppressed cocaine-seeking behavior, and this effect is blocked by AMPA receptor antagonists in the AcbSh. These findings suggest that the glutamatergic projection from the vmPFC to the AcbSh has the capacity to suppress specific goal-directed behaviors via AMPA-mediated activation of a specific "appetitive response-gating" neuron population. AMPA signaling in the vmPFC-AcbSh "limiter" circuit could engage a behavioral set that competes with or "interrupts" specific goal-directed appetitive behaviors.

C. vmPFC-μOR-mediated modulation of motivated behaviors: an integrated cellular and systems hypothesis

Based on the results from this thesis project, and on the literature reviewed above, we have developed a working model describing ILC-μOR physiological actions. A schematic diagram summarizing this working model is included below (Fig 1). We acknowledge that the presence of μORs on mPFC pyramidal cells is still controversial; thus, we have attempted to create a model that functions similarly with and without them. To summarize, μORs (represented by red triangles) are localized 1) on VIP-expressing interneurons (tan ovals), 2) the presynaptic axon terminals of glutamatergic thalamocortical projections, and 3) on pyramidal projections neurons (though these are faded to represent the inconclusive nature of their existence). VIP neurons are shown to have a primarily vertical innervation pattern with limited horizontal spread, consistent with their presumed role in controlling the excitability of cortical minicolumns. Our work in Chapter 4 as well as our review of DA D1 receptor-mediated modulation of PFC function has also allowed us to include D1 receptors (blue hexagons) in our model, with their expression on pyramidal cells and parvalbumin-containing interneurons (light blue rectangles). ILC pyramidal neurons are glutamatergic and project substantially to both the AcbSh and LH, with a small subpopulation that bifurcate and project to both structures (Gabbott *et al*, 2005). Medium spiny neurons in the AcbSh send inhibitory projections to the LH, where they directly oppose/interrupt feeding behavior (Stratford and Kelley, 1999).

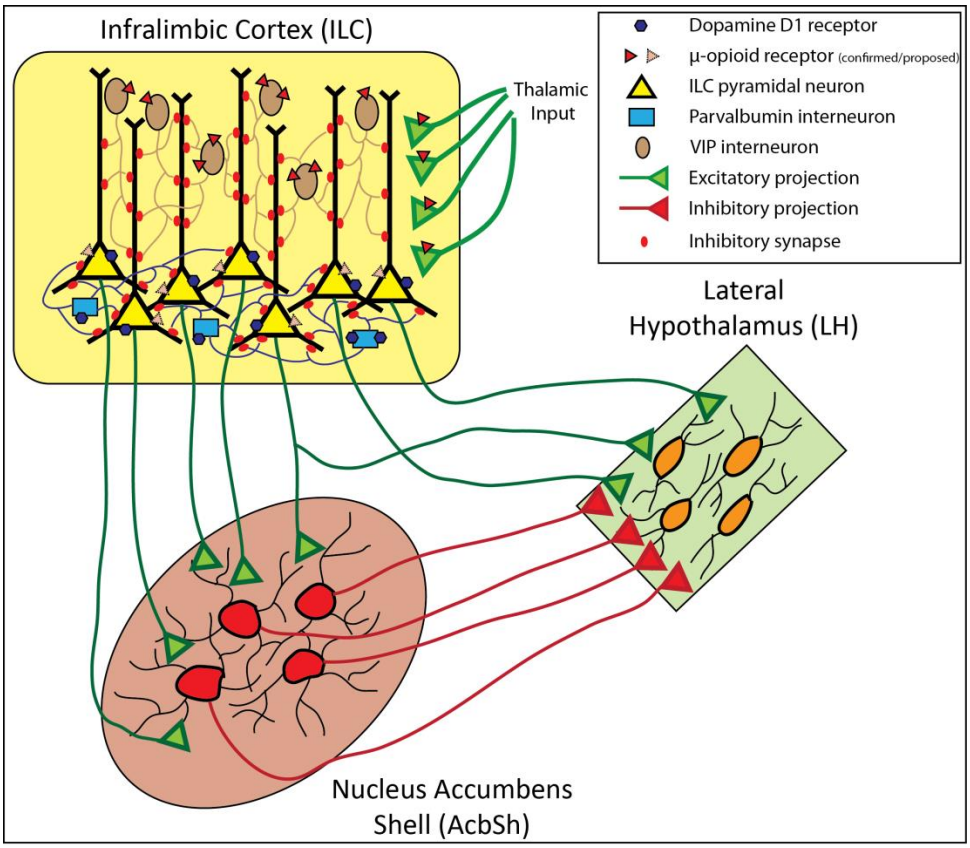


Figure 1. Schematic diagram illustrating a simplified infralimbic cortex microcircuit with efferent connections to prominent downstream projection targets (and their interconnectivity) included. The prefrontal cortex is a network of interconnected glutamatergic pyramidal cells (yellow triangles) and numerous types of GABAergic interneurons, including those expressing vasoactive intestinal peptide (VIP; gray ovals) and parvalbumin (blue rectangles). μ-opioid receptors (small red triangles) are found on VIP interneurons, presynaptic terminals of corticothalamic projections, and possibly on pyramidal cells. D1 receptors are on parvalbumin interneurons and pyramidal cells. ILC sends excitatory, glutamateric projections onto both the nucleus accumbens shell (AcbSh) and lateral hypothalamus (LH). The AcbSh sends inhibitory, GABAergic projections to the LH.

DAMGO infusion into the ILC could result in μOR-stimulated activity at each of these neuroanatomical loci. Stimulation of μORs located on VIP-expressing interneurons would suppress their activity, removing a source of inhibition on pyramidal cells in cortical minicolumns. At the same time, μOR stimulation would blunt incoming signals from the thalamus, leaving disinhibited ILC pyramidal neurons increasingly responsive to signals from the amygdala and cortical areas involved in the control of feeding processes. Finally, MORs on ILC pyramidal cells could potentiate incoming signals from these areas, which are then passed along

in a disorganized way to downstream targets in the AcbSh and LH. Heightened glutamate release in the LH would engage appetitive drive (Li *et al*, 2011; Stanley *et al*, 1993), leading to increased feeding and food-seeking behavior, whereas glutamate transmission through AMPA receptors in the AcbSh would engage competing non-goal-directed behaviors that compete with or interrupt LH-mediated processes of goal-directed appetitive drive (Ikeda *et al*, 2003; Krause *et al*, 2012). The expected effects of μOR activity on PFC function are represented in Fig 1B, below.

This model would predict both heightened appetitive motivation (due to over-engaged ILC→LH projections) and impaired performance on inhibitory control tasks (due to aberrant, uncontrolled activity in ILC→AcbSh projections), which were observed in Chapter 2. Moreover, we would expect that simultaneous blockade of AMPA receptors in the AcbSh would potentiate increases in appetitive drive evoked by μOR activation of ILC→LH projections, which was observed in Chapter 3. Lastly, blockade of D1 receptors would prevent the transmission of μ-opioid mediated effects (as described in Chapter 4) by blocking activation of noise-suppressing parvalbumin-containing interneurons and suppressing the excitability of pyramidal cells, rendering the PFC less sensitive to incoming signals.

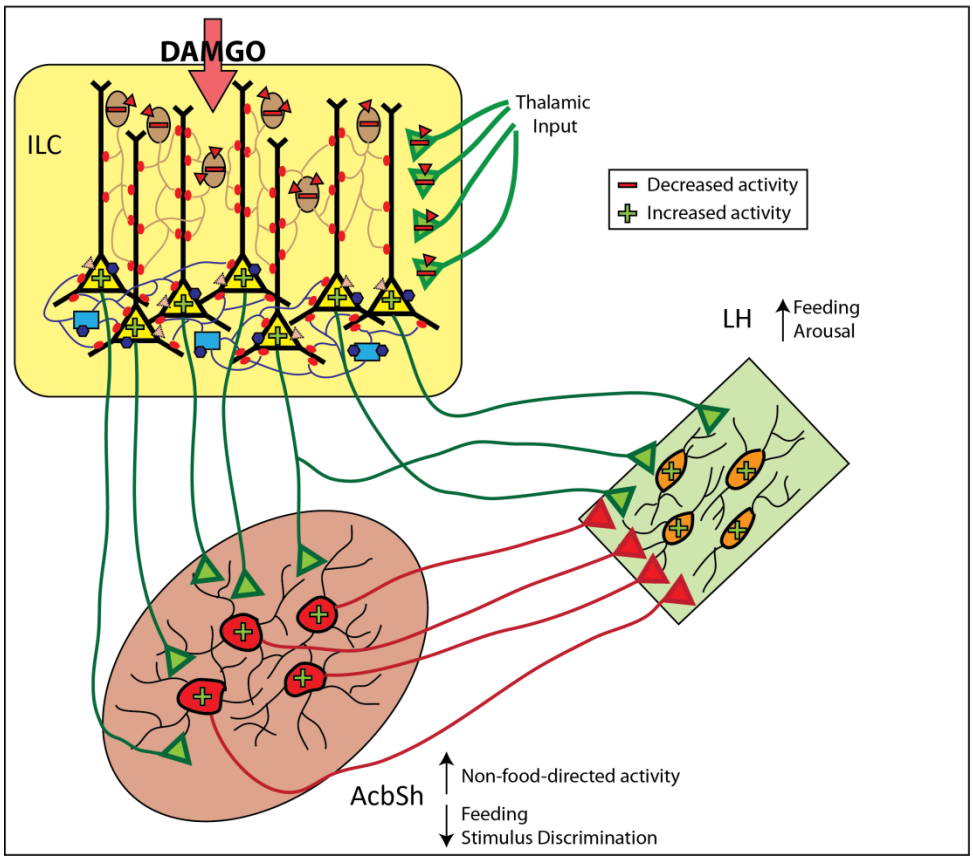


Figure 2. Changes in cellular activity and network function after μOR stimulation of ILC. Refer to Figure 1 for identification of individual network components. DAMGO infusion into the ILC would result in the inhibition of VIP interneurons, suppression of glutamate release from corticothalamic afferents, and (possibly) potentiation of D1-evoked excitatory signals via their location on pyramidal neurons. These summed actions would disinhibit PFC pyramidal neurons and increase their responsivity to non-thalamic inputs, resulting in increased firing and enhanced glutamate outflow onto PFC projection targets in the AcbSh and LH.

D. Cellular μOR localization: future directions

In order to more accurately depict how μ-opioid transmission modulates prefrontal function, the localization and physiological roles of opioid receptors within the vmPFC require a higher degree of characterization. While the model of prefrontal function proposed in this thesis is consistent with the best available information about cytoarchitecture and physiological function within the cortex, it should be noted very few of the pharmacological, physiological,

and cytoarchitectural studies used in constructing this model were actually conducted in mPFC tissue samples. For example, Ferezou *et al* (2007) and Taki *et al* (2000) convincingly localized μORs on cortical interneurons using a variety of methods, including morphological features, single-cell reverse transcription polymerase chain reaction (PCR), and immunohistochemistry. However, both studies were conducted in tissue slices outside of the mPFC in vaguely described frontoparietal cortex, including motor and somatosensory areas. While the neocortex as a whole does share certain gross anatomical characteristics, such as laminar organization, neurochemical architecture across the cortical mantle is not completely uniform. Dopamine D1 receptors, for example, are most highly expressed in frontal areas such as infralimbic, cingulate, and anterior insular cortex, with a more sparse distribution throughout the rest of the cortical mantle (Fremeau *et al*, 1991). As μOR stimulation has only been shown to induce behavioral changes in a small subset of primarily frontal cortical areas (Gorka *et al*, 2014; Mena *et al*, 2011), it is not unreasonable to assume that MOR distribution may display unique features in those areas relative to the rest of the cortex. Additional studies are needed to specifically address these potential differences.

E. μOR-mediated effects through frontal networks: future directions

Although this dissertation is specifically focused on prefrontal modulation of function in the Acb and LH, it should be noted that disinhibition of cortical output onto other PFC projection structures could also contribute to the behavioral effects observed after μOR stimulation of the vmPFC. A promising candidate arises when we revisit the work of Dawe and Loxton (2004), who parse impulsivity into two interrelated subcomponents: a predisposition toward rash-spontaneous behavior as well as an increased sensitivity to food and food-associated cues. As previously described, processing within the amygdala is important for associating the availability

of rewards with predictive stimuli in the environment and assigning incentive qualities to those cues that predict future rewards (Holland and Gallagher, 2004). The vmPFC as a whole sends substantial projections to the amygdalar complex, including the basolateral (BLA) and central nuclei (CeA; Gabbott *et al*, 2005; Vertes, 2004). Activity within the BLA is crucial for allowing food-predictive cues to override satiety and promote eating in sated rats (Petrovich *et al*, 2002). Moreover, stimulation of MORs in the central nucleus of the amygdala (CeA) produces a pronounced elevation in appetitive behavior directed at reward-predictive cues, likely through direct projections to the AcbSh (DiFeliceantonio and Berridge, 2012; Mahler and Berridge, 2009). Thus, μOR-mediated changes in prefrontal output onto the amygdalar complex could result in strengthened top-down influences on cue-encoding within multiple amygdala substrates, resulting in an over-strong attribution of motivational salience to food and food-related cues. Hence, the cue hypersensitivity that Dawe and Loxton (2004) describe as an additional distinguishing feature of binge-like behavior could also result from μOR-evoked disruption of normal communication between the ILC and the amygdala. Future studies are needed to address this possibility directly.

3. CLINICAL IMPLICATIONS

Aberrant activity in frontal areas including the vmPFC contributes to deficits in impulse control in a number of psychiatric disorders characterized by excessive appetitive motivation (Dong *et al*, 2016; Karhunen *et al*, 2000; Schienle *et al*, 2009; Seo *et al*, 2013; Uher *et al*, 2004). Numerous studies have suggested that these deficits arise from supernormal opioid transmission (Blasio *et al*, 2014; Gorelick *et al*, 2008; Love *et al*, 2009; Mitchell *et al*, 2012; Morganstern *et*

al, 2012; Selleck *et al*, 2015; Zubieta *et al*, 1996), and these studies are supported by clinical studies showing that opioid antagonists have some degree of clinical efficacy across several disorders characterized by loss of control over goal-seeking behavior (Cambridge *et al*, 2013; Kim *et al*, 2001; Mitchell *et al*, 2007; Volpicelli *et al*, 1992). However, the fact that these drugs have been limited in their usefulness (McElroy *et al*, 2013; Ziauddeen *et al*, 2013) suggests that further studies are needed to more thoroughly delineate opioid actions within the brain and how normal brain function is disrupted by opioid antagonists. The studies contained within the dissertation have made progress in this regard. We have characterized the vital role that opioid systems have in modulating both motivation and executive control processes, demonstrated that aberrant signaling in cortical areas can evoke disorganized behavioral repertoires in downstream structures, and shown powerful interactions between opioid and monoamine systems within the cortex.

Our results suggest that using poly-drug treatments may provide increased efficacy in treating disorders with loss-of-control features. Specifically, co-administering opioid and D1-specific antagonists may prove more effective at quelling impulsive, motivated tendencies in those with binge-like disorders, as opioid-mediated impairments in inhibitory control are dependent on intact D1 signaling in the PFC. While this would more effectively treat the presumptive source of the aberrant behavioral tendencies, we should keep in mind that systemic drug administration reaches all areas of the brain; hence, treatments utilizing opioid and dopamine antagonists run the risk of impairing normal reward function and inducing anhedonic or anergic behavioral states due to their effects in the ventral striatum. Non-specific effects could potentially be avoided by targeting neuropeptide systems that are co-expressed with cortical opioid systems (such as CCK, VIP, or CRF) but work in opposition to opioid-elicited effects.

Additional studies expanding our understanding of neuropeptide expression and modulation of prefrontal function can bring us closer to a solution.

Lastly, our working model for μ-opioid-mediated effects on prefrontal function is likely to have relevance outside of our stated focus on ingestive behavior. We were able to incorporate interactions between opioid and dopamine systems in the prefrontal cortex, suggesting that opioid function may evoke deficits in D1-mediated processes controlling working memory performance. Future studies exploring μ-opioid interactions with other prefrontal neuromodulatory systems may provide additional promising therapeutic targets or for μ-opioid modulation of other prefrontally-mediated processes.

4. CONCLUDING REMARKS

In this thesis, we presented novel behavioral and pharmacological evidence that demonstrates that μ-opioid signaling within the vmPFC plays a vital role in the modulation of goal-seeking and inhibitory control processes. In addition, we showed a functional relationship between prefrontal mechanisms of executive control and striatal processes limiting goal-seeking behavior. We generated novel data that begins to integrate the physiological effects of μORs into established models for monoamine modulation of prefrontal function. We refined earlier findings in the lab through the high-resolution mapping of opioid-sensitive areas for feeding and inhibitory control within the vmPFC, more precisely localizing where these effects can be elicited. We also proposed a working model to explain the potential cellular mechanisms through which μ-opioid receptors in the vmPFC produce the behavioral effects reported in this thesis. This model incorporates experimental findings discovered in our laboratory into the larger

literature describing pharmacological, anatomical, and behavioral characteristics of PFC control over feeding and inhibitory control as well as those arising from subcortical opioid-sensitive feeding sites. There is still much left to be done. Nevertheless, the results from this thesis provide a greater understanding of the neurological control over ingestive behavior, and, more broadly, motivational processes in general, and provide a step toward the development of more effective pharmacological and behavioral treatments for psychiatric disorders with binge-like features. Our findings provide a deeper understanding of the neurochemical processes mediating impulse control and binge-like behavior, and hopefully provide novel therapeutic strategies for future interventions aimed at helping those suffering from binge-like disorders regain control of themselves and their lives.

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