NEUROPHARMACOLOGICAL MECHANISMS UNDERLYING

INCENTIVE SALIENCE AND STRESS

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"If I have seen further, it is by standing on the shoulder of giants."

-Sir Isaac Newton

To my mother, father, and grandmother – thank you for being my giants.

I dedicate this piece of work to you.

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ABSTRACT

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Anhedonia, the lost of interest or pleasure in normally enjoyable activities, is a core

clinical feature of major depressive disorder (MDD). Although the term encompasses

both consummatory and appetitive components, empirical findings suggest that

anhedonia is more closely associated with deficits in motivational processing as

opposed to alterations in hedonic valuation of rewards. A growing body of evidence

points to a role for the mesolimbic dopamine (DA) system in the etiology of motivational

deficits. However, the neurobiological mechanisms underlying the manifestation of

anhedonic behavior are still not well understood. Given the frequent presentation of

anhedonia in stress-related psychiatric disorders, we hypothesized that exposure to

stressors disrupts brain signaling pathways that would typically facilitate reward

processing. The experiments in this thesis utilized the novelty-induced hypophagia

(NIH) test, a motivational conflict paradigm, to examine the effects of stress on neural

processes associated with the attribution of incentive salience to salient stimuli. Using in

vivo microdialysis, we assessed the role of nucleus accumbens (NAc) DA transmission

in mediating conditioned approach behavior for food reward, and established that stress

exposure blunts NAc DA response to palatable food and reduces incentive salience for

the reward, as defined by increased latency to approach. Pretreatment with the mixed

action opioid drug buprenorphine prevented the behavioral and neurochemical effects of stress in this paradigm, implying a role for the brain opioid system in mitigating the negative effects of stress on incentive behavior. Using a combination of genetic and pharmacological tools, the role of individual opioid receptors in restoring approach behavior suppressed by stress in the NIH test was next dissected. These studies identified the mu opioid receptor as a critical mediator of approach behavior and a potential pharmacological target for alleviating prodepressive behaviors during stress. Lastly, the interaction between elevated stress hormones and treatment with the selective serotonin reuptake inhibitor fluoxetine, a commonly used antidepressant, was investigated on incentive behavior in the NIH paradigm. We found that exogenous corticosterone exposure facilitated reduced approach behavior caused by exposure to chronic fluoxetine in C57BL/6 mice. Thus, exposure to stress hormones enabled fluoxetine to produce a behavior modeling a therapeutic response in a strain of mice that are otherwise insensitive to this antidepressant drug. Collectively, these findings further expand our understanding of the mechanisms underlying stress-induced affective behavior and antidepressant drug treatments.

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CHAPTER 1: GENERAL INTRODUCTION

Overview of depression

Major Depressive Disorder (MDD) is one of the most common forms of psychiatric illness existing today, with a lifetime prevalence of 17% in the US and 4% worldwide (Kessler et al., 2003). MDD is characterized by the presence of at least one of two major symptoms, depressed mood or the lost of interest or pleasure in daily activities, in addition to at least five secondary symptoms for a period of two or more weeks (DSM-V-TR, American Psychiatric Association). Secondary symptoms of MDD span a wide range of somatic, cognitive, and emotional deficits, including psychomotor agitation or retardation, diminished ability to concentrate, and feelings of worthlessness or excessive guilt. This combination of symptomology is incredibly disruptive to daily functioning and is consistently associated with impaired productivity and reduced job retention (Beck et al., 2011). The economic impact of lost productivity due to depression is estimated to amount to over $30 billion per year in the United States alone (Stewart et al., 2003). The chronicity of depression further adds to the debilitating nature of this condition. As many as 50% of people who recover from a depressive episode will experience an additional episode, typically within five years of the initial episode (Belsher and Costello, 1988; Kupfer and Frank, 2001; Burcusa and Iacono, 2007). Moreover, individuals diagnosed with MDD are at increased risk to develop other serious medical illnesses, such as heart disease (Lett et al., 2004) and stroke (Williams et al., 2004). In terms of years lost to ill-health or early death, MDD is considered one of the most disabling medical conditions and is predicted to be among the top three contributors to the worldwide burden of disease by 2030 (Mathers and Loncar, 2006).

Despite the availability of antidepressant medications, MDD continues to be a challenging disorder to treat. Results from the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study, the largest longitudinal randomized study of antidepressant efficacy to date, showed that the frequently used selective serotonin reuptake inhibitor (SSRI) citalopram produced a remission rate of around 30% (Trivedi et al., 2006). Furthermore, many of the participants who achieved remission did so after 8 weeks of treatment or later. Approximately 30-60% of depressed individuals do not respond to the first antidepressant prescribed and an estimated 15-30% fail to respond to multiple treatments and are considered treatment-resistant (Cain, 2007; Berlim et al., 2008). These alarming statistics highlight the importance of advancing our understanding of the biological mechanisms underlying depression to develop more efficacious and faster-acting therapies for the treatment of MDD.

Anhedonia as a depressive endophenotype

The investigation into the neurobiological mechanisms that govern the onset and maintenance of depression has been hindered in part by the broad diagnostic characterization of MDD. The currently used system of classification criteria for MDD encompasses a wide range of symptoms that differ in severity, onset, and recurrence (DSM-V-TR, American Psychiatric Association) and are likely mediated by different, though possibly related, physiological processes (Charney and Manji, 2004). For example, patients diagnosed with depression may either sleep more than normal or suffer from insomnia and report their appetite for food is greater or less than normal. The heterogeneous nature of MDD, as it is presently defined, severely limits the likelihood of discovering an etiological course consistent between all depressed individuals. A more appropriate strategy may be to focus research efforts on endophenotypes, narrowly

defined behavioral dimensions and biological measures that span traditional diagnostic criteria. The recently described research domain criterion (RDoC) framework emphasizes deconstruction of psychiatric syndromes into fundamental neurobiological components that can then be mapped onto genetic, molecular, cellular, and behavioral processes (Cuthbert, 2014) This approach allows for identification of the "downstream" mechanisms underlying specific clinical phenotypes and the "upstream" consequences of physiological processes (Hasler et al., 2004). The endophenotype system operates on the assumption that the biological processes regulating the manifestation of individual endophenotypes are fewer and less multifaceted than those of the psychiatric entity as a whole. Whether or not this is the case is yet to be determined. Nevertheless, closer examination into endophenotypes of depression has the potential to yield important findings and implications for the classification and subsequent treatment of depressive disorders (Berghorst and Pizzagalli, 2010).

The evaluation of endophenotypes is based on several criteria including heritability, specificity, and biological and clinical plausibility (Tsuang et al., 1993). Of the many psychopathological endophenotypes associated with depression (cognitive deficits, REM sleep abnormalities, and psychomotor changes to name a few), impaired reward function has the most compelling empirical evidence and broadest applicability (Hasler et al., 2004; Pizzagalli et al., 2005). Anhedonia, clinically defined as diminished interest or pleasure in response to stimuli that were previously perceived as rewarding in a premorbid state, is one of two required symptoms for the diagnosis of MDD (DSM-V-TR, American Psychiatric Association). Approximately 37% of individuals with depression experience clinically significant anhedonia (Pelizza and Ferrari, 2009).. Blunted reward sensitivity and diminished motivational drive are strong predictors for the diagnosis of MDD (Zimmerman et al., 2006; Treadway and Zald, 2013; Pizzagalli, 2014).

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Moreover, clinical studies indicate a familial association and heritability of anhedonic endophenotypes in depression (Bogdan and Pizzagalli, 2009; Sieradzka et al., 2015). Although hedonic deficits are commonly observed in other psychiatric disorders, such as schizophrenia and post-traumatic stress disorder, anhedonia within these illnesses is often associated with the presentation of depressive-like symptoms (Loas et al., 1999; Kashdan et al., 2006; Volkow et al., 2011). In regards to the biological underpinnings of anhedonia, multiple lines of evidence from both clinical and preclinical sources support a role for the brain reward system.

Reward sensitivity and processing in anhedonia

The most well-characterized reward circuit of the brain is comprised of dopaminergic neurons originating in the ventral tegmental area (VTA) that project to and innervate several limbic and cortical structures regions, including the nucleus accumbens (NAc), amygdala, hippocampus, and medial prefrontal cortex (mPFC). These structures are interconnected through reciprocal glutamatergic connections that are further modulated by GABAergic interneurons. Notably, dopaminergic projections from the VTA to the NAc have been shown to mediate the reinforcing properties of both natural rewards and drugs of abuse. Presentation of a rewarding stimulus produces phasic elevation of dopamine (DA) levels in the NAc, whereas depletion of DA in this region reduces reward responsiveness and reward-seeking behavior (Ikemoto and Panksepp, 1999). DA acts primarily on two subfamilies of G-protein coupled receptors (GPCRs) known as D1-like and D2-like receptors (Cooper et al., 2003). Stimulation of D1-like receptors increases adenylate cyclase activity, while stimulation of D2-like receptors decreases adenylate cyclase activity (Surmeier et al., 2007). Activation of DA receptors within the striatum modulates the sensitivity of medium spiny neurons to excitatory glutamatergic input from

corticolimbic structures (Hernandez-Lopez et al., 2000; Surmeier et al., 2007).

The proposed functional role of the midbrain DA system in reward processing has deviated over time. Early studies suggested that DA directly produced the hedonic impact, or "liking", of a pleasant stimulus (Wise, 1985; Koob and Le Moal, 1997). This concept was later challenged by taste reactivity studies which demonstrated that depletion of DA in rodent forebrain and striatal regions does not alter hedonic impact to rewards, suggesting that mesocorticolimbic DA activity is not necessary for "liking" (Berridge et al., 1989; Berridge, 2000). Seminal work conducted by Schultz et al. (1997) established a role for DA in the learning processes associated with reward response. In these studies, monkeys presented with appetitive stimuli, but not aversive, exhibited phasic activation of midbrain neurons. Repeated pairings of environmental cues with a rewarding stimulus shifted the temporal onset of phasic activation from the time of reward delivery to the time of cue presentation. Moreover, the basal firing of DA neurons was distinctly suppressed in trials that the reward was not presented after presentation of the conditioned cue. These observations gave rise to the reward prediction error hypothesis, which posits that DA encodes the discrepancy between predicted and experienced reward, thus regulating reinforcement-dependent learning (Schultz et al., 1997; Hyman et al., 2006; Wise, 2008; Glimcher, 2011). Building upon these electrophysiological observations, Berridge and Robinson (1998) proposed incentive salience theory, which describes a role for DA in mediating the learned reward associations that attribute motivational "wanting", but not "liking", to a previously neutral stimulus (Berridge, 2012). Indeed, several studies have demonstrated dissociation between motivational behavior and hedonic valuation in response to rewarding stimuli. For example, highly rewarding drugs of abuse have repeatedly been shown to induce conditioned taste aversion in rodents at doses that would be readily self-administered

(Berger, 1972; Cappell and Le Blanc, 1973; Cappell et al., 1973; Wise et al., 1976; White et al., 1977). Although, more recent studies have argued that avoidance of cues associated with drugs of abuse is independent of taste aversion, and may instead reflect conditioned fear (Parker, 2003). On the other hand, DA antagonists and NAc DA depletion have been shown to distinctly suppress effort-related behavioral responding for food reward without diminishing food consumption in low-effort conditions (Aberman and Salamone, 1999; Salamone et al., 2002). Thus, mesolimbic DA activity within the NAc is likely involved in the effort-based and goal-directed behavior that underlies motivational "wanting" of an attractive stimulus.

Diagnostic assessments of anhedonia in depressed individuals have traditionally emphasized the aspect of "loss of pleasure" (Treadway and Zald, 2013). Although, MDD patients often report diminished general positive affect and reduced intensity of emotional experiences (Germans and Kring, 2000; Naragon-Gainey et al., 2009), several studies indicate that individuals presenting with anhedonia do not differ from healthy controls in perceiving the pleasantness of an emotionally salient stimulus (Berenbaum and Oltmans, 1992; Burbridge and Barch, 2007; Etain et al., 2007; Sherdell et al., 2012). Instead, clinical evidence suggests that anhedonia in depression is characterized primarily by deficits in affective processing that may underlie reduced motivational drive for reward (Pizzagalli, 2014). Laboratory studies utilizing reinforcement paradigms demonstrate that MDD patients fail to develop a response bias towards more rewarding stimuli (Pizzagalli et al., 2005; Pizzagalli et al., 2008), an effect that has been shown to be associated with striatal function in monkeys (Lauwereyns et al., 2002). Neuroimaging studies have linked MDD to reduced grey matter volume within striatal regions (Kim et al., 2008; Matsuo et al., 2008; Pizzagalli et al., 2008; Wacker et al., 2009) and diminished activation of the ventral striatum to anticipation of rewards

(Forbes, 2011; Smoski et al., 2011), reward prediction errors (Steele et al., 2007; Kumar et al., 2008), and receipt of reward (McCabe et al., 2009; Pizzagalli et al., 2009; Smoski et al., 2009; Wacker et al., 2009). Moreover, severity of anhedonic symptoms is positively correlated with reduced ventral striatum response to rewarding stimuli (Keedwell et al., 2005; Epstein et al., 2006). Altered striatal activity in MDD may be a result of generally reduced DA activity. MDD patients exhibit lower levels of cerebrospinal homovanilic acid, the primary metabolite of DA, signifying reduced basal levels of DA in depression (Lambert et al., 2000; Mitani et al., 2006). Furthermore, positron emission topography imaging studies report reduced DA transporter binding in the striatum of depressed patients with anhedonic symptoms (Sarchiapone et al., 2006). Altogether, these findings suggest a link between alterations in mesocorticolimbic DA functioning and impaired reward processing in anhedonia.

Interaction of brain stress and reward systems in depression

A substantial body of evidence supports a pathophysiological role of stress in depression. Approximately 80% of depressive episodes are reported to be preceded by a stressful life event (Mazure, 1998; Hammen, 2005). However, prospective longitudinal studies have also found exposure to chronic stressors (i.e. persisting for more than 12 months) rather than acute stressful episodes to have a significant influence on the onset and course of depression (Caspi et al., 2003; Harkness and Monroe, 2006). Exposure to chronic stress is associated with more frequent recurrences (Lethbridge and Allen, 2008), increased resistance to treatment (Amital et al., 2008), and more severe depressive symptoms (Leskela et al., 2006). Interestingly, the association between stress and depression weakens after multiple depressive episodes (Kendler et al., 1998), suggesting that factors aside from stress reactivity are more closely associated

with recurrent rather than initial depressive episodes.

Stress is also strongly associated with the manifestation of anhedonic behavior. Acute stress has been shown to impair reward responsiveness within healthy populations, particularly in individuals who exhibited greater cortisol reactivity to the stressor (Berghorst et al., 2013) or self-reported higher levels of anhedonia (Bogdan and Pizzagalli, 2006). Moreover, both acute and chronic stressors are reported to produce hedonic blunting, predominantly in individuals with a family history of depression (Berenbaum and Connelly, 1993; Al'absi et al., 2012). Conversely, pronounced anhedonic symptoms are correlated with increased sensitivity to stress (Horan et al., 2007). These observations indicate an interaction between brain stress and reward systems in the emergence of depressive phenotypes.

Hypothalamic-pituitary-adrenal (HPA) axis

The hypothalamic-pituitary-adrenal (HPA) axis is an integral component of the body's homeostatic and stress response system. Exposure to either a physical or psychological stressor facilitates synthesis and secretion of corticotrophin-releasing factor (CRF) from the hypothalamus. CRF stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland, which in turn triggers the adrenal glands to release glucocorticoid hormones, commonly referred to as cortisol in humans and corticosterone (CORT) in rodents (Sapolsky et al., 2000). Glucocorticoids mobilize the body's energy stores and regulate a variety of cardiovascular, metabolic, and immunological functions to effectively prepare the body to respond to a stressor and facilitate recovery to homeostasis (Kadmiel and Cidlowski, 2013). Over a longer time scale, glucocorticoids regulate the expression of genes and promote memory formation, effects thought to mediate adaptive modulations to the system for future events (de Kloet et al., 2005;

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Groeneweg et al., 2011).

Glucocorticoids enact their effects through molecular events that trigger genomic and non-genomic mechanisms that involve the dual activation of intracellular mineralocorticoid (MR) and glucocorticoid receptors (GR), collectively referred to as corticosteroid receptors. Once diffused through the cellular membrane, glucocorticoids bind to the receptor complex and translocate into the nucleus to act on DNA response elements to activate gene transcription (Ringold, 1985). Alternatively, the receptor complex can repress expression of genes by inhibiting other transcription factors from binding to their target genes (De Bosscher et al., 2003). More recently, a non-classical corticosteroid pathway involving membrane bound GRs and MRs has been shown to mediate the rapid non-genomic mechanisms of stress response (Tasker et al., 2006).

Within the central nervous system, GRs and MRs differ in both their affinity for glucocorticoids and regional pattern of expression. MRs exhibit a higher affinity for glucocorticoids and regulate basal HPA tone, whereas GRs are only activated during conditions involving high circulating levels of CORT and facilitate termination of the stress response (De Kloet et al., 1998). GRs are widely distributed throughout the brain, but exhibit high levels of expression in the hypothalamus and corticolimbic structures, including the hippocampus, prefrontal cortex, and amygdala. MRs, on the other hand, are less ubiquitous in the brain, but are highly expressed in the hippocampus (Young et al., 2003; Joels et al., 2012). Elevated circulating levels of glucocorticoids serve as a negative feedback signal to the HPA axis by binding to GRs at the level of the hypothalamus and pituitary to inhibit further secretion of CRF and ACTH (Miller et al., 1992). The HPA axis also receives strong regulatory inputs from the hippocampus. Activation of corticosteroid receptors on hippocampal neurons signal for the termination of HPA axis activity (Sapolsky et al., 1984; Jacobson and Sapolsky, 1991).

Under normal conditions, glucocorticoids serve to facilitate the physiological adaptations required to appropriately respond to stressors. Excessive activation of the HPA axis, however, is thought to contribute to the onset of depressive symptoms. A substantial proportion of MDD patients demonstrate signs of HPA axis dysfunction, as evidenced by flattening of cortisol diurnal rhythms, augmented circulating concentrations of cortisol and CRF (Nemeroff et al., 1984) and insensitivity to dexamethasone, an exogenous steroid that suppresses cortisol in healthy controls (Holsboer et al., 1987). Both the expression and function of GRs are significantly reduced in depressed individuals (López et al., 1998; Pariante, 2004). Moreover, early-life stress exposure is predictive of structural reductions in regions involved in regulation of the HPA axis (Sahay and Hen, 2007; Treadway et al., 2009), indicating that HPA axis dysregulation in depression likely occurs via diminished inhibitory feedback signals. Notably, MDD is consistently associated with reductions in hippocampal volume (Campbell et al., 2004; Videbech et al., 2004). In addition to its role in glucocorticoid inhibitory feedback mechanisms, the hippocampus is also a key mediator of cognitive and emotional processes. Thus, altered hippocampal neuronal activity may contribute to the psychopathologies of depression.

Outside of the HPA axis, CRF-containing neurons and CRF receptors are widely distributed throughout brain regions implicated in stress response (Swanson et al., 1983; Sakanaka et al., 1987; Griebel and Holsboer, 2012), suggesting that CRF can act directly at these regions independent of HPA axis activation (Koob et al., 1993; Dautzenberg and Hauger, 2002). . The effects of CRF are mediated through activation of two subfamilies of GPCRs, CRF-R1 and CRF-R2, though CRF shows greater binding affinity for CRF-R1 (Dautzenberg and Hauger, 2002). Exogenous administration of CRF mimics many of the behavioral and autonomic effects of acute stress exposure (Dunn

and Berridge, 1987), and has been shown to induce depressive and anxiety-like behaviors in rodents (Britton et al., 1982; Owens and Nemeroff, 1991; Liang et al., 1992; Swiergiel et al., 2008). Notably, antagonism of CRF receptors attenuates many of the affective behavioral responses to stress (Britton et al., 1986; Heinrichs et al., 1994; Bale and Vale, 2004). Hypersecretion of CRF has been implemented in the onset of depressive disorders. MDD patients exhibit elevated cerebrospinal fluid levels of CRF (Nemeroff et al., 1984) and enhanced neuroendocrine response to the combined dexamethasone/CRF test (Steckler et al., 1999) that is normalized after successful antidepressant treatment (Heuser et al., 1998; Ising et al., 2005). Additionally, port-mortem studies have revealed increased CRF expression and reduced binding in the brains of depressed individuals. (Nemeroff et al., 1988; Purba et al., 1996).

Effects of Stress on Mesocorticolimbic Dopaminergic Pathways

Brain stress response systems interact with the midbrain DA system to produce complex state-dependent responses to aversive stimuli (Cabib and Puglisi-Allegra, 2012). Corticosteroid receptors and CRF receptors are expressed throughout the mesocorticolimbic system, specifically in the prefrontal cortex, amygdala, NAc, and VTA (Harfstrand et al., 1986; Van Pett et al., 2000; Dautzenberg and Hauger, 2002; Butts et al., 2011; Hensleigh and Pritchard, 2013). CRF in the VTA reduces operant responding for food reward and attenuates NAc DA release to food reward (Wanat et al., 2013). Electrophysiological studies report inhibition of VTA DA neurons in response to stress (Ungless et al., 2004), however distinct anatomical regions within the VTA have been shown to contain DA neurons that differ in their molecular properties and response to aversive versus rewarding stimuli. For example, DA neurons in the dorsal VTA are inhibited by acute stress, whereas DA neurons in the ventral VTA are excited by

aversive footshocks (Brischoux et al., 2008). Moreover, aversive stimuli selectively modify synapses projecting to the mPFC, whereas rewarding stimuli modifies synapses on DA cells projecting to NAc medial shell (Lammel et al., 2011). Additionally, GABAergic neurons in the rostromedial tegmental nucleus (RMTg) project to VTA DA neurons and are selectively activated in response to stress (Jhou et al., 2009). Thus, aversive inputs from various brain regions implicated in stress response may converge onto the RMTg to inhibit DA transmission in the NAc.

Several studies have reported robust DA release in the mPFC following acute stress (Imperato et al., 1989) or acute administration of CORT (Piazza et al., 1996; Butts et al., 2011). Chronic administration of exogenous CORT flattens glucocorticoid rhythm and enhances basal and depolarization evoked DA release in the mPFC in a GR-dependent manner (Ago et al., 2008; Minton et al., 2009). Mesocortical DA neurons exert top-down control over striatal DA release (Deutch and Roth, 1990; King et al., 1997; Ventura et al., 2002). Therefore, stress-induced potentiation of mPFC DA activity may facilitate blunted DA release in mesolimbic pathways. Additionally, NAc responsiveness to excitatory input from the mPFC has been shown to be regulated by the hippocampus (O'Donnell and Grace, 1995). Hence, motivational states are likely influenced by glucocorticoid-mediated alteration of hippocampal function. Interestingly, stress-induced stimulation of mesoaccumbens DA release is associated with short-lasting or controllable aversive experiences (Abercrombie et al., 1989; Ventura et al., 2002; Cabib and Puglisi-Allegra, 2012), whereas exposure to chronic or uncontrollable stressors selectively activates the HPA axis (De Boer et al., 1989) and promotes inhibition of mesoaccumbens DA (Puglisi-Allegra et al., 1991; Imperato et al., 1992; Rossetti et al., 1993; Cabib and Puglisi-Allegra, 2012). Reduced mesoaccumbens DA activity after chronic or uncontrollable stress is posited to contribute to coping failure and

the development of behavioral despair (Cabib and Puglisi-Allegra, 2012). This holds

particular relevance for the etiology of psychiatric disease states seeing as exposure to

chronic stressors perceived as uncontrollable or unresolvable is more associated with

depressive behavior (Kendler et al., 2003).

FIGURE 1.1
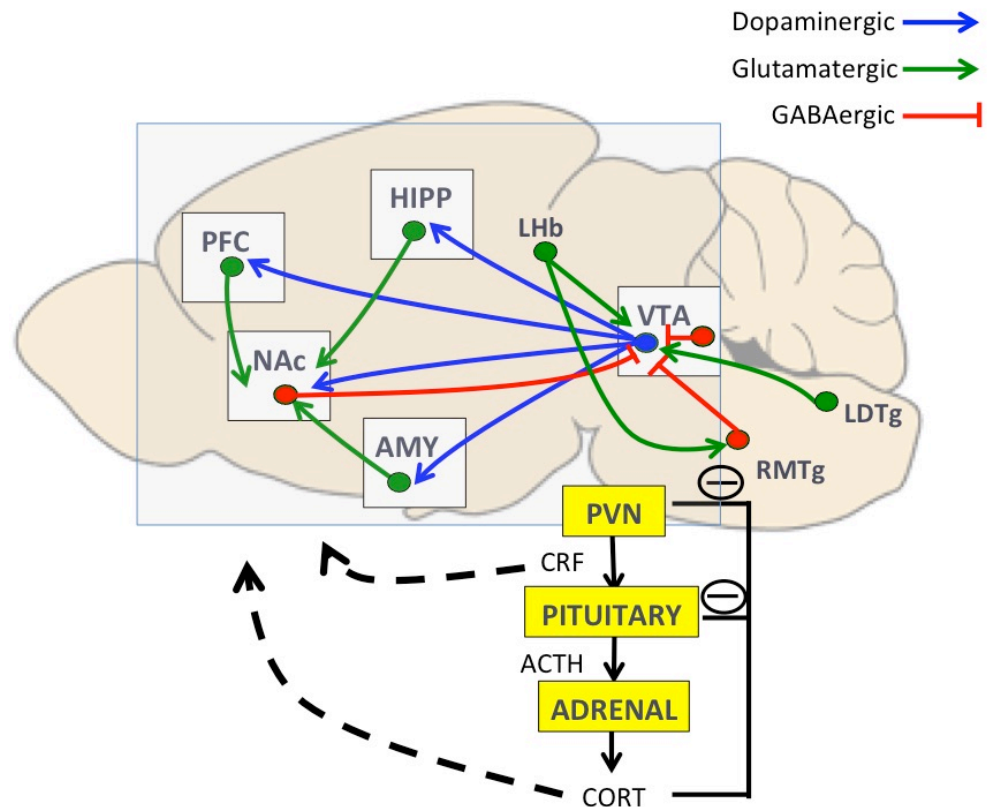
VTA-NAc reward circuit and HPA axis. A simplified schematic of the major connections within the mesocorticolimbic dopamine system and hypothalamic-pituitary-adrenal axis. DA neurons originating from the VTA project to and innervate subcortical and cortical regions. This pathway is further modulated by reciprocal glutamatergic and GABAergic signaling. Exposure to stress activates secretion of CRF from the PVN, which stimulates secretion of ACTH from the pituitary, which in turn triggers release of CORT from the adrenals. CORT serves as a negative feedback signal at the level of the pituitary and PVN. CRF and CORT act on brain regions that overlap with brain reward circuitry to modulate dopamine response to both rewarding and aversive stimuli. ACTH, adrenocorticotropic hormone; AMY, amygdala; CORT, corticosterone; CRF, corticotropin-releasing factor; HIPP, hippocampus; LHb, lateral habenula; LDTg, lateral dorsal tegmentum; NAc, nucleus accumbens; PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus; RMTg, rostromedial tegmentum; VTA, ventral tegmental area.

Opioid System

In addition to its well-established role in nociception and analgesia (Dickenson, 1991), the opioid system is also highly implicated in the regulation of reward processing (Koob, 1992) and stress response (Bruchas et al., 2010). The endogenous opioid system is composed of three principal families of G-protein coupled receptors (GPCRs), mu (MOR), kappa (KOR), and delta (DOR), which interact with the peptides β-endorphin, dynorphin, and enkephalin, respectively. Opioid receptors and their peptides are widely expressed throughout the peripheral and central nervous system (Mansour et al., 1994; Wittert et al., 1996). Within the brain, opioid receptors are primarily distributed in the cortex and limbic system (Mansour et al., 1988). Opioid receptors localized to mesocorticolimbic DA pathway are thought to mediate both the reinforcing properties of rewards and the aversive properties of stressful experiences.

Activation of MORs expressed by GABAergic interneurons in the VTA disinhibit the activation of DA neurons and facilitate DA release in the NAc (Johnson and North, 1992; Spanagel et al., 1992b), an essential molecular component of reward processing (Contet et al., 2004). Studies in mice with genetic deletion of opioid receptors have helped to establish that MORs mediate the reinforcing properties of addictive substances, including morphine, nicotine, and ethanol (Matthes et al., 1996; Kieffer and Gaveriaux-Ruff, 2002). Importantly, MORs also influence the processing of natural rewards, as evidenced by reduced operant responding for both standard chow and sucrose pellets in MOR knockout animals compared to wildtype (Papaleo et al., 2007). Furthermore, activation of MORs in hedonic "hot-spots" within the NAc shell stimulates positive orofacial reactions to food and increases consumption (Pecina and Berridge, 2005). The role of DORs in reward processing is not as clear, however deletion of DORs or its endogenous ligand results in increased levels of anxiety and depressive-like

behavior, suggesting that these receptors are more involved in mood-enhancing processes (Konig et al., 1996; Filliol et al., 2000; Ragnauth et al., 2001).

In contrast, to the MOR and DOR, the KOR is more implicated in mediating the aversive properties of stress or stimuli with negative emotional valence (McLaughlin et al., 2003; McLaughlin et al., 2006a; McLaughlin et al., 2006b; Lutz and Kieffer, 2013). Prolonged exposure to environmental stress can give rise to dysregulation of the dynorphin-KOR modulatory system resulting in increased levels of dynorphins in mesocorticolimbic regions (Shirayama et al., 2004; McLaughlin et al., 2006b). Activation of KORs induce depressive-like and anxiety-like behaviors in rodents (Carlezon et al., 2006; Van't Veer and Carlezon, 2013) and reduce DA release in key brain regions involved in the processing of reward and motivation,(Svingos et al., 1999; Shirayama et al., 2004; Flaisher-Grinberg et al., 2012). Notably, stimulation of KORs in the NAc region decreases DA transmission, whereas administration of a KOR antagonist increases DA transmission (Spanagel et al., 1992a). Moreover, infusion of a KOR agonist into the NAc produces robust conditioned place aversion (Bals-Kubik et al., 1993). Facilitation of stress-like effects by KOR activation is mediated in part by interactions with the neuroendocrine system as evidenced by the finding that the behavioral and neurochemical effects of environmental stress or exogenous CRF exposure are blocked by treatment with KOR antagonists (McLaughlin et al., 2003; Land et al., 2008).

FIGURE 1.2
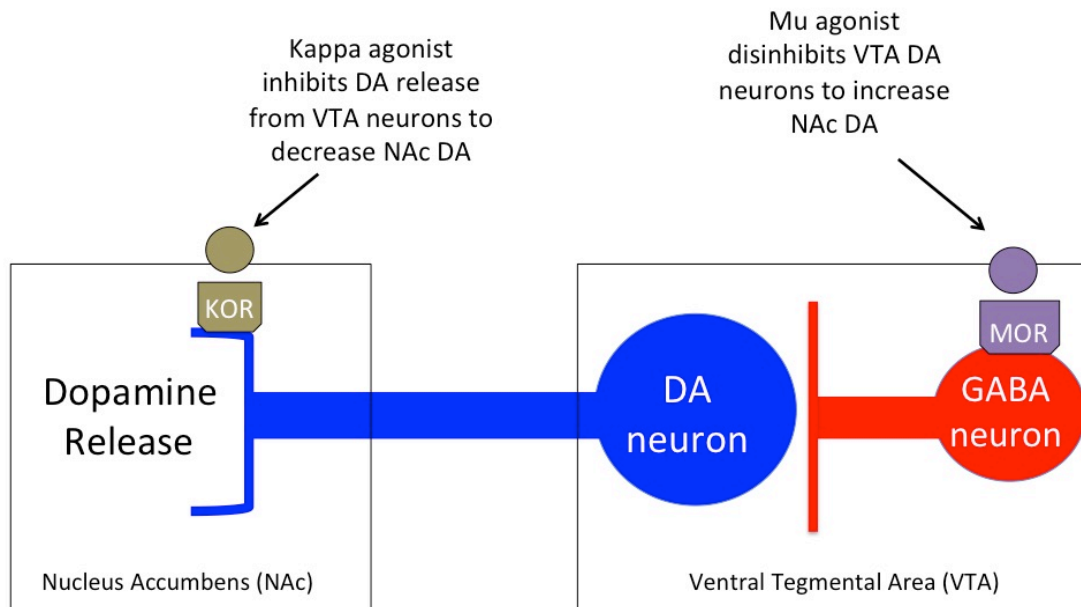
The endogenous opioid system modulates DA transmission within the mesolimbic pathway. KOR, kappa opioid receptor; MOR, mu opioid receptor. Adapted from Carroll & Carlezon (2013).

Rodent models of stress-induced depression

There is no singular animal model or paradigm that perfectly recapitulates the complex symptomology of depression. However, converging lines of evidence support stress-induced molecular changes as a key mediator of the onset and progression of depressive symptoms in humans. To that end, stress models of depressive behavior in animals have utilized realistic and relevant inducing conditions to simulate critical physiological components of the psychiatric condition (construct validity). Moreover, these models have been shown to demonstrate face validity by producing behavioral and physiological changes that parallel the symptom profile of depression. Finally, animal models relevant to depression can be shown to demonstrate predictive validity by responding appropriately to antidepressant drugs that are effective in humans).

Chronic Mild Stress (CMS)

The theoretical rationale for the CMS paradigm is derived from the clinical observation that depressive episodes are often precipitated by an accumulation of life stressors, and that stressors perceived as unpredictable or uncontrollable stressors are more associated with the onset of depression (Kendler et al., 2003). The procedure, first developed by Katz (1981), involved exposing subjects to a variety of severe stressors for a period of three weeks. In the late 1980s, Willner and colleagues modified the paradigm to avoid the use of severe stressors and instead utilize mild stressors (e.g. change of cage mate, overnight illumination, food/water deprivation) presented in a semi-randomized sequence to model unpredictability and prevent habituation to the stressors (Willner et al., 1987). The procedure was originally targeted at modeling anhedonia, due to the fact that chronically stressed animals consistently exhibited reduced preference

for a sweetened solution, suggesting stress-induced modulation of reward sensitivity. Additional studies have gone on to show that CMS exposure also elicits a wide variety of behavioral and pathophysiological effects, including anxiogenic and depressive-like behaviors, neurochemical changes and alterations in neuroplasticity (Willner, 2005). Importantly, many of these effects have been shown to be reversed by antidepressant treatment.

The many pathophysiological changes resulting from CMS provide a comprehensive model of symptomologies that parallel the human condition when exposed to stress and serve as an invaluable tool for the evaluation of neurobiological mechanisms underlying affective behavior. The disadvantage of this model, however, is the long duration and labor-intensive aspects of the procedure. Moreover, many researchers have expressed frustration in the reproducibility of CMS-induced effects between laboratories. Adoption of a standardized CMS protocol may help to increase consistency in findings in the future.

Chronic CORT Treatment

The chronic CORT paradigm was developed as an etiologically relevant model of depressive disorders characterized by hyper-activation of HPA axis activity. Indeed, hypercortisolemia is frequently observed in MDD patients exhibit, which is thought to be associated with some of the behavioral and cognitive deficits observed in depression. In this paradigm exogenous CORT is delivered directly via oral administration, slow-release CORT pellets, or serial injections for a period of 14 or more days. Along with elevating circulating levels of CORT, chronic CORT treatment has been shown to reduce sucrose preference and operant responding for rewards (Gourley and Taylor, 2009), increase

behavioral despair as measured by immobility in the forced swim test and tail-suspension test (Gourley et al., 2008; David et al., 2009) and increase anxiety-like behavior in the light-dark emergence task (Ardayfio and Kim, 2006). In addition, chronic CORT treatment consistently reduces hippocampal cell proliferation and survival while reducing expression of neurotrophins such as BDNF (Czeh et al., 2001; Bilsland et al., 2006; Murray et al., 2008). Notably, many of the neurochemical and behavioral changes produced by chronic CORT treatment are reversed by antidepressant treatment, thus lending to the predictive validity of this model.

The advantage of chronic CORT treatment is several-fold. The procedure is significantly less complex compared to the CMS protocol, and can easily be replicated and confirmed by independent research groups. Whereas the CMS procedure may produce varying degrees of CORT response in individual animals, the exogenous administration strategy ensures that all animals are exposed to identical levels of CORT. Although, it is possible that individual variations in HPA axis reactivity can influence the behavioral and physiological consequences of exogenous CORT exposure. A limitation of this method is that the HPA axis is only one of many systems that respond to stress. Thus, the pathophysiological effects of CORT treatment alone are not necessarily reflective of total exposure to physiological stress. There is also some variation between laboratories in the way that CORT exposure is accomplished. Nonetheless, exogenous administration of CORT enables investigation into how CORT specifically contributes to the development of stress-induced changes in behavior and physiology

Behavioral tests of anhedonia

Sucrose Preference Test

The sucrose preference test is a measure of hedonic sensitivity to rewarding stimuli. In this procedure, animals are given the choice of consuming either water or a saccharin/sucrose solution. Non-stressed mice exhibit increased consumption of the sweetened solution, whereas animals exposed to stress exhibit substantial reductions in preference for the sweetened solution. This finding has long been hypothesized to model reduced perception of rewards, and is thus often cited as an animal measure of anhedonia. A hallmark of the CMS procedure is the demonstration of reduced sucrose preference among stressed animals (Willner, 2005). Chronic treatment of stressed animals with antidepressants restores sucrose preference, reflecting the way that antidepressants have been shown to work in humans. Reduction in sucrose preference after CMS is a controversial endpoint, however. Since stressed animals also experience significant weight loss compared to non-stressed animals, sucrose intake data may be adjusted to include weight differences between the two groups. In some instances, differences in sucrose preference sometimes become insignificant when this is done (Matthews et al., 1995; Forbes et al., 1996). However, other laboratories have shown this finding to hold true irrespective of changes in body weight (Willner, 1997). Another limitation of this test is that it doesn't take into consideration the motivational aspects of anhedonia, which appears to be the principal deficit in human populations. In fact, when asked to rate the pleasantness of different concentrations of sucrose solutions, there is no difference in hedonic value between depressed individuals and healthy controls (Amsterdam et al., 1987; Berlin et al., 1998; Dichter et al., 2010). Regardless, the sucrose preference test continues to be a widely used behavioral measure of anhedonia in animals.

Conditioning Paradigms

Approach motivation, the mobilization of energy by or toward a positively valenced stimulus, is a fundamental component of reward-seeking behavior (Lewin, 1935). Pavlovian conditioning theory posits that temporal pairing of a neutral conditioned stimulus, such as an environmental cue, with an appetitive unconditioned stimulus, such as food, can result in the acquisition of an approach response to the conditioned stimulus in subsequent events. Appetitive reactions evoked by the conditioned stimulus signal attribution of incentive salience, or "wanting", thus maintaining learned behaviors that facilitate acquisition of the rewarding or desired outcome. Alterations in conditioned approach behavior may signal dysregulation of the neural mechanisms that translate incentive learning into motivated behavioral output.

Operant Responding

Operant responding, also known as instrumental learning, is an oft-used measure of motivational drive for rewarding stimuli in rodents. Rodents are trained to associate an instrumental response (e.g. lever pressing) with a conditioned stimulus. Presentation of the conditioned stimulus leads to an increase in instrumental responding. This paradigm can be further adapted to utilize a progressive ration (PR) procedure in which the experimenter progressively increases the number of lever presses required to obtain each subsequent award (Hodos, 1961). The final ratio achieved by the animal before discontinuing lever presses is referred to as the "break point" and is reflective of the subject's motivation or willingness to work for a reward. Operant paradigms provide a more sensitive measure of motivational output compared to consummatory measures, such as the sucrose preference test. It is important to note, however, that under a PR

schedule animals periodically receive the reward as they successfully progress to the next fixed ratio. The intermittent reinforcement of reward may contribute to sustained levels of operant behavior, thus potentially obscuring the effects of experimental manipulations on motivational drive in this paradigm.

Intracranial self-stimulation (ICSS) is an operant conditioning based behavioral paradigm in which animals learn to self-deliver brief electrical pulses into specific brain regions implicated in brain reward circuitry, most notably the lateral hypothalamus (LH) (Olds and Olds, 1963) and VTA (Miliaressis et al., 1975). Dopaminergic fibers from the VTA to the NAc pass through the LH and are innervated by peptidergic projections from the LH that are thought to mediate rewarding responses to drugs of abuse (Fulton et al., 2000; Kelley and Berridge, 2002; Kiefer and Wiedemann, 2004; Harris et al., 2005). Acute administration of drugs of abuse lower ICSS thresholds (Vlachou and Markou, 2011), whereas CMS exposure increases ICSS thresholds, suggesting reduced sensitivity to rewards reflective of an anhedonic state (Moreau et al., 1995). Similar to traditional operant responding paradigms, ICSS allows for quantitative assessment of behavioral output for rewarding stimuli. However, ICSS directly activates reward-processing systems while bypassing sensory modulatory inputs (e.g. palatability) that may be involved in motivational salience.

Conditioned Place Preference (CPP)

The conditioned place preference paradigm utilizes Pavlovian conditioning to assess the rewarding or aversive effects of a particular treatment or event (Tzschentke, 2007). In this procedure, a drug or non-drug treatment is repeatedly paired with distinctive environmental cues. Eventually the animal learns to associate the contextual cues with the rewarding (or aversive) properties of the treatment and will choose to spend more (or

less) time in the conditioned environment even in the absence of the original stimulus. The CPP test is a powerful tool for investigating the neurobiological mechanisms underlying the reinforcing properties of salient stimuli. However, because animals passively receive the treatment, this paradigm may not fully address the effort-based mechanisms associated with motivated behavioral output.

Novelty-induced hypophagia (NIH) test

The unconditioned suppression of feeding in a novel environment, commonly referred to as hyponeophagia, is a well-documented phenomenon in rodents. Thus the novelty-induced hypophagia (NIH) test taps into stress-induced changes in conditioned approach behavior. In this paradigm animals are trained over a period of days to rapidly approach and consume a highly palatable food reward in their home cage environment. Once stable approach latencies for the food are established, animals are examined for their appetitive and consummatory behavior in a brightly lit and differently scented novel arena. Untreated animals consistently exhibit a robust increase in latency to approach the food and reduced food consumed in the novel arena compared to the home cage. Chronic treatment with antidepressant drugs reverses this effect. Interestingly, acute treatment with an anxiolytic drug also reduced approach latency in this paradigm (without altering home cage behavior), which led researchers to conclude that this paradigm is a measure of anxiety-like behavior in rodents (Bodnoff et al., 1988; Bodnoff et al., 1989; Dulawa and Hen, 2005). Alternatively, approach latency in the novel arena may reflect altered motivational drive for rewarding stimuli. The distinct suppression of approach behavior in the novel arena enables investigation into how aversive cues contribute to diminished incentive salience for natural rewards.

Goals of research

The overarching goals of this thesis research were to further elucidate the mechanisms

underlying stress-induced changes in motivational drive and in the process identify

potential pharmacological targets for the prevention or reversal of affective behavior. The

majority of studies examining the neurobiological underpinnings of motivated behavior

for natural rewards has been conducted in rats and has at times conflated hedonic

processes and motivational output in their assessment of anhedonic behavior. In this

thesis work we employed a single conditioned approach behavioral paradigm in mice in

which "liking" (amount of food consumed) and "wanting" (latency to approach the food)

behaviors could de easily dissociated so as to simultaneously measure the motivational

aspects and neurochemical substrates underlying incentive salience for a salient

stimulus. Once characterizing the core neurochemical phenotype associated with stable

approach behavior, we uncovered the effects of stress on these same neural processes,

thus establishing a unique role for NAc DA in the onset and maintenance of incentive

behavior in mice. Next, using the same behavioral paradigm we identified distinct

pharmacological targets within the brain opioid system as potential mediators of

conditioned approach behavior under stress. Lastly, we sought to address the role of

stress hormones in mediating behavioral response to the selective serotonin reuptake

inhibitor (SSRI) fluoxetine in the NIH paradigm. Together, these findings help to broaden

our understanding of the mechanisms underlying anhedonic behavior in stress related

psychiatric disorders.

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CHAPTER 2: BUPRENORPHINE PREVENTS STRESS-INDUCED BLUNTING OF NUCLEUS ACCUMBENS DOPAMINE RESPONSE AND APPROACH BEHAVIOR TO FOOD REWARD IN MICE

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Abstract

Anhedonia is a hallmark symptom of several psychiatric disorders, including depression, schizophrenia and post-traumatic stress disorder. Given the frequent presentation of anhedonia in stress-related psychiatric disorders, we assessed the effects of stress on the motivational and neurochemical correlates underlying conditioned approach behavior for palatable food in the mouse. The effect of palatable food exposure was measured on dopamine release in the nucleus accumbens (NAc), a region known to play a prominent role in food reward behavior in awake freely moving C57BL/6J mice using in vivo microdialysis. Animals trained to consume food in a familiar environment exhibited a significant 30% elevation in NAc dopamine release coincident with the presentation of food. This effect was not seen in animals that consumed the food in an unfamiliar environment or interacted with the food reward for the first time and was region specific. Presentation of an acute stressor (bright light and novel scent) during food exposure blocked dopamine release and delayed approach behavior in response to the food reward in trained animals. These effects were reversed in animals pretreated with buprenorphine, a mixed opioid drug shown previously to produce antidepressant-like and anxiolytic effects. Together, these data indicate that exposure to mild stress reduces incentive drive to approach palatable food via alterations in NAc dopamine responsiveness to the food reward. Moreover, they implicate the brain opioid system as a potential pharmacological target for treatment of anhedonia

Introduction

Anhedonia, the lost of interest or pleasure in typically enjoyable or rewarding activities, is a hallmark symptom of several psychiatric disorders including depression, schizophrenia and post-traumatic stress disorder. Although trait anhedonia encompasses both consummatory and motivational components, clinical studies indicate that the major deficit lies in motivational output as opposed to processing of hedonic value (Burbridge and Barch, 2007; Sherdell et al., 2012; Argyropoulos and Nutt, 2013). Dopamine (DA) neurotransmission has long been known to be involved in the processing of reward and motivation, and more recently shown to modulate depressive-like behavior in rodents (Chaudhury et al., 2013; Tye et al., 2013). Yet, the manner in which altered DA activity influences changes in behavior is still not well understood.

The brain's reward system is composed in part of mesolimbic DA neurons originating in the ventral tegmental area (VTA) that project to and innervate several limbic structures including the nucleus accumbens (NAc). Dopaminergic innervation of the NAc is thought to play a critical role in mediating the reinforcing properties of both drugs of abuse and natural rewards. Studies conducted largely in rats have measured phasic increases in extracellular concentrations of DA in the NAc in response to both initial consumption of a palatable food reward and the instrumental responding for food (Bassareo and Di Chiara, 1997; Ahn and Phillips, 2007). On the other hand, depletion or inhibition of DA activity in the NAc reduces goal-directed behavior towards food, but does not diminish hedonic reactions to food (Berridge, 2007, 2012a). Thus, dopaminergic signaling in the NAc is likely involved in both initiating unconditioned appetitive behavioral responses and eliciting cue-triggered incentive salience (Berridge and Robinson, 1998; Cardinal et al., 2002; Parkinson et al., 2002; Cone et al., 2016).

However, very little work has been done in evaluating the relationship between NAc DA reactivity to food stimuli and approach behavior in other species, such as mice, which exhibit different approach and appetitive behaviors to novel and familiar foods.

Given the frequent presentation of anhedonia in stress-related psychiatric disorders, exposure to stress may contribute to the dysregulation of reward circuitry. The endogenous opioid system has been implicated in mediating the aversive properties of stress (McLaughlin et al., 2003; McLaughlin et al., 2006b; Lutz and Kieffer, 2013b) and has been shown to either facilitate or reduce DA release in key brain regions involved in processing of reward and motivation, including the NAc (Svingos et al., 1999; Shirayama et al., 2004; Bruchas et al., 2009; Flaisher-Grinberg et al., 2012a). Recent studies in animals have revealed promising effects of opioid drugs, such as buprenorphine (BPN), in ameliorating anxiety and depressive like behaviors induced by stress (Almatroudi et al., 2015; Browne et al., 2015; Falcon et al., 2015; Falcon et al., 2016b). Moreover, clinical trials have shown BPN to have therapeutic effects in treatment-resistant human populations (Bodkin et al., 1995a; Nyhuis et al., 2008; Ehrich et al., 2014).

The goal of the present study was to use a single behavioral paradigm in mice to simultaneously measure the motivational aspects and neurochemical substrates underlying approach behavior for palatable food reward. Changes in extracellular DA levels in the NAc and behavior were measured during the presentation of palatable food to non-deprived C57BL/6 mice using in vivo microdialysis. Comparison of the effects of conditioned versus unconditioned food stimuli on NAc DA release and approach behavior established that prior exposure to food reward was necessary to produce food-cued DA reactivity in the NAc and that this response reflected both anticipatory and consummatory signals. DA levels in the striatum were unaffected. Once the core

neurochemical phenotype associated with stable approach behavior was established,

we demonstrated that exposure to an acute stressor during food presentation inhibited

the NAc DA response to a conditioned food stimulus and reduced motivational salience

for the reward, as measured by increased latency to approach. Lastly, we showed that

pretreatment with the mixed-opioid drug BPN prevented both the neurochemical and

behavioral effects of stress in this paradigm.

Materials and Methods

Animals

Male C57BL/6 J mice, 7-8 weeks old upon arrival, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in pairs in polycarbonate cages and maintained under a 12 hour light-dark cycle (lights on at 0700 hours) in a temperature (22 °C)- and humidity-controlled environment. Chow and water were available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Drugs

Buprenorphine hydrochloride (Sigma, St. Louis, MO) was dissolved in distilled water and injected intraperitoneally (i.p.). The dose was calculated according to the base weight of the drug and administered in a volume of 10 ml/kg.

Surgery

Microdialysis probes were custom-made and surgically implanted in mice as described previously (Knobelman et al., 2001). Under isoflurane anesthesia, the probe was implanted in the nucleus accumbens [+1.2 mm anteroposterior (AP), ±0.5 mm mediolateral (ML) from bregma, −4.5 mm dorsoventral (DV) from dura] or dorsal striatum (+1.0 mm AP, ±1.7 mm ML fro bregma, −4.5 mm DV from dura) (Franklin and Paxinos, 1997) using a stereotaxic instrument. BPN treated mice were injected with drug immediately after completion of surgery while still anesthetized. Untreated animals received no injection. Following surgery, the mice were placed into a 21.5 cm high, clear polycarbonate cylindrical in vivo microdialysis chamber with a counterbalance arm

holding a liquid swivel (Instech Laboratories, Plymouth Meeting, PA) and allowed to recover overnight with the pump flow rate set to 0.9 µl/min. For testing the flow rate was set to 2.2 µl/min. Dialysate samples were collected into polypropylene microcentrifuge vials and stored at −80 °C until analyzed for dopamine as described previously (Andrews and Lucki, 2001) using a Shimadzu Prominence HPLC system (including a LC-20 AD pump and Sil-20 AC refrigerated microsampler) using a Unijet microbore column (3 µM ODS/ 100 × 1 mm) coupled with an Antec Decade II electrochemical detector. DA levels were identified by comparing their elution times with those of reference standards and quantified from their respective peak heights using a linear regression analysis of the peak heights obtained from a series of reference standards.

Experimental procedures

Mice were trained to consume three (~1.4g) Reese's peanut butter chips (The Hershey Company, Hershey, PA) presented in a small, clear petri dish in a home cage environment in daily 15-minute sessions. Opaque, black, plastic dividers were placed inside each home cage to separate the mice during training sessions. Mice were allowed to habituate to the dividers for 1 hour before the start of the training session. Sessions continued until animals approached the food reward in less than 30 seconds for three consecutive days. Mice assigned to the "test cage" trained group received five additional training sessions in the microdialysis chamber. Mice assigned to the "home cage" trained group underwent surgery after meeting criteria in the home cage and were tested in the microdialysis procedure without prior food exposure in the microdialysis chamber. Mice assigned to the "no training" group were naïve to the food reward and received no training prior to surgery and testing. All animals were tested in the microdialysis chamber.

Microdialysis experiments started 17–20 hours after surgery. The pump flow rate was increased to 2.2 µl/min two hours prior to testing. Baseline dialysate samples were collected at five-minute intervals starting one hour prior to food exposure. The final three samples were used to establish a baseline value before food exposure. During food exposure, a small clear petri dish with three peanut butter chips was placed in the microdialysis chamber for fifteen minutes and 3 samples were collected. Three additional samples were collected after the food dish had been removed. At the completion of the experiment, the mice were sacrificed and their brains were removed, placed in cold isopentane, and frozen at −80 °C. The brains were then sectioned (35 µm) with a refrigerated cryostat and the tissue examined for the location of the dialysis probe.

Behavioral recordings

Mice were evaluated for their responses to food presentation in the microdialysis chamber. The latency to eat the peanut butter chips and the amount consumed during the 15-minute feeding period was live recorded for each animal. Mice were also monitored for defensive burying (aggressive shoveling movement of bedding material with forepaws), rearing (standing on hindpaws), grooming (nose/face/body washing), and idle (absence of active moments) behavior before, during, and after food presentation. For each behavioral category, mice received a score of either "1" if they engaged in the behavior within the 5 minute sampling time or "0" if they did not. The scores were averaged among animals in each group for each time point, thereby reflecting the proportion of animals engaging in a particular behavior at a given time.

Data analysis

One-way and two-way ANOVA were performed to examine the significance of differences between experimental groups. Significant overall main effects were followed by Dunnett's or Holm-Sidak's multiple comparisons test where appropriate. Microdialysis data were expressed as a percentage of baseline values determined by the mean of three samples collected immediately before food presentation. A repeated measures two-way ANOVA was used to compare variations in DA levels within experimental groups and significant differences were followed by Dunnett's test. Variations in DA levels between groups were compared at individual time points using Tukey's test. For all tests, $p < 0.05$ was considered statistically significant. Data are expressed as mean ±SEM.

Results

The effects of training experience on DA response and approach behavior to palatable food

To determine whether familiarity with the food reward and feeding environment impacts DA response and approach behavior to palatable food in C57BL/6J mice, we first evaluated the effects of training experience on NAc DA reactivity and latency to approach and consume the food. Repeated-measures two-way ANOVA revealed a significant effect of training experience [$F(2, 15)=9.884$, $p=0.002$] and an interaction between training experience and time [$F(16, 120) = 2.713$, $p=0.001$] on extracellular DA levels in response to the food presentation. Multiple comparisons indicated that food presentation significantly increased extracellular DA levels (~30%) in the NAc of animals exposed to food in the test cage ($p<0.05$). DA levels remained high after the food was removed. This response was attenuated in animals allowed to consume the food in feeding cages but not in the test cage (home cage trained animals) and returned to baseline levels after removal of the food. Animals that were naïve to the peanut butter chips did not demonstrate significant variation of DA levels during or after presentation of the food (Figure 2.1A).

Differing approach latencies between groups [$F(2,15)=84.98$, $p <0.001$] paralleled the association between DA levels and food presentation (Figure 2.1B). Animals interacting with the food reward for the first time had a significantly higher approach latency compared to test cage and home trained animals ($p <0.001$). Similarly, the amount of food consumed differed between groups [$F(2,15)=14.92$, $p=0.003$]. As illustrated in Figure 2.1C, test cage trained and home cage trained animals consumed

more of the peanut butter chips compared to untrained animals (p < 0.01).

Determination of a behavioral profile, by measuring burying, rearing, grooming, and idle behavior before, during, and after food presentation, provided additional information on the differential effects of training experience. Animals with no previous experience with the food reward showed significantly more burying behavior during food presentation compared to test cage and home cage trained animals. A two-way ANOVA revealed a significant main effect of time $[F(8,120)=8.658, p<0.0001]$ and an interaction between training experience and time $[F(16, 120)=2.401, p=0.016]$ on the amount of burying behavior (Figure 2.2A). A significant main effect of time was also observed for rearing $[F(8, 120)=4.074, p<0.003]$ (Figure 2.2B) and grooming $[F(8,120)=2.562, p<0.013]$ (Figure 2.2C) behaviors. Animals from all groups exhibited more rearing and grooming behavior following food presentation. Analysis of the time spent idle showed a significant main effect of time $F(8,120)=12.05, p<0.0001]$ and an interaction between groups $F(16,120)=3.072, p<0.0002]$. Home cage trained and animals without training spent more time idle prior to food exposure compared to test cage trained animals (Figure 2.2D). Moreover, untrained animals spent more time idle during food presentation compared to test cage and home cage trained animals.

NAc DA is responsive to anticipatory and consummatory cues for palatable food

Having demonstrated that mice trained to feed in the test cage exhibit DA reactivity to food exposure, we next sought to address whether the NAc DA response is an appetitive signal or dependent on consummation of the food reward. To test this, we presented test cage trained animals with empty petri dishes (in which the peanut butter chips were previously stored) while sampling from the NAc (Figure 2.3A). In the absence of food, we

observed a 40% elevation in DA in response to presentation of the food dish F(8,32)=5.184, p=0.003].

NAc DA response to palatable food is not reflective of generalized striatal activity

To examine the regional specificity of the food-induced DA response, a separate cohort of test cage trained animals was implanted with microdialysis probes in the dorsal striatum. As seen in Figure 2.3B, food presentation had no effect on extracellular DA release in this region F(8,24)= 1.663, p=0.160], although these animals exhibited similar approach latencies and food consumption to animals who received probe implants in the NAc (data not shown).

BPN prevents effects of stress on NAc DA response and approach behavior

The next experiment measured how stress exposure influences the NAc DA response to a familiar food reward and approach behavior. Repeated measures ANOVA revealed a significant effect of treatment [F(2,19)=9.848, p=0.001], time [F(8,152)=3.454, p=0.001], and an interaction [F(16, 152)=2.541, p=0.002]. Animals exposed to acute stress, a bright light and novel scent during food presentation, exhibited no change in extracellular DA in response to the food reward (Figure 2.4A). In contrast, animals treated with BPN 24 hours prior to testing showed restored DA response to the palatable food reward.

Exposure to stress also increased latency to approach the food compared to unstressed animals (Figure 4B). A one way ANOVA also revealed a significant effect of stress on latency to approach the food reward [F(2,19)=4.735, p=0.021]. This effect was reversed in animals pretreated with BPN. Interestingly, there was no significant difference between groups in amount of food consumed [F(2,19)=0.040, p=0.961]

(Figure 2.4C).

Examination of the behavioral profile indicated differences in burying and rearing behaviors before, during, and after food presentation between groups. There was a significant main effect of time on burying behavior [$F(8,152)=6.068$, $p=0.001$] (Figure 2.5A). For rearing behavior, there was a significant main effect of time [$F(8,152)=6.958$, $p=0.001$] and an interaction [$F(16, 152)=2.242$, $p=0.006$]. Untreated animals exposed to the stressor exhibited increased rearing during and after the feeding period compared to unstressed animals (Figure 2.5B). There were also significant main effects of time on grooming [$F(8,152)=2.816$, $p=0.006$] and idle [$F(8,152)=6.848$, $p=0.001$] behavior.

Discussion

The present study is the first to establish in mice that repeated exposure to palatable food elicits DA efflux in the NAc, and that the major component of the DA response is associated with the conditioned incentive salient effects, as measured by the decreased latency to approach the food. Because mice were not food deprived prior to testing, approach behavior likely represents motivational "wanting" as opposed to a homeostatic drive to feed. Presentation of the palatable food in an unfamiliar feeding environment, or to mice that were naïve to the food reward, failed to elicit a NAc DA response. The DA response was limited to the NAc and did not occur in the striatum. Altogether, these findings indicate a critical role for NAc DA transmission as a substrate for facilitating the emergence and maintenance of motivated behavioral output for food reward. Furthermore, we showed that exposure to stressful stimuli during food presentation diminishes the NAc DA response and increases approach latency to the conditioned food stimulus. Remarkably, pretreatment with BPN 24 hours prior to testing blocked the effects of stress on NAc DA reactivity and restored approach behavior for the food, suggesting a role for opioid systems in mitigating the aversive effects of stress on incentive salience for natural rewards.

There is an expansive body of literature implicating a role for DA in mediating reward-seeking behavior for natural reinforcers, the majority of which have been conducted in rats (Ikemoto and Panksepp, 1999). Although the present study design is similar in some respects to those done in rats, our findings reveal key disparities in the correlational relationship between NAc DA response to food and incentive behavior. Notably, in rats, consumption of a novel food, but not a familiar food, stimulates DA release in the NAc (Bassareo and Di Chiara, 1997; Gambarana et al., 2003). Thus, DA

response to a novel food in rats likely serves as a learning cue that is subject to habituation following repeated exposure. In contrast, we show that repeated exposure to a food stimulus in a familiar feeding environment is necessary to induce a DA response in the mouse NAc. Furthermore naïve mice exhibited more burying and idleness during food presentation, suggesting that the animals found the novel food aversive rather than appealing. Overall, these findings highlight the importance of environmental context in the emergence of conditioned neurochemical and behavioral responses to rewards in the mouse.

In rats, stimuli that predict a reward consistently promote DA release in the NAc, whereas the DA response to physical consumption of the reward is variable (Cenci et al., 1992; Salamone et al., 1994; Martel and Fantino, 1996; Hajnal and Norgren, 2001). In the current study, test cage trained mice presented with the predictive stimulus (empty food dish) exhibited a sharp rise in NAc DA efflux that rapidly returned to baseline. However, in animals allowed to feed DA efflux persisted throughout food presentation and after removal of the food. Therefore, NAc DA transmission in mice is responsive to both anticipatory and consummatory cues.

The chronic mild stress (CMS) paradigm has repeatedly been shown to reduce preference for a sucrose solution in rodents. (Papp et al., 1991; Willner et al., 1992; Rygula et al., 2005; Willner, 2005). Optogenetic activation of VTA DA neurons restores sucrose preference in mice after CMS exposure, implicating a role for the mesolimbic DA system in mediating stress-induced changes in reward processing (Tye et al., 2013). Although reduced sucrose preference is often cited as an anhedonic effect, the translational value of this test is limited in that it more closely models changes in hedonic value of a reward, which is not typically observed in anhedonic patients (Berlin et al.,

1998; Dichter et al., 2010). CMS has also been shown to blunt NAc DA reactivity to palatable food in rats (Di Chiara and Tanda, 1997; Di Chiara et al., 1999; Gambarana et al., 2003). However, because stress did not alter approach behavior or food consumption in these studies, it is difficult to interpret how diminished NAc DA response influenced motivational salience.

In the present study we adapted a hyponeophagia-based paradigm to model how stress affects the neurochemical underpinnings of motivational drive. The suppression of feeding in a novel environment is a well-documented phenomenon in rodents and often used to assess emotionality and anxiety. Chronic treatment with antidepressants or acute treatment with anxiolytic drugs reduces approach latencies for food in a novel environment, suggesting a restoration of incentive salience for the food (Bodnoff et al., 1988; Bodnoff et al., 1989; Dulawa and Hen, 2005). We hypothesized that reduction in NAc DA transmission in response to food presentation by a stressful or unfamiliar environment may be associated with changes in approach behavior, and possibly serve as a neurochemical substrate of a component of anhedonia. Indeed, presentation of a bright light and novel scent during food exposure blunted NAc DA reactivity to the conditioned food stimulus and significantly increased the time to consume the food. Interestingly, the amount of food consumed was not altered in animals exposed to the novel stimuli, signifying that stress specifically alters neurocircuitry associated with motivational drive as opposed to hedonic value. These findings are in line with the incentive salience theory which postulates that hedonic "liking" and motivational "wanting" are independent psychological processes controlled by different neural mechanisms (Berridge, 2007; Wise, 2008a). Specifically, the theory posits a role for DA in mediating appetitive goal-directed behavior. Hence, anhedonic behavior may derive

from alterations in dopaminergic activity that would normally promote motivational salience.

The brain opioid system has long been known to play a role in mediating reward seeing-behavior. Activation of mu-opioid receptors (MORs) in the ventral tegmental area results in increased DA release in the NAc, an effect that is associated with increased intake of palatable food (Spanagel et al., 1992; Zhang et al., 2003; Pecina and Berridge, 2005; Katsuura and Taha, 2010; Murray et al., 2014). In contrast, activation of kappa opioid receptors (KORs) in the NAc inhibits DA neurotransmission and induces prodepressive behaviors in rodents (Maisonneuve et al., 1994; McLaughlin et al., 2006b). Moreover, exposure to stress increases levels of dynorphins (the endogenous ligand of KORs) in limbic regions. Consequently, increased activation of KOR is a potential mechanism in which stress diminishes DA reactivity to food reward. It is important to note, however, that physical stressors can also increase DA efflux in the NAc (Thierry et al., 1976; Abercrombie et al., 1989; Imperato et al., 1992; Kalivas and Duffy, 1995a). Thus, environmental stress may differentially affect NAc DA neurotransmission in response to expected rewards.

BPN is a mixed-pharmacology opiate drug, with potent activity as a partial agonist at the mu-opioid receptor (MOR) and antagonist at kappa-opioid receptors (KOR) (Lutfy and Cowan, 2004; Cowan, 2007). Our laboratory and others have shown that low doses of BPN treatment reduce latency to approach palatable food in a novel environment (Almatroudi et al., 2015; Falcon et al., 2015). In the present study BPN was effective in restoring approach behavior and NAc DA reactivity to food in animals exposed to an acute stressor. Animals were tested 24 hours after BPN administration, a time at which the drug is no longer activating MOR, but may still have antagonist activity

at KOR (Paronis and Bergman, 2011). Moreover, the dose of BPN used in the current study does not alter extracellular DA levels in the NAc when injected 1 hour prior to sampling (Falcon et al., 2015); therefore, it is unlikely that DA levels would be altered 24 hours later. Thus, BPN's effect in this paradigm may be mediated by inhibition of KOR-induced reduction in NAc DA, though additional studies are needed to confirm this.

DA neurotransmission in the NAc is only one facet of the complex and interconnected circuitries that govern the brain reward system. Dopaminergic VTA neurons also innervate the medial prefrontal cortex (mPFC) and basolateral amygdala (BLA), structures known to mediate the processing of both rewarding and aversive stimuli. The mPFC and BLA also send dense glutamatergic inputs to NAc DA terminals, which can synaptically facilitate or depress DA efflux in the NAc (Floresco et al., 1998; Jackson and Moghaddam, 2001). Moreover, DA transmission in the NAc has been shown to interact with corticolimbic glutamate signaling to facilitate the emergence of incentive salience for a food reward (Faure et al., 2008). Additional studies are needed to determine how stress-induced changes in glutamate transmission modulate DA-mediated incentive salience.

In conclusion, our findings with a mouse model measuring the motivational aspects and neurochemical substrates underlying approach behavior for palatable food reward suggest a unique role of NAc DA transmission in establishing and mediating incentive salience for a palatable food reward in mice. Moreover, we show that exposure to an acute stressor is sufficient to alter NAc DA responsiveness to a food reward, which in turn diminishes approach behavior. The ability of BPN to prevent changes in neurotransmission and behavior caused by stress exposure supports the opioid system as a potential target for the development of novel therapeutic treatments of anhedonia

and emotional dysfunction.

FIGURE 2.1
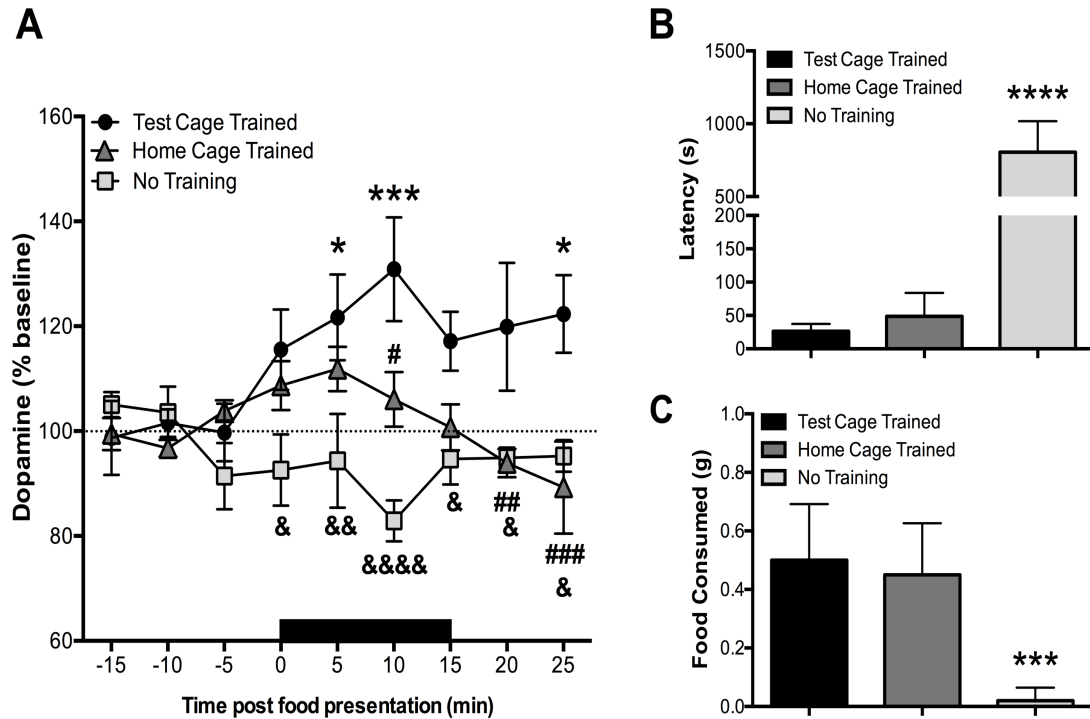
Effects of training experience on NAc DA response and approach behavior to palatable food. A) NAc DA response to food presentation in test cage trained (n=7), home cage trained (n=6), and naïve animals (n=5). Black bar denotes duration of food exposure. Test cage trained animals were the only group to exhibit a significant change from baseline in DA levels in response to the food reward. The asterisk denotes significant differences compared to baseline values within the test cage trained group (*p<0.05, ***p<0.001). The symbol **#** denotes significant differences in DA release between test cage trained and home cage trained animals (#p<0.05). The symbol **&** denotes significant differences in DA release between test cage trained and naïve animals (&p<0.05). **B)** Naïve animals took longer to approach the food and **C)** consumed less food compared to test cage trained animals. The asterisk denotes a significant difference compared to test cage trained animals (***p<0.001, ****p<0.0001). Data is depicted as mean ± SEM.

FIGURE 2.2
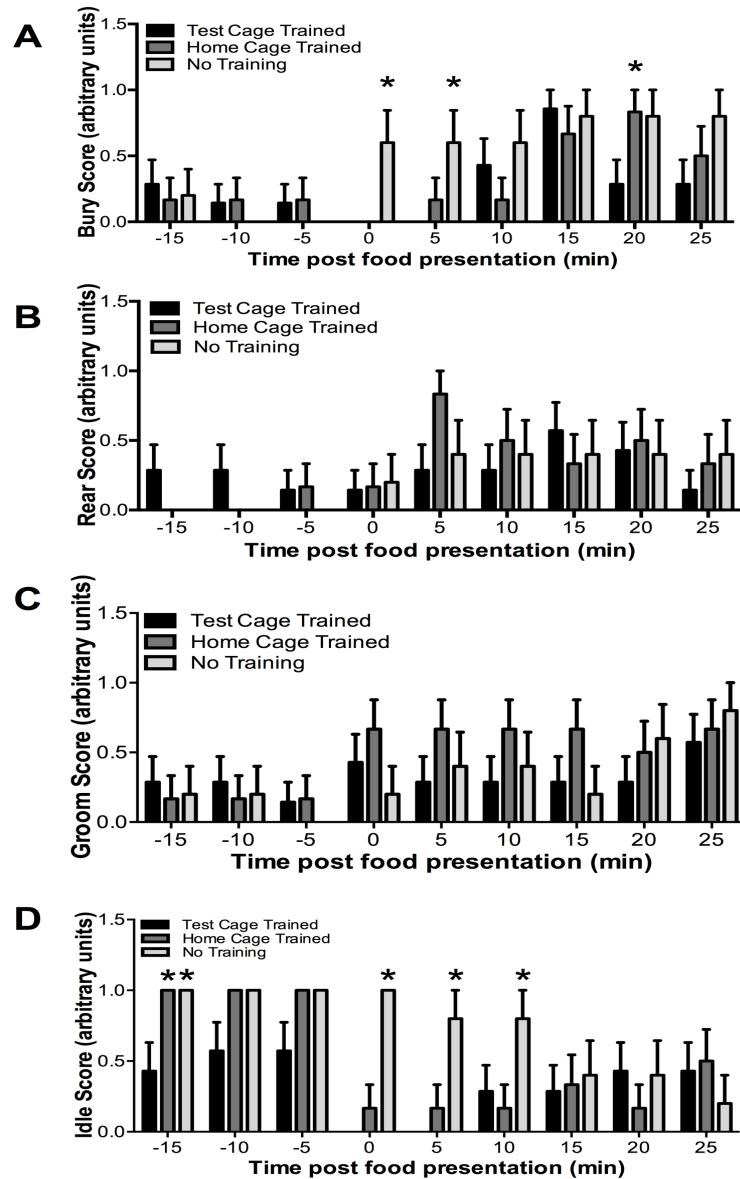
Effects of training experience on behavioral profile. Time course of behavioral profiles of test cage trained (n=7), home cage trained (n=6), and naïve animals (n=5). Behavior was scored in blocks of 5 minutes and measured before, during, and after food exposure. Animals were evaluated for changes in **A)** burying, **B)** rearing, **C)** grooming and **D)** idle behavior. Naïve animals exhibited more burying behavior and idle behavior during food presentation. The asterisk denotes a significant difference compared to test cage trained animals. (*p<0.05). Data is depicted as mean ± SEM.

FIGURE 2.3
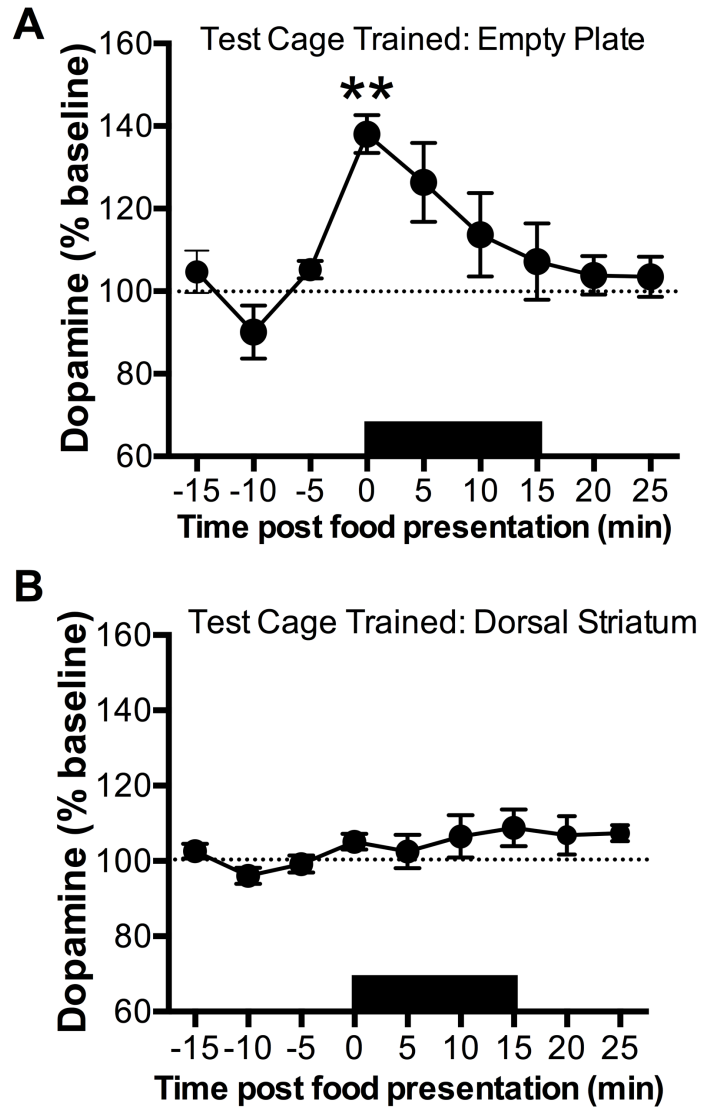
DA response to conditioned cues for food reward and effects of food exposure on general striatal activity. Black bar denotes duration of conditioned cue or food exposure. **A)** NAc DA response to presentation of a conditioned cue (empty food plate) in in test cage trained animals exposed to an empty food plate (n=5). Exposure to the empty plate significantly increased NAc DA levels from baseline. The asterisk denotes significant differences compared to baseline values (**p<0.01). **B)** Dorsal striatum DA response to food presentation in test cage trained animals (n=4). Food presentation did not alter DA transmission in the dorsal striatum. Data is depicted as mean ± SEM.

FIGURE 2.4

A

Dopamine (% baseline)

160
140
120
100
80
60

- Test Cage Trained
- Test Cage Trained + Stressor
- Test Cage Trained + Stressor + BPN

*

&&
&
&
##
##

-15 -10 -5 0 5 10 15 20 25

Time post food presentation (min)

B

- Test Cage Trained
- Test Cage Trained + Stressor
- Test Cage Trained + Stress + BPN

Latency (s)

100
50
0

*

#

C

- Test Cage Trained
- Test Cage Trained + Stressor
- Test Cage Trained +Stress + BPN

Food Consumed (g)

1.0
0.8
0.6
0.4
0.2
0.0
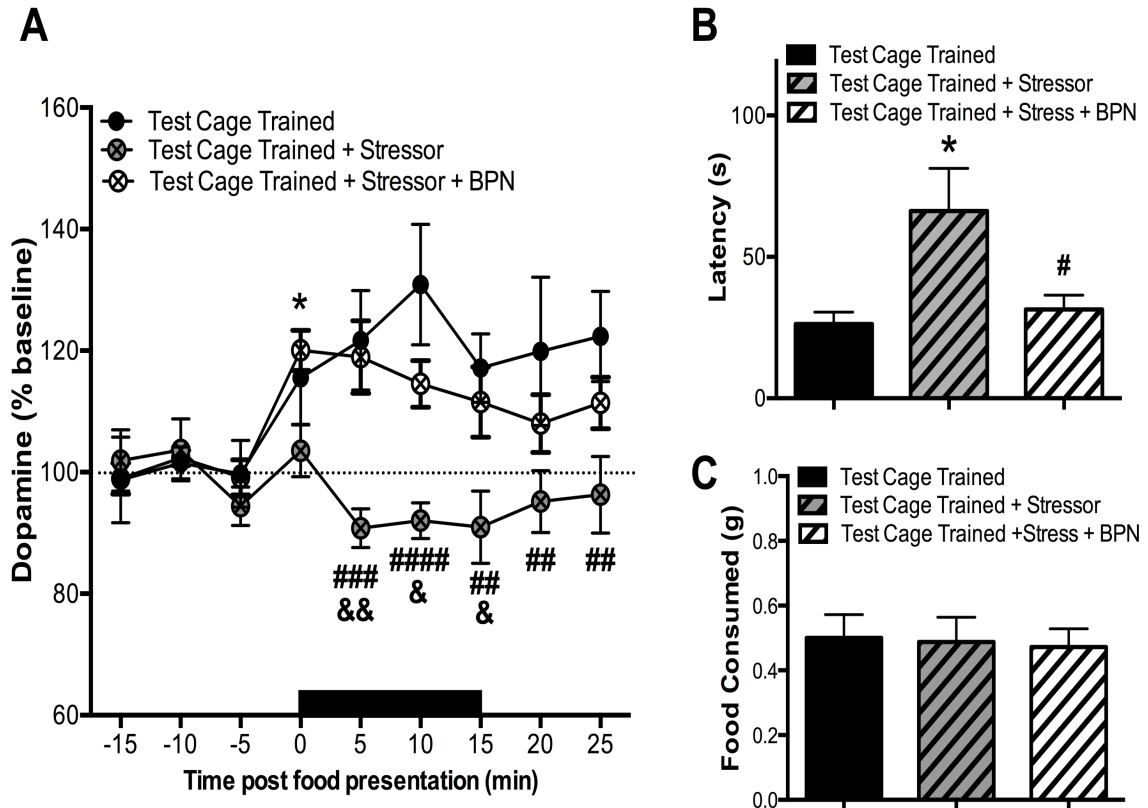
Effects of stress exposure and BPN treatment on NAc DA response and approach behavior to palatable food. A) NAc DA response to food presentation in test cage trained animals exposed to a stressor (bright light and novel vanilla scent) during food presentation (n=8), and test cage trained animals treated with BPN and exposed to a stressor during food presentation (n=7). Black bar denotes duration of food and stress exposure. BPN treatment prevented stress-induced inhibition of NAc DA response to food. The asterisk denotes significant differences compared to baseline values within the BPN treated group (*p<0.05). The symbol **#** denotes significant differences in DA release between test cage trained animals and untreated test cage trained animals exposed to the stressor (##p<0.01, ###p<0.001, ####p<0.0001, analysis includes data from Figure 2.1A). The symbol **&** denotes significant differences in DA release between untreated and BPN treated test cage trained animals exposed to the stressor (&p<0.05, &&p<0.01). **B)** Untreated test cage trained animals exposed to the stressor took longer to approach the food compared to non-stressed test cage trained animals. Approach latency was restored to control values in BPN treated animals. The asterisk denotes a significant difference compared to test cage trained animals (*p<0.05, analysis includes data from Figure 2.1B). The symbol **#** denotes a significant difference between untreated and BPN treated test cage trained animals exposed to the stressor (#p<0.05). **C)** There was no significant effect of group condition on amount of food consumed. Data is depicted as mean ± SEM.

FIGURE 2.5
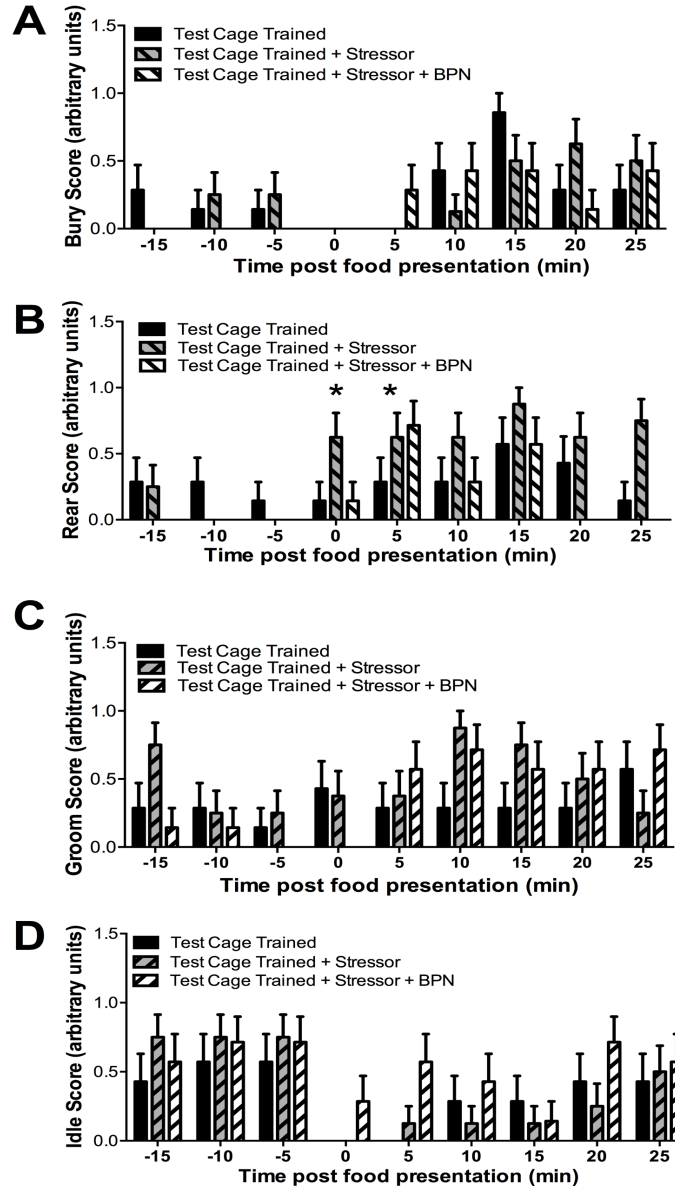
Effects of stress exposure and BPN pretreatment on behavioral profile. Time course of behavioral profiles of test cage trained animals exposed to a stressor during food presentation (n=8), and test cage trained animals treated with BPN and exposed to a stressor during food presentation (n=7). Behavior was scored in blocks of 5 minutes and measured before, during, and after food exposure. Animals were evaluated for changes in **A)** burying, **B)** rearing, **C)** grooming and **D)** idle behavior. Untreated animals exposed to stress displayed more rearing behavior during food presentation compared to non-stressed test cage trained animals. The asterisk denotes a significant difference compared to test cage trained animals (*p<0.05, analysis includes data from Figure 2.2). Data is depicted as mean ± SEM.

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CHAPTER 3: A ROLE FOR THE MU OPIOID RECEPTOR IN THE ANTIDEPRESSANT-LIKE EFFECTS OF BUPRENORPHINE

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Abstract

Buprenorphine (BPN), a mixed opioid drug with high affinity for mu (MOR) and kappa
(KOR) opioid receptors, has been shown to produce behavioral responses in rodents
that are similar to those of antidepressant and anxiolytic drugs. Recent studies have
identified KORs as the primary mediator of BPN's effects in rodent models of
depressive-like behavior. However, the role of MORs in BPN's behavioral effects has not
been as well explored. The current studies investigated the role of MORs in mediating
conditioned approach behavior in the novelty-induced hypophagia (NIH) test, a
behavioral measure previously shown to be sensitive to BPN treatment. The effects of
BPN were evaluated in the NIH test 24 hr post-administration in mice with genetic
deletion of MOR (Oprm1$^{-/-}$) or KORs (Oprk1$^{-/-}$), or after pharmacological blockade with
the non-selective opioid receptor antagonist naltrexone. Behavioral responses to BPN in
the NIH test were blocked in Oprm1$^{-/-}$ mice, but not Oprk1$^{-/-}$ mice. To further elucidate
the opioidergic mechanisms underlying behavioral response in the NIH paradigm,
animals were treated with the MOR agonist morphine, the KOR antagonist nor-BNI, or
the selective MOR antagonist cyprodime and assessed for approach behavior in the
novel arena. The antagonist cyprodime significantly reduced approach latency, whereas
morphine and nor-BNI were both ineffective. Moreover, antinociceptive studies with
morphine confirmed MOR antagonist effects of cyprodime and revealed MOR antagonist
properties of BPN 24 hr post-administration. Interestingly, the combined treatment of
naltrexone with BPN did not block BPN's ability to reduce approach latency in the novel

arena. Altogether, these data support modulation of MOR activity as a key component of

BPN's antidepressant-like effects in the NIH paradigm.

Introduction

Opioid receptors and their endogenous ligands are important modulators of neural pathways involved in the regulation of mood and emotional states (Lutz and Kieffer, 2013a). The kappa opioid receptor (KOR) in particular is thought to contribute to the etiology of depression and anxiety (Bruchas et al., 2010). Exposure to stress increases expression of dynorphin, a neuropeptide that acts primarily at KORs (Shirayama et al., 2004). Activation of KORs by dynorphin or a KOR agonist has been shown to elicit dysphoria and depressive-like behavior in rodents (Carlezon et al., 2006; McLaughlin et al., 2006c; McLaughlin et al., 2006a), whereas treatment with a selective KOR antagonist mitigates stress-induced affective behavior (Mague et al., 2003; McLaughlin et al., 2003; Carr et al., 2010). These preclinical findings have lent support to pharmacological modulation of the opioid system as a potential target for the development of novel antidepressants. Buprenorphine (BPN), a mixed-opioid drug, has emerged as a promising new candidate as an antidepressant drug. Clinical studies have shown low doses of BPN to be effective in alleviating depressive symptoms in treatment-resistant patients and reducing suicidal ideation in severely suicidal patients (Bodkin et al., 1995b; Nyhuis et al., 2008; Yovell et al., 2015). Most notably, mood-elevating effects were observed within a couple weeks of treatment, in stark contrast to first-line treatments that often take 4-6 weeks to enhance mood.

BPN is a high affinity partial agonist at the MOR and an antagonist at the KOR. Preclinical studies have identified KOR antagonism as a potential mediator of BPN's therapeutic effects in the FST (Almatroudi et al., 2015; Falcon et al., 2015). Whether

BPN's effects at the MOR significantly contribute to its effects on other behavioral tests for antidepressant activity is not as well understood. Our laboratory has previously shown that a single administration of low dose BPN ameliorates stress-induced suppression of approach behavior for palatable food when tested 24 h post-administration (Falcon et al., 2015). The goal of the present study was to investigate the role of individual opioid receptors in mediating BPN's restoration of conditioned approach behavior in the novelty-induced hypophagia test. To that end, we employed two approaches, genetic deletion and selective pharmacological modulation of opioid receptors, to distinguish the MOR from the KOR as the primary mediator of BPN's effects in this paradigm. The results of this study show that BPN was effective in reducing approach latency in the novel arena in Oprk1$^{-/-}$ mice but not Oprm1$^{-/-}$ mice. Administration of the selective MOR antagonist cyprodime, but not the MOR agonist morphine or KOR antagonist nor-BNI, replicated the effects of BPN in the NIH test. Moreover, BPN's effects were not blocked by combination treatment with naltrexone. These studies suggest a role for the MOR in mediating the antidepressant-like response of BPN in the NIH test as a behavioral measure of motivational conflict.

Materials and Methods

Animals

Male C57BL/6J mice, 7 weeks upon arrival and purchased from Jackson Laboratories (Bar Harbor, ME) were used for the majority of the studies. Male Oprm1$^{-/-}$ mice and littermate wild-type (WT) controls were generated using heterozygous breeding. Male Oprk1$^{-/-}$ mice used in this study were generated from mating pairs of Oprk1$^{-/-}$ purchased from Jackson Laboratories and maintained via homozygous breeding. Male C57BL/6J mice, originally from Jackson Laboratories but generated within the colony, were used as their wild type (WT) controls because this was the background strain used in all of the genetic lines. Mice were housed up to 5 per cage (or in pairs for NIH experiments) in polycarbonate cages and maintained under a 12 h light-dark cycle (lights on at 0700 hours) in a temperature (22 °C) and humidity-controlled environment. Food and water were available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Drugs

Buprenorphine hydrochloride (Sigma, St. Louis, MO and NIDA) and norbinaltorphimine (nor-BNI; Tocris Bioscience, Ellisville, MO) were administered at doses previously found to have antidepressant effects in C57BL/6J mice (Falcon et al., 2015). Doses of morphine sulfate (Spectrum Chemical, New Brunswick, NJ), U50,488 (Sigma), naltrexone hydrochloride (Sigma), and cyprodime hydrochloride (Tocris) were chosen

based on results from pilot studies investigating their antinociceptive effects. Buprenorphine and nor-BNI were dissolved in distilled water and injected intraperitoneally (i.p.). Morphine and naltrexone were dissolved in physiological saline and injected i.p. Cyprodime was dissolved in 1% ethanol and delivered by i.p. injection. Mice in the control groups were injected with vehicle or saline where appropriate. All doses were calculated according to the base weight of the drug and administered in a volume of 10 ml/kg.

Hot plate test

Mice were individually placed onto a hot plate heated to 55°C and enclosed by a Plexiglas container. The latency for the mouse to lick the hindpaw or jump was recorded. An 80-second cut-off was imposed to avoid tissue damage. The non-selective opioid antagonist naltrexone (1 mg/kg) (n = 6-7 per group) and selective MOR antagonist cyprodime (10 mg/kg) (n= 8-16 per group) were injected 1 hour prior to morphine or U50,488 administration and animals were tested 30 minutes later to determine the MOR and KOR antagonist properties of each drug. To assess the protracted effects of BPN on MOR activity, animals were examined for baseline antinociceptive response 24 hours after BPN (0.25 mg/kg) and then tested 30 minutes after morphine (10 mg/kg) administration (n = 9 per group). Data for this experiment is expressed as percentage of baseline (pre-morphine) response.

Novelty-induced hypophagia (NIH) test

Mice were pair housed and trained to eat a palatable food (three peanut butter chips presented in a small, clear petri dish) in a home feeding cage. Opaque, black, plastic dividers were placed inside each cage to separate the mice during home cage training

sessions. Mice were allowed to habituate to the dividers for 1 hr before the start of the training session. Animals were trained daily in 15-min sessions until they met the criteria of three consecutive days with approach latencies of 30 seconds or below. For novel cage testing, mice were placed in an empty, clear polycarbonate cage (25.5 × 46 × 20 cm) with bright lighting (60 W light bulb) and scented with lemon (20% Lemon Joy solution). There was no food deprivation or habituation period prior to the novel cage test. The novel cage test session was videotaped, and the latency to approach during the 15-min test session was measured. The approach latency was defined by the time it took the mouse to approach the dish in the center of the arena and begin feeding.

In Experiment 1, mice were treated with BPN (0.25 mg/kg) immediately after their last training session and tested 24 hr later in the novel environment (n = 8-19 per group). Similarly, in Experiment 2 mice were injected with morphine (10 mg/kg) or nor-BNI (10 mg/kg) and tested 24 hr later (n = 10-22 per group). In Experiment 3 mice were injected with cyprodime (10 mg/kg) 24 hr or 1 hr before testing (n = 10-25 per group). In Experiment 4, mice were either pre-treated with naltrexone (1 mg/kg) 1 hr before BPN (0.25 mg/kg) and tested 24 hr later or treated with naltrexone 24 hr after BPN and tested 1 hr later (n = 9-19 per group).

Data Analysis

One-way and two-way ANOVAs were performed to determine the significance of differences between experimental groups. Significant overall main effects or interactions were followed by Holms-Sidak's post hoc test. Unpaired two- tailed Student's t tests were applied where appropriate. For all tests, $p < 0.05$ was considered statistically significant. Data are expressed as mean ± SEM.

Results

Effects of MOR and KOR disruption on BPN's behavioral effects in the NIH test

Acquisition of conditioned approach behavior to the palatable food was significantly diminished in Oprm1^{-/-} mice (Figure 3.1). Repeated measures two-way ANOVA revealed a significant main effect of genotype [$F_{(2, 71)}$ = 12.89, $p < 0.001$] and time [$F_{(13, 923)}$ = 89.57, $p < 0.001$], in addition to a significant genotype*time interaction [$F_{(26, 923)}$ = 3.724, $p < 0.001$]. Between the second and tenth day of training Oprm1^{-/-} took significantly longer to reach stable approach latencies compared to WT and Oprk1^{-/-} mice ($p < 0.05$). A main effect of genotype was also observed in terms of days taken to reach criteria [$F_{(2, 68)}$ = 6.102, $p = 0.004$]. Oprm1^{-/-} were slowest to meet the criteria of three consecutive days with approach latencies under 30 seconds ($p < 0.01$).

The behavioral effects of BPN (0.25 mg/kg) in the NIH test 24 h post-administration were assessed in Oprm1^{-/-}, Oprk1^{-/-}, and WT mice (Figure 3.2). A significant genotype*treatment interaction was observed for latency to approach the food [$F_{(2, 70)}$ = 3.573, $p = 0.033$] and amount of food consumed [$F_{(2, 69)}$ = 3.882, $p = 0.025$] in the novel arena. Post-hoc tests revealed BPN significantly reduced approach latencies and increased food consumption in WT ($p < 0.05$) and Oprk1^{-/-} ($p < 0.001$) mice, but not Oprm1^{-/-} mice.

Effects of nor-BNI and morphine in the NIH test 24 h post-administration

The effects of nor-BNI (10 mg/kg) and morphine (10 mg/kg) treatment on approach behavior in the NIH test 24 hr post-administration were assessed in WT animals (Figure

3.3). One-way ANOVA revealed no significant effects of treatment on approach latencies [F (2, 44) = 0.4651, p = 0.631] or food consumption [F (2, 44) = 1.972, p = 0.151].

Effect of selective MOR antagonist cyprodime in the NIH test 1 hr and 24 hr post-administration

The effects of cyprodime (10 mg/kg) treatment on approach behavior in the NIH test 1 h and 24 h post-administration was evaluated in WT animals (Figure 3.4). One-way ANOVA revealed a significant effect of treatment on latency to approach [F (2, 46) = 7.710 P = 0.001 and food consumption F (2, 43) = 5.708 P = 0.006] in the novel arena. Cyprodime administered 1 hr prior to testing significantly reduced latency to approach the food reward (p < 0.01) and increased the amount of food consumed (p < 0.05). This effect was not observed when cyprodime was administered 24 hr prior to testing.

Cyprodime's selectivity for MOR antagonism was confirmed using the hot plate test (Figure 3.5). One-way ANOVA revealed a significant effect of treatment on antinociceptive response [F (4, 46) = 7.513, p < 0.0001]. Antinociception was significantly increased in animals treated with morphine (10 mg/kg, p < 0.0001) or U50,488 (10 mg/kg, p < 0.05). Pre-treatment with cyprodime (10 mg/kg) significantly blocked antinociception induced by both morphine (p < 0.001), but not U50,488.

Pharmacological blockade of opioid receptors does not block BPN's behavioral effects in the NIH test.

Next, we determined the effects of pharmacological blockade of opioid receptors on BPN's behavioral effects in the NIH test (Figure 3.6). The effects of naltrexone (1 mg/kg) pre-treatment or post-treatment in combination with BPN (0.25 mg/kg) were measured in WT mice. Two-way ANOVA revealed a main effect of BPN [F (1, 70) = 11.20 P = 0.0013]

and naltrexone [$F_{(2, 70)} = 9.023$ $P = 0.0003$] on approach latency in the NIH test. Treatment with naltrexone alone significantly reduced approach latency in the NIH test BPN's ability to reduce latency to approach in the novel arena was not affected by either pre-treatment (1 hr prior to BPN administration, 24 hr prior to test) or post-treatment (24 h after BPN administration, 1 hr prior to testing). Similarly, food consumption in the novel arena was increased by both BPN [$F_{(1, 71)} = 3.615$, $p = 0.0613$] and naltrexone [$F_{(2, 71)} = 8.421$, $p = 0.0005$] regardless of time of naltrexone treatment.

The ability of naltrexone to effectively block MOR and KOR agonist activity was confirmed using the hot plate test (Figure 3.7). One-way ANOVA revealed a significant effect of treatment on antinociceptive response [$F_{(4, 28)} = 8.892$, $p < 0.001$]. The latency for animals to lick their hindpaw was significantly increased in animals treated with the selective MOR agonist morphine (10 mg/kg, $p < 0.001$) or the selective KOR agonist U50,488 (10 mg/kg, $p < 0.01$). Pre-treatment with naltrexone (1 mg/kg) significantly blocked antinociception induced by both morphine ($p < 0.001$) and U50,488.

Effect of BPN treatment on morphine-induced antinociception 24 hr post-administration

BPN's activity at the MOR 24 h post-administration was determined using the hot plate test (Figure 3.8). BPN (0.25 mg/kg) administered 24 h prior to morphine significantly reduced morphine-induced antinociception compared to vehicle treated animals ($t = 2.971$, $p < 0.01$).

Discussion

We report here for the first time two important findings concerning the pharmacological properties underlying BPN's mechanism of antidepressant action and the involvement of the opioid system in mediating behavioral states etiologically relevant to human depression. First, utilizing animals with genetic knockdown of individual opioid receptors, we established that the effects of BPN in the NIH test are dependent on the MOR. Second, we demonstrate that BPN acts predominantly as a MOR antagonist at the time of testing (24 h post-administration), and that selective antagonism of the MOR is sufficient to alleviate the aversive effects of novelty on conditioned approach behavior for palatable food. Altogether, these data provide supportive preclinical evidence for the modulation of MOR activity as an integral component of BPN's therapeutic effects.

The NIH test is a conflict-based paradigm that assesses stress-induced changes in motivational salience for a palatable food reward. Chronic treatment with selective serotonin reuptake inhibitors is reported to reduce approach latency for food in a novel environment in certain strains of rodents (Bodnoff et al., 1988; Bodnoff et al., 1989; Dulawa and Hen, 2005; Bechtholt et al., 2008). C57BL/6 mice, however, demonstrate a refractory response to SSRIs in this paradigm (Balu et al., 2009a; Robinson et al., 2016). Our laboratory has previously shown a single administration of low dose BPN to reduce novel arena approach latency in C57BL/6 mice when tested 24 hr post-administration (Falcon et al., 2015). This time point was chosen because BPN treatment induces MOR dependent hyperactivity in C57BL/6 mice that could obfuscate measures of behavioral output in the NIH test (Marquez et al., 2007; Falcon et al., 2015). Due to its complex

pharmacology (Lutfy and Cowan, 2004), the role of individual opioid receptors in mediating BPN's behavioral response in the NIH test are not well understood. Thus, the primary aim of these studies was to systematically dissociate MOR versus KOR mediated mechanisms of action in the NIH paradigm.

The present study is the first to show that genetic deletion of MOR abolishes the behavioral effects of BPN in the NIH test. MORs are well known to play an important role in mediating the reinforcing properties of rewarding stimuli. Oprm1$^{-/-}$ mice fail to show conditioned place preference for drugs of abuse (Matthes et al., 1996; Kieffer and Gaveriaux-Ruff, 2002) in addition to reduced operant and hedonic responding for palatable food rewards (Papaleo et al., 2007). In the current study, Oprm1$^{-/-}$ mice displayed a marked deficit in acquiring conditioned approach behavior towards the peanut butter chips during training sessions. Interestingly, these animals did eventually develop stable approach latencies, suggesting that attribution of incentive salience to palatable food can occur independently of MOR activity, possibly through adoption of other feeding-related pathways (Menzies et al., 2013). Oprm1$^{-/-}$ and Oprk1$^{-/-}$ did not significantly differ from WT in their final baseline approach latency in the novel arena. Thus, the pure absence of individual receptors does not appear to mitigate the effects of stress in this paradigm. This is in contrast to previous studies that have reported increased behavioral resistance to stress in Oprk1$^{-/-}$ mice (Redila and Chavkin, 2008; Falcon et al., 2016a) and reduced anxiogenic behavior in Oprm1$^{-/-}$ mice (Filliol et al., 2000). However, the present study is not directly comparable due to the differences in the behavioral tests measured. Interestingly, BPN efficacy in the NIH test was more pronounced in Oprk1$^{-/-}$ compared to WT mice. This may be due to compensatory upregulation of delta opioid receptors (Slowe et al., 1999), which have been shown to heterodimerize with MOR (Gomes et al., 2000).

Pre-treatment with the non-selective opioid antagonist naltrexone did not alter behavioral response to BPN in the NIH test. Similarly, naltrexone administered 24 hr post BPN (1 hr prior to testing) did not block BPN's effect on approach behavior in the novel arena. Naltrexone was administered at a dose experimentally confirmed to antagonize opioid activity at both the MOR and KOR. Thus, MOR activation by BPN is not required to obtain subsequent behavioral effects measured at 24 hr post-administration. This was confirmed in a separate experiment showing that administration of morphine had no effect in the NIH test when assessed 24 hr post-administration. Interestingly, treatment with the KOR antagonist nor-BNI was also ineffective in reducing approach latency in the NIH test. Although this data corroborates our findings with knockout animals, it is contradictory to other studies demonstrating behavioral response to nor-BNI in the NIH test conducted in mice or rats (Almatroudi et al., 2015; Huang et al., 2016). This discrepancy may be attributed to differences in procedural execution or strain differences in behavioral response to KOR antagonists in this paradigm.

One of the more striking findings arising from the present study is that treatment with the selective MOR antagonist cyprodime was sufficient to reduce approach latency in the NIH test. Notably, cyprodime's effects were observed 1 hr post-administration but not 24 hr post-administration, indicating acute, but not protracted, behavioral effects of this drug. Studies conducted by Almatroudi et al. (2015) showed no behavioral effect of the irreversible MOR antagonist CCAM in the NIH test when administrated 48 hr prior to testing. However, CCAM may more closely model genetic deletion of MORs rather than the competitive blockade of MOR activity by cyprodime described here. Studies characterizing the antinociceptive effects of BPN have shown it to exhibit a rapid onset of action at the MOR followed by slow receptor dissociation, resulting in a prolonged

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blockade of MOR for hours following injection (Cowan et al., 1977; Walker et al., 1995). In the present study we confirmed that pretreatment of BPN at the dose used in NIH testing antagonized the antinociceptive effects of morphine when tested 24 h post-administration of BPN. Thus, the eventual blockade of MORs, as opposed to the brief period of MOR activation, likely mediates the protracted effects of BPN in the NIH test.

Given the role of MOR activation in mediating appetitive control, it is somewhat counterintuitive that blockade of MORs reinstated motivational behavior for palatable food. However, MORs are also known to modulate stress-induced changes in dopamine outflow in key brain regions involved in the processing of reward. Activation of MOR in the ventral tegmental area (VTA) has been shown to reduce dopamine transmission in the nucleus accumbens (NAc), a region highly implicated in the presentation of goal-directed behavior (Berridge, 2012a; Latagliata et al., 2014). Moreover, local infusion of a MOR agonist into the central amygdala reduced open arm time in the elevated plus maze, suggesting a MOR mediated induction of anxiety-like behavior during exposure to novelty stress (Wilson and Junor, 2008). Thus, MOR activity may exert context-specific regulation of circuitries involved in reward processing, so as to promote reward-seeking behavior under basal conditions but not stressful conditions. Consequently, antagonism of MORs during exposure to acute stress may prevent stress-induced decreases in affective behavior.

In conclusion, these studies propose a novel mechanism of action for BPN's antidepressant like effects in the novelty-induced hypophagia test, a behavioral measure of stress-induced motivational conflict. Utilizing genetic and pharmacological tools, we present a systematic approach to characterizing the role of opioid receptors in mediating affective behavior. The results of this study encourage further investigation into the

identification of neurobiological mechanisms underlying the effects of BPN in specific

behavioral domains.

Acknowledgements

FIGURE 3.1

A

B
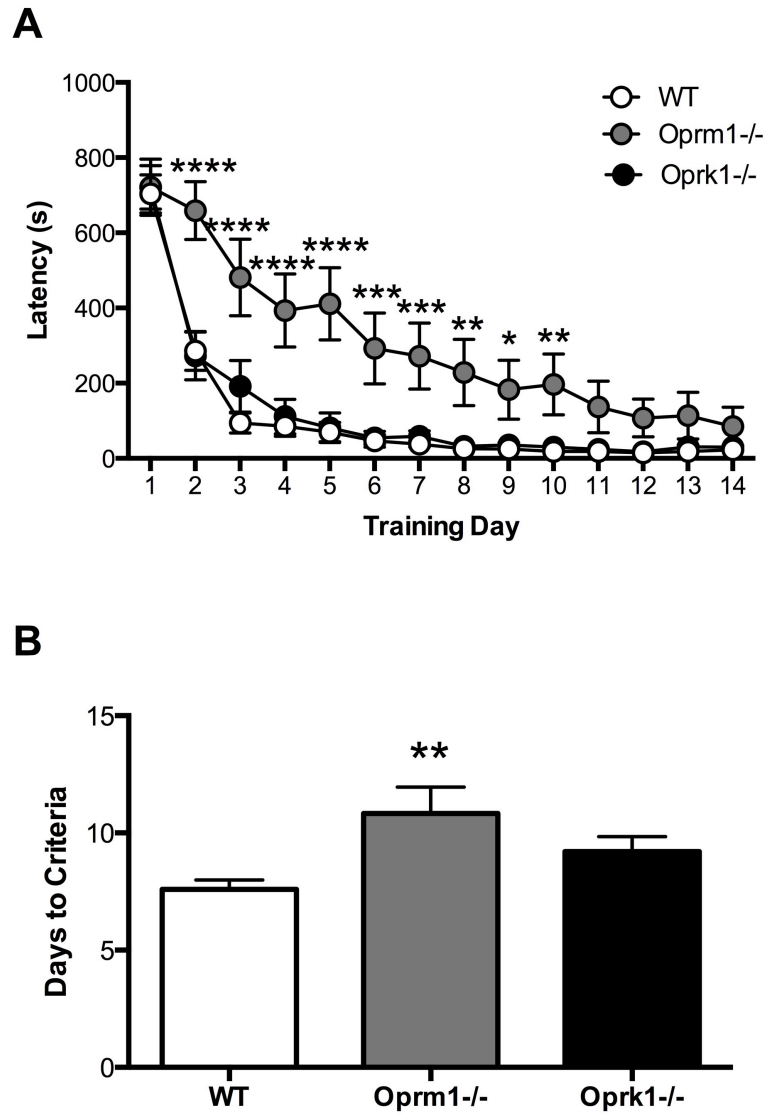
Effect of genotype on home cage training. A) Oprm1-/- mice exhibited higher approach latencies compared to WT mice during training and B) took significantly longer to reach criteria compared to WT mice. n = 18-36 per group. (* p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.00001 compared to WT). Data is depicted as mean ± SEM.

FIGURE 3. 2
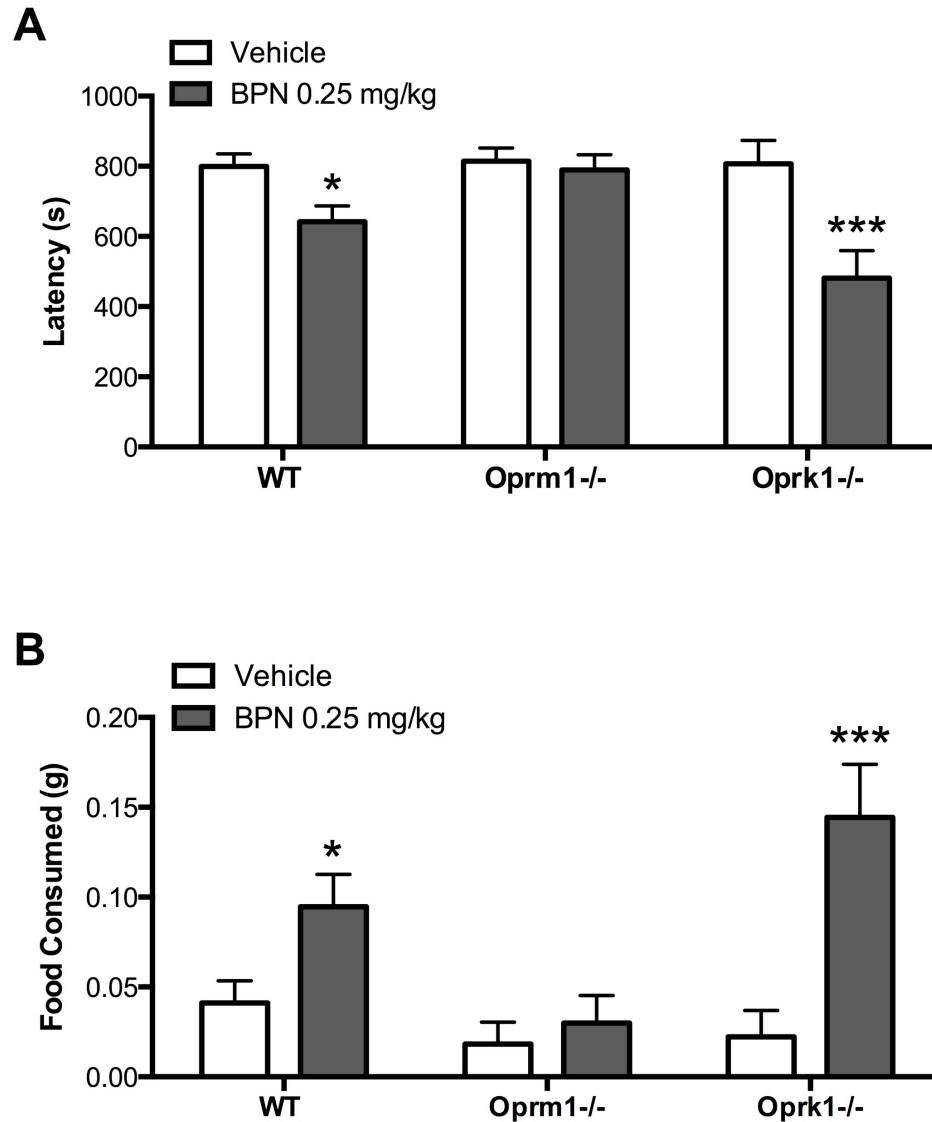
BPN's behavioral effects in the NIH test are blocked in Oprm1$^{-/-}$ mice. A). BPN reduced latency to approach and B) increased food consumed in WT and Oprk1$^{-/-}$ mice but not Oprm1$^{-/-}$ mice. n = 8-19 per group. (*p < 0.05, ***p < 0.001 compared to vehicle). Data are depicted as mean ±SEM.

FIGURE 3.3
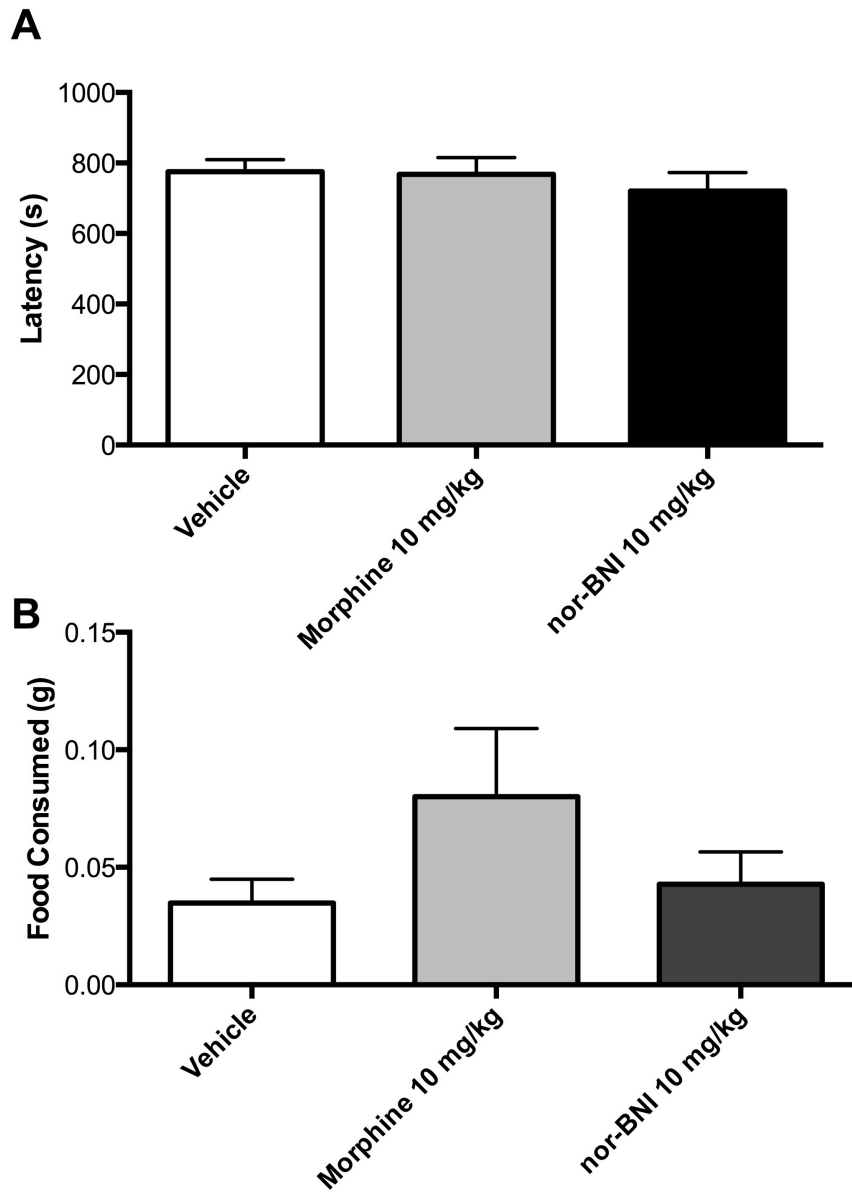
Treatment with morphine or nor-BNI does not affect behavior in the NIH test 24 h post administration. Treatment with a MOR agonist or KOR antagonist had no effect on A) approach latency in the NIH test or B) amount of food consumed in the novel arena. n = 10-22 Data are depicted as mean ±SEM.

FIGURE 3.4

A

B
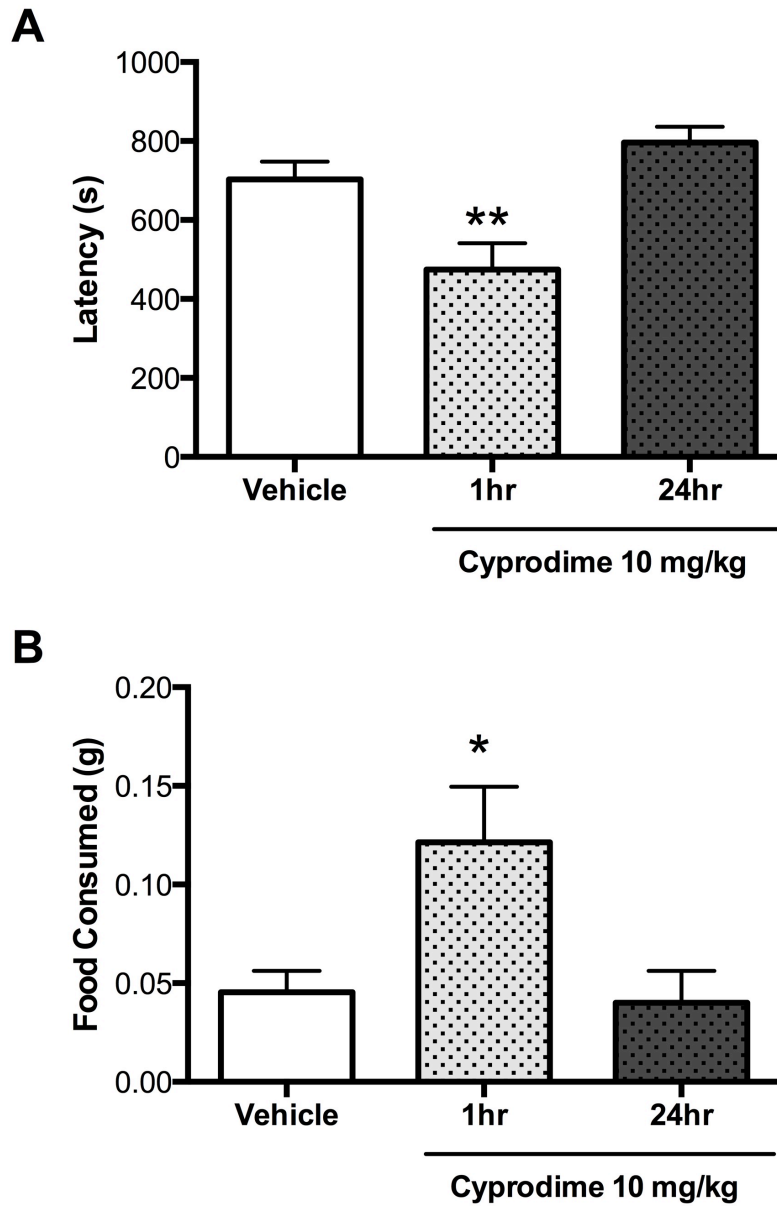
Treatment with cyprodime reduced approach latency in the NIH test 1 h post administration. Treatment with a MOR antagonist was sufficient to A) reduce approach latency and B) increase food consumption 1 h post administration but not 24 h post administration. n = 10-25. (**p < 0.01 compared to vehicle). Data are depicted as mean ±SEM.

FIGURE 3.5
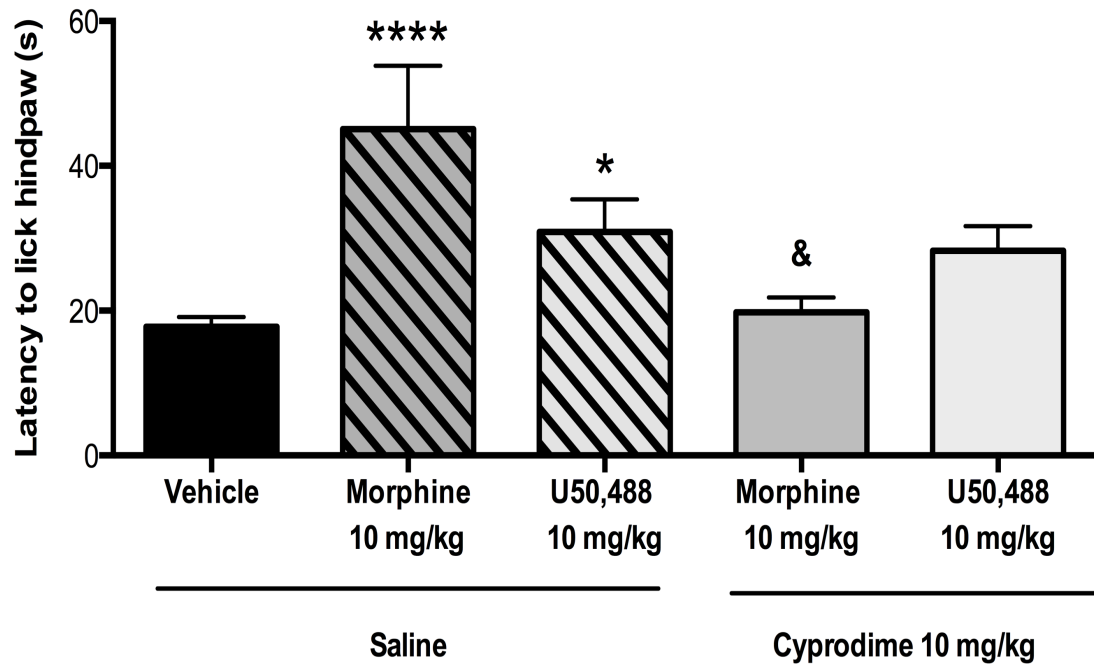
Cyprodime selectively blocks morphine-induced antinociception. Pretreatment with cyprodime blocked morphine induced antinociception but not U50,488. n= 8 -16 per group. (*p < 0.05, ****p < 0.0001 compared to saline + vehicle, &p < 0.05 compared to saline + morphine). Data are depicted as mean ±SEM.

FIGURE 3.6
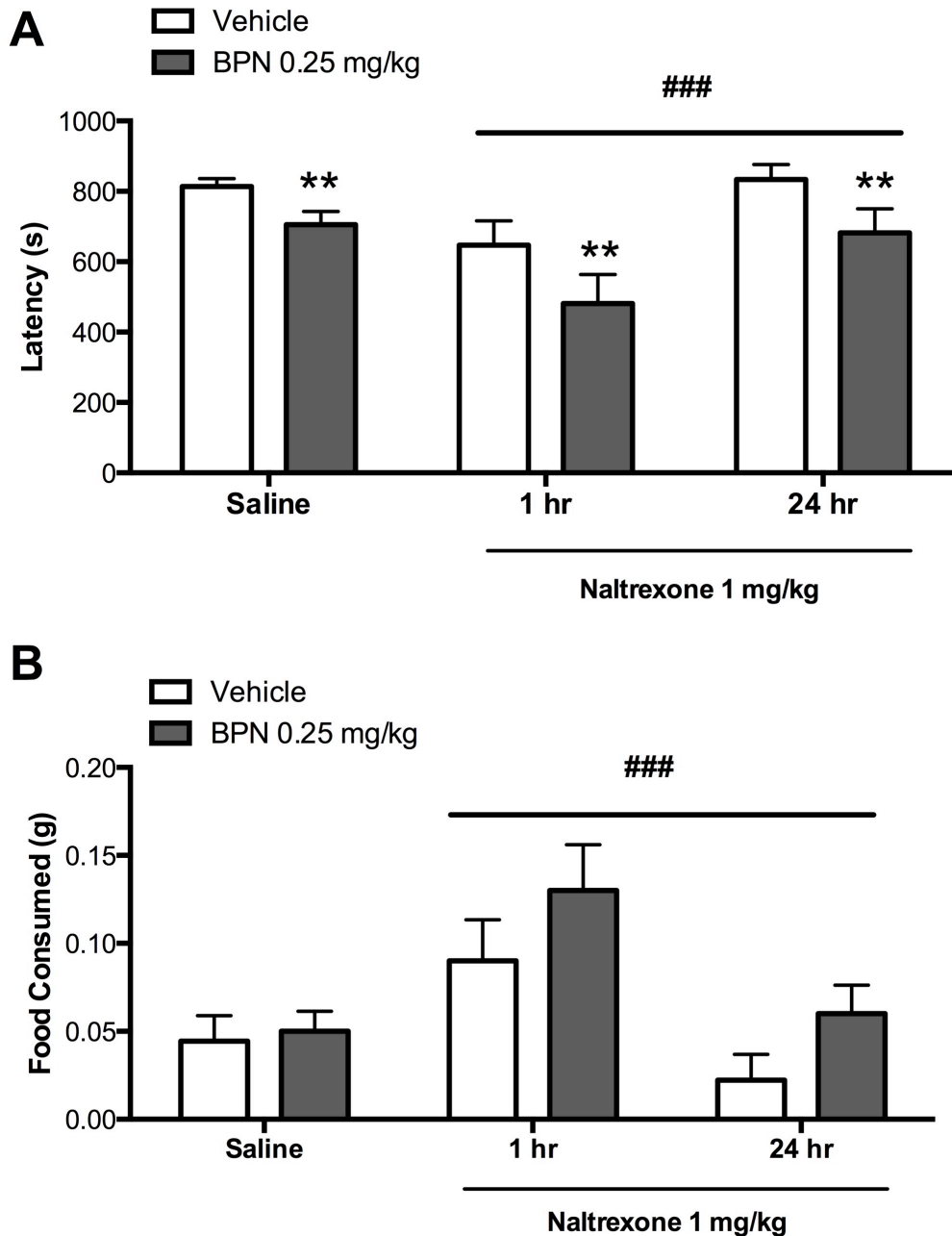
Pharmacological blockade of opioid receptors does not block BPN's behavioral effects in the NIH test. A) Combination treatment of BPN and naltrexone reduced approach latency in the novel arena. Naltrexone given alone also reduced approach latency in the NIH test. B) Naltrexone treatment increased food consumption in the novel arena. n = 9-19. (**p < 0.01 compared to vehicle, ###p < 0.001 compared to saline).

FIGURE 3.7
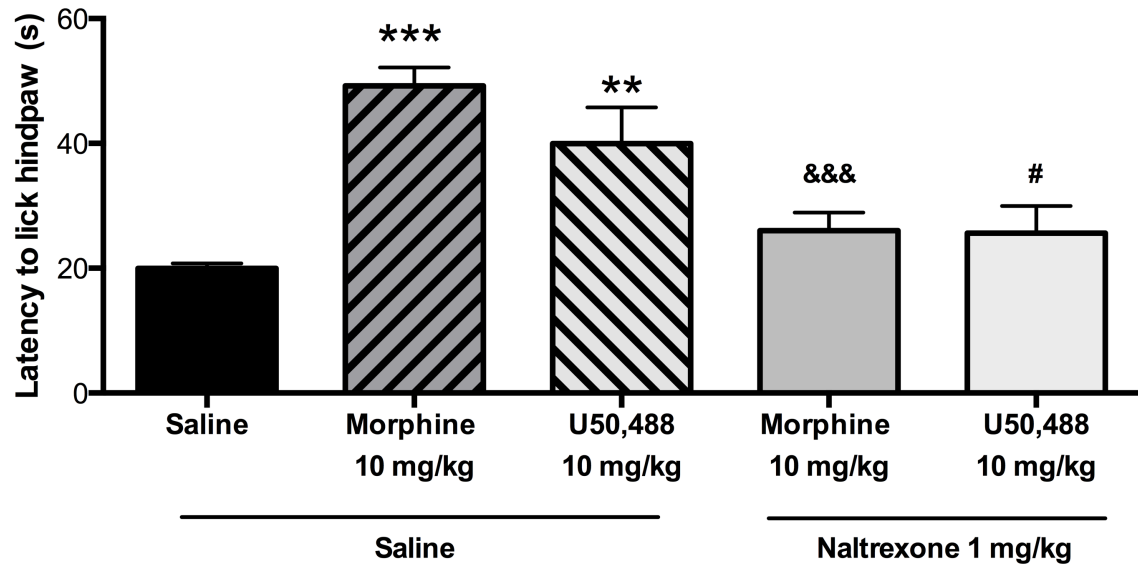
Naltrexone blocks morphine and U50,488-induced antinociception. Naltrexone pretreatment blocked antinociceptive effects of morphine and U50,488. n = 6-7 per group. (**p < 0.01, ***p < 0.001 compared to saline + saline, &&&p < 0.001 compared to saline + morphine, #p < 0.05 compared to saline + U50). Data are depicted as mean ±SEM.

FIGURE 3.8
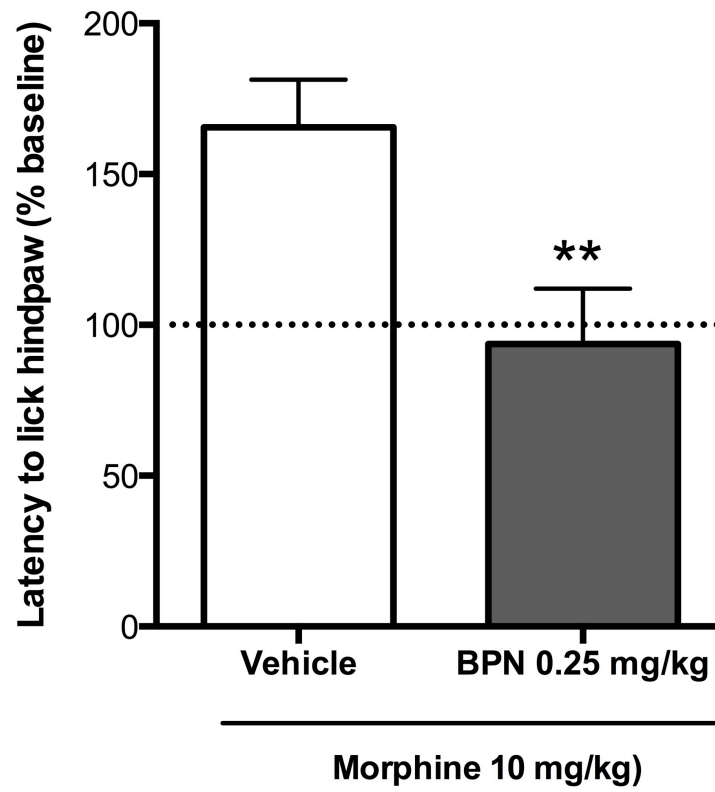
Pretreatment with BPN blocks morphine-induced antinociception. (**p < 0.01 compared to vehicle). n = 9 per group. Data are depicted as mean ±SEM.

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CHAPTER 4: CORTICOSTERONE EXPOSRE AUGMENTS SENSITIVITY TO THE BEHAVIORAL AND NEUROPLASTIC EFFECTS OF FLUOXETINE IN C57BL/6 MICE

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Abstract

Both genetic background and pre-existing stress play critical roles in the effects of antidepressant drugs. The current studies showed this principal by demonstrating that exposure to the stress hormone corticosterone (CORT) allowed behavioral and neurogenic effects to emerge following chronic treatment with fluoxetine of C57BL/6 mice, a strain ordinarily resistant to these effects. Adult male mice were implanted subcutaneously with 21-day slow-release CORT pellets (10 mg) or placebo and then co-treated with 5 mg/kg fluoxetine (b.i.d., i.p.) or saline for 14 days. Animals were then assessed for approach behavior in the novelty-induced hypophagia (NIH) test, hippocampal cell proliferation, corticosteroid receptor expression, and CORT plasma levels. Co-treatment of CORT with fluoxetine significantly reduced approach behavior in the novel environment of the NIH test and increased hippocampal cell proliferation whereas fluoxetine given alone was ineffective. CORT given alone did not alter approach behavior in the novel environment and caused a smaller increase of cell proliferation. The CORT effect was blocked by adrenalectomy and was likely due to increased adrenal feedback. Cell proliferation in CORT-treated animals was associated with reduced mineralocorticoid, but not glucocorticoid, receptor mRNA expression. Although the pellets were advertised to release CORT for 21 days, plasma CORT levels were increased at 1 day after implantation but were not sustained when measured at 7 days or longer intervals. Nevertheless, the transient CORT increase was sufficient to induce long-lasting behavioral and molecular changes when followed by fluoxetine treatment. These studies warrant further investigation into the role of glucocorticoids and environmental stress as adjunctive facilitators of the response to antidepressants, especially for treatment-resistant patients.

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Introduction

Major Depressive Disorder (MDD) is one of the most common psychiatric disorders, with a lifetime prevalence of 17% in the United States and 4% worldwide (Kessler et al., 2005; Eaton et al., 2008). In terms of years lost to disability, MDD is considered one of the most disabling medical conditions and is predicted to be a leading contributor to the worldwide burden of disease by 2030 (Mathers and Loncar, 2006). The majority of pharmacotherapies developed for the treatment of MDD target brain monoamine systems, primarily serotonin (5-HT), norepinephrine, and dopamine. The most common of these, the selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (SNRIs), comprise a large proportion of pharmaceutical sales and are considered first line treatment for MDD. Unfortunately, an estimated 40% of patients fail to respond to these therapies (Cipriani et al., 2009; Culpepper, 2010). Further insight into the neurobiological mechanisms underlying antidepressant response is needed for the development of more efficacious antidepressant regimens.

The combination of genetic vulnerabilities and environmental factors, such as stress, are thought to be significant contributors to the onset of depression in humans (Charney and Manji, 2004). The likelihood of experiencing a depressive episode is greatly increased following a stressful life event or after accumulation of chronic minor stresses (Caspi et al., 2003; Harkness and Monroe, 2006). Moreover, many patients suffering from depression exhibit signs of dysfunctional hypothalamic-pituitary-adrenal (HPA) axis activity, as demonstrated by elevated basal cortisol levels and resistance to

dexamethasone, an exogenous steroid that suppresses cortisol in healthy individuals (Pariante and Miller, 2001b; Gillespie and Nemeroff, 2005). Interestingly, successful antidepressant treatment is often associated with restored suppression of HPA axis response (Schule, 2007). Together, these findings suggest a potential role of stress hormones, such as cortisol (corticosterone (CORT) in rodents), in the pathology and treatment of depression.

CORT produces its effects in the central nervous system via activation of glucocorticoid (GR) and mineralocorticoid (MR) receptors. Though these receptors are ubiquitous throughout the brain, they are highly abundant in the hippocampus, where they provide crucial inhibitory feedback signals to the HPA axis (Sapolsky et al., 1984; Jacobson and Sapolsky, 1991). A reduction or absence of these inhibitory signals can promote hyperactivation of the axis and augmented secretion of glucocorticoids (Anacker et al., 2011; McEwen et al., 2012). In a healthy individual, elevated corticosteroid activity helps facilitate the physiological and behavioral adaptations required to appropriately respond to stressors and reinstate homeostasis. However, prolonged exposure to CORT can inhibit the proliferation and survival of adult-born hippocampal neurons, which have been shown to play an important role in the behavioral and neuroendocrine components of stress responses in rodents (Gould and Tanapat, 1999; Snyder et al., 2011). Conversely, chronic treatment of normal rodents with SSRIs, such as fluoxetine, increases hippocampal neurogenesis and neurotrophins such as brain derived neurotrophic factor (BDNF) (Duman and Monteggia, 2006; Schmidt and Duman, 2006; Krishnan and Nestler, 2008). Increased hippocampal neurogenesis is associated with behavioral indications of antidepressant efficacy in

rodents, such as reduced hyponeophagia and increased performance in the forced swim test (Dranovsky and Hen, 2006).

Our laboratory has previously shown that not all strains of mice respond to antidepressant treatments. Normal C57BL/6 mice are insensitive to the effects of chronic fluoxetine treatment, measured in the novelty-induced hypophagia (NIH) test, a conflict-based test in which motivation to approach a highly palatable food is suppressed by a novel environment. Nor did C57BL/6 mice exhibit increase hippocampal cell proliferation in response to the antidepressant treatment (Balu et al., 2009a). In a small clinical study, Dinan et al. (1997) found that 4-day dexamethasone therapy significantly enhanced antidepressant response to SSRIs in treatment-resistant patients. Therefore, we hypothesized that activation of stress circuitry might be important to reveal the behavioral and neurogenic effects of fluoxetine in this non-responsive mouse strain.

CORT is a vital component of the central nervous system's stress response circuitry. Although corticosteroids alone do not encompass all aspects of stress exposure (Belzung, 2014), previous studies have shown that chronic CORT exposure can induce a depressive-like motivational state in rodents that is similar to that produced by a chronic mild stress paradigm (Gourley et al., 2008). Moreover, CORT treatment alone is sufficient to alter molecular targets that are implicated in depression and antidepressant efficacy, such as hippocampal neurogenesis (Bilsland et al., 2006; Gourley and Taylor, 2009). In the current study we investigated the effects of exposure to commercial CORT pellets for 21 days in augmenting fluoxetine's behavioral and proliferative effects in C57BL/6 mice. The results of this study showed that fluoxetine produced behavioral effects in the NIH test only in mice exposed to CORT. Furthermore, CORT administration with fluoxetine co-treatment augmented hippocampal cell

proliferation, an effect potentially mediated by alterations in hippocampal corticosteroid receptor expression. Interestingly, analysis of plasma at the end of treatment revealed a paradoxical decrease in CORT levels in animals treated with the pellets, suggesting that the CORT pellets did not work as advertised. Adrenalectomized animals implanted with CORT pellets revealed a sharp drop in CORT plasma levels by day 7 of treatment, indicating that this method of CORT exposure produced transiently elevated, but not sustained, CORT levels. Nevertheless, these experiments revealed the important finding that CORT exposure potentiates the behavioral and neurogenic effects of chronic fluoxetine administration in a mouse strain that is otherwise non-responsive to this antidepressant treatment.

Materials and Methods

Animals

Intact and adrenalectomized male C57BL/6J, 7-8 weeks old upon arrival, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in groups of 4 (except for those used in the NIH test whom were housed in in pairs) in polycarbonate cages and maintained on a 12 hour light-dark cycle (lights on at 0700 hours) in a temperature (22 °C)- and humidity-controlled environment. Food and water were available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Experimental Design

Experiment 1: Intact animals were implanted with CORT pellets (10 mg) or placebo pellets. Beginning on day 7 of CORT treatment, animals were dosed with either fluoxetine (5 mg/kg b.i.d., i.p.) or saline daily for the remaining 14 days of the experiment. Cohort 1: Animals were tested in the NIH and home cage test on the last two days of drug treatment (n =8-10 per group). Cohort 2: Animals received a single injection of BrdU on the last day of drug treatment and were sacrificed 24 hours later. In these animals hippocampal tissue was dissected and analyzed for BrdU positive cells and corticosteroid mRNA expression. Trunk blood was collected at time of sacrifice and analyzed for plasma CORT levels (n = 15-19 per group).

Experiment 2: Adrenalectomized animals were implanted with CORT pellets (10 mg) or placebo pellets and received chronic fluoxetine treatment as described in *Experiment 1.* All mice received additional CORT replacement through the drinking water (25 μg/ml in

0.9% saline) to prevent the loss of electrolyte homeostasis (Funder, 2006) and eliminate the confounding effects of adrenalectomy alone on neurogenesis (Cameron and Gould, 1994). Animals received a single injection of BrdU on the last day of drug treatment and were sacrificed 24 hours later. Hippocampal tissue was dissected and analyzed for BrdU positive cells (n =7-10 per group).

Experiment 3: Intact animals were implanted with CORT pellets (2.5 mg) or placebo pellets and received chronic fluoxetine treatment as described in *Experiment 1.* Animals received a single injection of BrdU on the last day of drug treatment and were sacrificed 24 hours later. Hippocampal tissue was dissected and analyzed for BrdU positive cells (n = 9-10 per group).

Experiment 4: Adrenalectomized animals were implanted with CORT pellets (10 mg) or placebo pellets and then sacrificed 1, 7, 14, or 21 days after implantation. Trunk blood was collected at time of sacrifice and analyzed for plasma CORT levels (n = 5-6 per group).

Drug Formulation

CORT pellets (2.5 mg and 10 mg, 21 day release, Innovative Research of America, Sarasota, FL, USA) were composed of a proprietary matrix of cholesterol, cellulose, lactose, phosphates and stearates designed to facilitate continuous and sustained diffusion of CORT over a period of 21 days. Placebo pellets consisted of the same matrix without the active product. Fluoxetine hydrochloride (5 mg/kg; Anawa, Zurich) was dissolved in distilled water and delivered by intraperitoneal (i.p.) injection in a volume of 10 ml/kg. Fluoxetine was administered twice daily because, due to its half-life, this dosing strategy results in relatively stable plasma levels (Hodes et al., 2010) and

occupation of brain serotonin transporters (Hirano et al., 2004). Control animals received saline (0.9% NaCl). 5-Bromo-deoxyuridine (BrdU; Roche Applied Sciences Indianapolis, IN) was dissolved in warm saline at a dose of 200 mg/kg and administered i.p. in a volume of 10 ml/kg.

Novelty Induced Hypophagia (NIH) Test

Mice were pair housed and trained to eat a palatable food (three peanut butter chips presented in a small, clear petri dish) in a home cage environment. Animals were trained daily in 15-minunte sessions until they met the criteria of three consecutive days with approach latencies of 30 seconds or less. Opaque, black, plastic dividers were placed inside each cage to separate the mice during training of home cage training sessions. Mice were allowed to habituate to the dividers for 1 hour before the start of the training session. Once all animals had met criteria, training sessions were suspended and drug treatments were initiated. Three days before novel testing all animals were re-exposed to the peanut butter chips through additional training sessions. For novel cage testing, peanut butter chips were presented in the center of an empty, clear polycarbonate cage (25.5 × 46 × 20 cm) with bright lighting (60 W light bulb) and scented with lemon (20% Lemon Joy solution). Novel cage testing was videotaped. Mice were placed into the test cage and the latency to approach during the 15-minute test session was measured. The approach latency was defined as the time to ingestion. There was no food deprivation or habituation period prior to the novel cage test. All behavioral testing took place during the light phase. The home cage test was performed the day after the novel cage test.

BrdU Incorporation Using Flow Cytometry

Flow cytometry is a frequently used method for analyzing newly dividing cells in the

hippocampus. This method has been previously validated by our lab and others, and compared to results obtained from immunostaining (Bilsland et al., 2006; Balu et al., 2009b; Spoelgen et al., 2011). BrdU labeling was measured in cells displaying the nuclear marker 7-aminoactinomycin D (7-AAD) by flow cytometry as previously described (Balu et al., 2009b). Briefly, mice were decapitated 24 hours following BrdU injection, their brains quickly removed, and the hippocampus dissected. Hippocampal tissue was manually minced, digested using an enzymatic mixture (1 mg/ml papain, Roche Applied Science; 0.1 M L-cysteine, Sigma-Aldrich, St. Louis, MO), and then mechanically triturated to form a single cell suspension. Cells were fixed, permeabilized, and stained using the flourescein isothiocyanate (FITC) BrdU Flow Kit (BD Biosciences, San Jose, CA). Data were collected on the same day using a BD FACS Canto System (BD Biosciences) at the University of Pennsylvania Flow Cytometry Core Facility. Background signals were controlled for by collecting data from a BrdU-free control. All data were analyzed using BD FACSDiva Software (BD Biosciences).

Analysis of corticosteroid receptor expression using quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted with Trizol reagent (Gibco BRL, Life Technologies, NY) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufactures' instructions. RNA concentrations were measured and 300 ng/µl RNA was used as a template to synthesize c-DNA using the Superscript Vilo c-DNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). All reactions were performed with a master mix of SYBR green (Applied Biosystems, Austin, TX) and 300 nM primers (final concentration). Quantitative real-time polymerase chain reactions (qRT-PCR) were run using the Stratagene MX3000 and MXPro QPCR software. Cycling parameters were as follows: 95°C for 10

min, 40 cycles at 95°C (30 s) and 60°C (1 min), ending with a melting curve analysis to control for amplification. All reactions were performed in triplicate and the mean cycle threshold was used for analysis. The mRNA levels of target genes were normalized to the house- keeping gene TATA binding protein (TBP) using the $2^{\Delta ct}$ method. Primer sequences are available upon request.

Analysis of plasma CORT using enzyme-linked immunosorbent assay (ELISA)

Trunk blood was collected at time of sacrifice, which occurred between 8-10 am for all experiments. Blood was stored in 0.5 mL heparin and centrifuged at 3000 rpm for 20 minutes. Plasma was removed and stored frozen (–80º C) until analysis. The amount of CORT in the plasma from each sample was measured in duplicate by ELISA following the manufactures instructions (Immunodiagnostic Systems, Fountain Hills, AZ). Intra-assay variability for the CORT kit ranged from 5.9%-7.0%, inter-assay variability ranged from 8.2-8.9%; mean assay sensitivity was 0.17 ng/ml.

Data Analysis

One-way and two-way ANOVA were performed to examine the significance of differences between treatments. Significant overall main effects ($p < 0.05$) or interactions showing a trend ($p < 0.10$) were followed by Tukey or Bonferroni post-hoc tests. For all follow-up tests, $p < 0.05$ was considered statistically significant. Data are expressed as mean ± SEM.

Results

The effects of 10 mg CORT pellet exposure and fluoxetine treatment on behavior

Mice were randomly assigned to either placebo or CORT pellet exposure, and then further separated into either saline or fluoxetine treatment groups. As seen in Figure 4.1A, a two-way repeated measures ANOVA revealed a significant interaction [$F_{(9,102)}$ = 3.761, $p < 0.001$] and main effect [$F_{(3,102)}$ = 12.68, $p < 0.001$] of time on body weight during drug treatment. Placebo-treated animals exhibited significant weight gain by day 14 ($p < 0.05$). Although CORT-exposed animals failed to gain weight within the initial 7 days of treatment, animals subsequently treated with fluoxetine showed significant weight gain by day 21 ($p < 0.001$) whereas saline treated animals continued to show inhibited weight gain. The overall change in body weight (from day 1 to day 21) shown in Figure 4.1B illustrates a significant main effect of treatment with fluoxetine [$F_{(1, 34)}$ = 8.830, $p < 0.01$].

The behavioral effects of CORT and fluoxetine treatment were then measured in the NIH test. Exposure to a novel environment increased approach latency [$F_{(1,65)}$ = 972.4, $p < 0.0001$] and reduced the amount of food consumed [$F_{(1,68)}$ = 136.0, $p < 0.0001$] compared to home cage in all treatment groups. There was a significant interaction between CORT exposure and fluoxetine treatment in approach latencies in the novel environment [$F_{(1,33)}$ = 8.041, $p < 0.01$]. CORT-exposed animals treated with fluoxetine displayed significantly lower approach latencies compared to CORT-exposed animals treated with saline. Moreover, fluoxetine treatment had no effect on approach

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latency in placebo-treated animals in the novel environment (Figure 4.2A). There were

no significant differences in food consumption in the novel environment between drug

treatment groups (Figure 4.2B). In the home cage, CORT treatment significantly reduced

latency to approach [$F(1, 33) = 4.772$, $p < 0.05$] and increased the amount of food

consumed compared to placebo treated animals [$F(1, 34) = 4.956$, $p < 0.05$] (Figure

4.2C and 4.2D).

The effects of 10 mg CORT pellet exposure and fluoxetine treatment on hippocampal

cell proliferation, CORT plasma levels and corticosteroid receptor expression

In a separate cohort, animals received a single injection of BrdU on the last day of drug

treatment and were sacrificed 24 hours later. Hippocampal tissue was analyzed for BrdU

positive cells and corticosteroid mRNA expression. Additionally, trunk blood was

collected at time of sacrifice and analyzed for plasma CORT levels. As seen in Figure

4.3A, flow cytometric analysis of hippocampal tissue revealed that in placebo treated

animals, fluoxetine had no effect on hippocampal cell proliferation. Interestingly, CORT-

exposure significantly increased hippocampal cell proliferation compared to placebo

treated animals [$F(1,59) = 50.87$, $p < 0.001$]. Moreover, there was a significant

interaction between CORT exposure and fluoxetine treatment on neurogenesis [$F(1, 59)$

$= 6.702$, $p < 0.05$]. Post hoc multiple comparisons revealed that CORT-exposed animals

treated with fluoxetine displayed significantly higher hippocampal cell proliferation

compared to CORT-exposed animals treated with saline.

Analysis of circulating CORT levels at the time of sacrifice revealed that

exposure to CORT pellets significantly reduced CORT plasma levels in both saline and

fluoxetine treated animals by approximately 50% when measured on day 21 [F(1, 66) = 36.06, p < 0.001] (Figure 4.3B). Fluoxetine treatment did not alter CORT levels.

Glucocorticoid (GR) and mineralocorticoid (MR) receptor transcription was examined in the hippocampus as a potential molecular mechanism underlying the CORT-induced neurogenic response to fluoxetine in C57BL/6 mice. There was no significant effect of CORT or fluoxetine on GR mRNA expression (Figure 4.3C). However, exposure to CORT significantly reduced hippocampal MR mRNA expression in both saline and fluoxetine treated animals [F(1,68) = 4.276, p < 0.05] (Figure 4.3D).

The effects of adrenalectomy on 10 mg CORT pellet exposure induced hippocampal cell proliferation

To investigate the mechanisms underlying the increase in hippocampal cell proliferation by CORT pellets, adrenalectomized animals were used to examine the effects of 10 mg CORT pellet exposure and fluoxetine treatment on hippocampal neurogenesis in the absence of adrenal feedback. There was a significant main effect of CORT on cell proliferation [F(1, 30) = 5.298, p < 0.05] and a trend towards an interaction [F (1, 30) = 3.372 p = 0.08]. As illustrated in Figure 4.4A, adrenalectomized CORT-exposed animals treated with fluoxetine, but not saline, displayed a significant two-fold increase in cell proliferation compared to placebo treated animals (p < 0.05). However, CORT treatment did not increase cell proliferation in adrenalectomized animals.

The effects of 2.5 mg CORT pellet exposure and fluoxetine treatment on hippocampal cell proliferation

We next examined whether a lower dose of CORT pellet exposure combined with fluoxetine treatment would induce an increase in hippocampal neurogenesis in intact

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animals. There was a significant main effect of CORT pellet exposure [$F_{(1, 35)} = 6.477$, $p < 0.05$] and a significant interaction between CORT pellet exposure and fluoxetine treatment [$F_{(1, 35)} = 4.705$, $p < 0.05$] on hippocampal cell proliferation. As shown in Figure 4.4B, CORT-exposed animals treated with saline exhibited a significant increase in cell proliferation compared to placebo treated animals, as in prior studies. In contrast, the lower dose of CORT pellet was incapable of increasing hippocampal cell proliferation.

Evaluation of the sustained effects of 10 mg CORT pellet treatment on plasma CORT levels

To determine whether 10 mg CORT pellets maintain elevated plasma CORT levels for the advertised duration, adrenalectomized animals were implanted with 10 mg CORT pellets on day 0 and, CORT plasma levels were assessed on day 1, 7, 14, and 21 post-implantation. As shown in Figure 4.5, plasma CORT levels changed dramatically over time ($F_{(3,19)} = 16.18$, $p < 0.01$), and were no longer in the supraphysiological range by the seventh day of CORT pellet exposure.

Discussion

Activation of stress circuitry from implanted CORT pellets produced behavioral and neurogenic effects from chronic fluoxetine treatment in a strain of mice that would otherwise have been unresponsive to the effects of the antidepressant. Specifically, co-treatment of CORT with fluoxetine significantly reduced the effects of novelty stress measured on approach behavior in the NIH test and increased hippocampal cell proliferation. These two effects of antidepressant treatment have been linked together because the behavioral response to fluoxetine is blocked in mice that cannot increase hippocampal cell proliferation (Sahay and Hen, 2007). Although treatment with CORT alone unexpectedly increased cell proliferation to a lesser extent, this effect was absent in adrenalectomized mice while the augmented combination treatment effect was preserved. Measurement of plasma CORT levels revealed that the CORT pellet did not maintain elevated levels for more than a few days, even though it was expected to be active for 21 days, suggesting that the impact of the CORT treatment was likely the after-effect resulting from the supraphysiological levels of acute exposure. Overall, these findings reveal potential neurobiological mechanisms underlying effective antidepressant response in a unique model of treatment resistance.

Hyponeophagia, the unconditioned suppression of feeding in a novel environment, is a behavioral measure of stress that may be sensitive to the anxiolytic effects of chronic, but not acute, antidepressant treatment with SSRIs (Bodnoff et al., 1988; Bodnoff et al., 1989; Dulawa et al., 2004; Dulawa and Hen, 2005; Bechtholt et al., 2007). Fluoxetine's effect of reducing approach latency to food in a novel environment is abolished after focal irradiation of the hippocampus or genetic deletion of hippocampal precursor cells, indicating that hippocampal neurogenesis is a necessary component of

this behavioral antidepressant response (Santarelli et al., 2003; Surget et al., 2008; Wang et al., 2008; David et al., 2009). Intriguingly, unlike other mouse strains, C57BL/6 mice did not exhibit reduced hyponeophagia or increased hippocampal neurogenesis following chronic fluoxetine treatment (Balu et al., 2009a). However, in the present study, we showed that CORT-exposure via pellet implantation induced a behavioral response to fluoxetine in the NIH test in this unresponsive strain. Moreover, CORT-exposure in combination with fluoxetine treatment produced a robust increase in hippocampal neurogenesis that was not seen in placebo treated animals. Although correlative, the increased behavioral response to chronic fluoxetine treatment in CORT-treated mice could be attributed to heightened hippocampal cell proliferation.

Stress is a well-established robust inhibitor of adult neurogenesis (Gould and Tanapat, 1999; McEwen et al., 2012). Similarly, CORT exposure alone has been shown to be a negative regulator of hippocampal neurogenesis (Cameron and Gould, 1994; Wong and Herbert, 2004; Bilsland et al., 2006; Murray et al., 2008; Brummelte and Galea, 2010). Reduced hippocampal cell proliferation typically coincides with increased plasma CORT levels, signifying that circulating CORT levels at the time of testing underlie CORT-induced changes in proliferation (Wong and Herbert, 2006). Paradoxically, we observed dramatically reduced plasma CORT levels in all CORT-exposed animals following the 21-day pellet treatment, while hippocampal cell proliferation was increased. We suspected this might have been due to unanticipated changes in adrenal function. The adrenals operate through an inhibitory feedback system in which increased circulating CORT levels serve as a signal for reduced synthesis and secretion from the adrenals (Sapolsky et al., 1984; Herman and Cullinan, 1997). CORT pellet treatment may have increased internal negative feedback to the

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point of adrenal inactivation, resulting in reduced endogenous circulating CORT levels and disinhibition of cell proliferation. To test for this we examined the effects of CORT pellet and chronic fluoxetine co-treatment on hippocampal cell proliferation in adrenalectomized animals. Notably, in the absence of adrenal feedback, CORT-exposed animals treated with saline did not demonstrate increased neurogenesis whereas those given fluoxetine still exhibited a proliferative response. Therefore, the mechanisms underlying the augmented neurogenic effect seen in combination treated animals cannot be attributed to artifacts of adrenal feedback. It is important to note, however, that adrenalectomized animals in all treatment groups were supplemented with a low dose CORT-treatment (25μg/ml) delivered via drinking water. This mode of delivery produces small rhythmic changes in plasma CORT levels, which have been shown to be necessary for fluoxetine-stimulated neurogenesis in rats (Huang and Herbert, 2006). It is possible, then, that rhythmic fluctuations in CORT levels modulate sensitivity to the proliferative effects of fluoxetine. In spite of this, adrenalectomized placebo animals treated with fluoxetine did not exhibit increased proliferation, demonstrating that supplemental CORT alone was not sufficient to induce a neurogenic response to fluoxetine.

To determine whether a lower dose of CORT could elicit a neurogenic response in the presence of fluoxetine without increasing proliferation on its own, we evaluated hippocampal cell proliferation in intact animals treated with 2.5 mg CORT pellets. Similar to the 10 mg CORT pellet treatment, exposure to 2.5 mg CORT pellet treatment produced elevated cell proliferation. However, there was no additional effect in the presence of fluoxetine, suggesting that this dose is sufficient to produce CORT-induced increases in neurogenesis, but not sufficient to elicit an augmented proliferative

response when combined with fluoxetine. This finding is in contrast to David et al. (2009) who showed a low dose of 5 mg/kg/day CORT treatment to be effective in reducing hippocampal cell proliferation alone and stimulating proliferation when paired with fluoxetine treatment in C57BL/6 mice. However, whereas the current study utilized a three-week CORT pellet treatment, David and colleagues had CORT delivered though drinking water and animals were treated for a substantially longer period of time (7 weeks). Therefore, rhythmic low dose CORT treatment over a longer period of time may be sufficient to increase neuronal sensitization to fluoxetine in this strain.

Hippocampal MRs and GRs play a vital role in mediating stress responsiveness. Altered corticosteroid activity can dysregulate the stress response system and enhance the risk of development of stress-related disorders (Groeneweg et al., 2012). On the other hand, synergistic interactions between hippocampal corticosteroid receptors and serotonergic signaling pathways may mediate the effects of CORT exposure on enhancing the neurogenic responses to fluoxetine in C57BL/6 mice. For example, CORT treatment has been shown to facilitate fluoxetine-induced enhancement of dopaminergic modulation at the mossy fiber synapse (Kobayashi et al., 2013).

Consistent with recent findings, CORT treatment reduced hippocampal MR expression while having no effect on GR expression (Saenz del Burgo et al., 2013), suggesting that MR expression is more sensitive to the effects of CORT exposure. This may be due to the fact that MRs exhibit 10-fold higher affinity for CORT compared to GRs (Joels et al., 2008). Hippocampal MRs selectively contribute to neuronal stability and excitatory tone and have been shown to mediate behavioral reactivity to a novel environment (Oitzl et al., 1994; Berger et al., 2006), hence changes in MR expression and function are likely to impact both hippocampal plasticity and associated behaviors.

Interestingly, in the current study, MR expression was similar between CORT-exposed animals treated to either saline or fluoxetine treated, indicating that variations in expression alone cannot explain the augmented behavioral and neurogenic responses seen in CORT-exposed animals treated with fluoxetine. However, it is important to note that MR and GR expression exhibit a diurnal regulation that is modulated by circulating CORT levels (Herman et al., 1993; Holmes et al., 1995). Since exogenous CORT treatment has been reported to flatten the natural circadian rhythm of plasma CORT (Leitch et al., 2003), CORT pellet exposure could potentially alter rhythmic MR and GR occupancy throughout the day. Thus, the observed neurogenic effects of CORT and fluoxetine treatment might be mediated by changes in circadian expression of MR and GRs. Further studies are needed to confirm this.

A major caveat of this study is the lack of sustained CORT release from the pellet treatment. CORT pellets have been used to model chronically elevated CORT levels, a physiological indicator of dysregulated HPA axis functioning and risk factor for the onset of MDD (Goodyer et al., 2010; Owens et al., 2014). On the contrary, we found that CORT plasma levels dropped precipitously between day 1 and day 7 of CORT pellet treatment. Rather than a sustained release, the CORT pellets produced a rapid, but short-lived, elevation of CORT during the initial days of exposure and then became inactive, resulting in CORT levels falling to the normal physiological range at the time of the experimental studies. Similar findings were reported in another study evaluating the performance of pellets designed to release CORT for 7 days in birds. Muller et al. (2009) found that CORT plasma levels peaked 1-2 days after pellet implantation and reached placebo levels by day 3. The authors posited that the pellets, being originally designed for rodents, are not as effectively metabolized in other species. However, our data

corroborate the findings that the CORT pellets do not reliably produce sustained CORT release for the indicated length of treatment. In light of this, slow-release CORT pellets are not appropriate for modeling prolonged elevated CORT levels. Instead, these pellets may more closely model the effects of exposure to a strong acute stressor, as with post-traumatic stress disorder. Interestingly, the initial surge in CORT levels during the first few days of treatment was sufficient to induce long lasting molecular and behavioral changes in treated animals, suggesting that alterations in CORT levels, not necessarily at a pathological level, can impact the efficacy of fluoxetine.

In conclusion, this study found that exposure to exogenous CORT increases behavioral and neurogenic sensitivity to chronic fluoxetine treatment in C57BL/6 mice, a typically non-responsive strain of mice. These data recapitulate the general findings that genetic background and environment play a fundamental role in antidepressant response. Although slow-release CORT pellets did not model the effects of sustained elevated CORT exposure as anticipated, these studies effectively indicate that CORT exposure is sufficient to reveal the anxiolytic and neuroplastic effects of chronic fluoxetine treatment in a typically unresponsive strain and could model an augmentation strategy for treatment-resistant patients. These findings implicate corticosteroid receptor activity and modulation as a potential variable in the stratification of antidepressant response in patients with MDD and possibly as a mediator of the effects of environmental stress on the effects of antidepressants.

FIGURE 4. 1
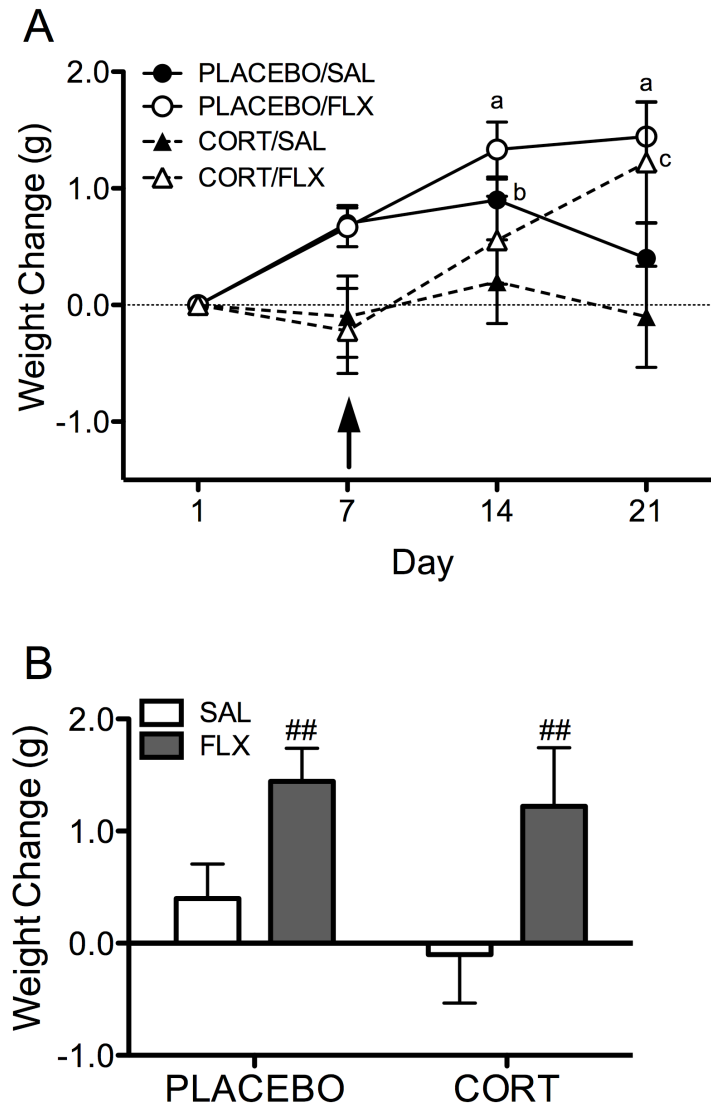
Effects of 10 mg CORT pellet and fluoxetine treatment on weight gain. SAL = Saline, FLX = Fluoxetine, CORT = Corticosterone. Arrow denotes start of fluoxetine treatment.   A) Weight change over time in each treatment group. Placebo treated animals gained weight over time. CORT/SAL animals displayed inhibited weight gain whereas CORT/FLX animals showed normal weight gain after beginning FLX treatment. Symbols represent significant differences compared to day 1: a (PLACEBO/FLX, $p < 0.05$) b (PLACEBO/SAL, $p < 0.01$) **c** (CORT/FLX, $p < 0.01$) (B) Overall weight change (from day 1 to day 21) showed that fluoxetine treatment increased weight gain in both placebo and CORT treated animals (n = 9-10 per group). Data is depicted as mean ± SEM. ##$p < 0.01$ within placebo or CORT treated groups.

FIGURE 4. 2
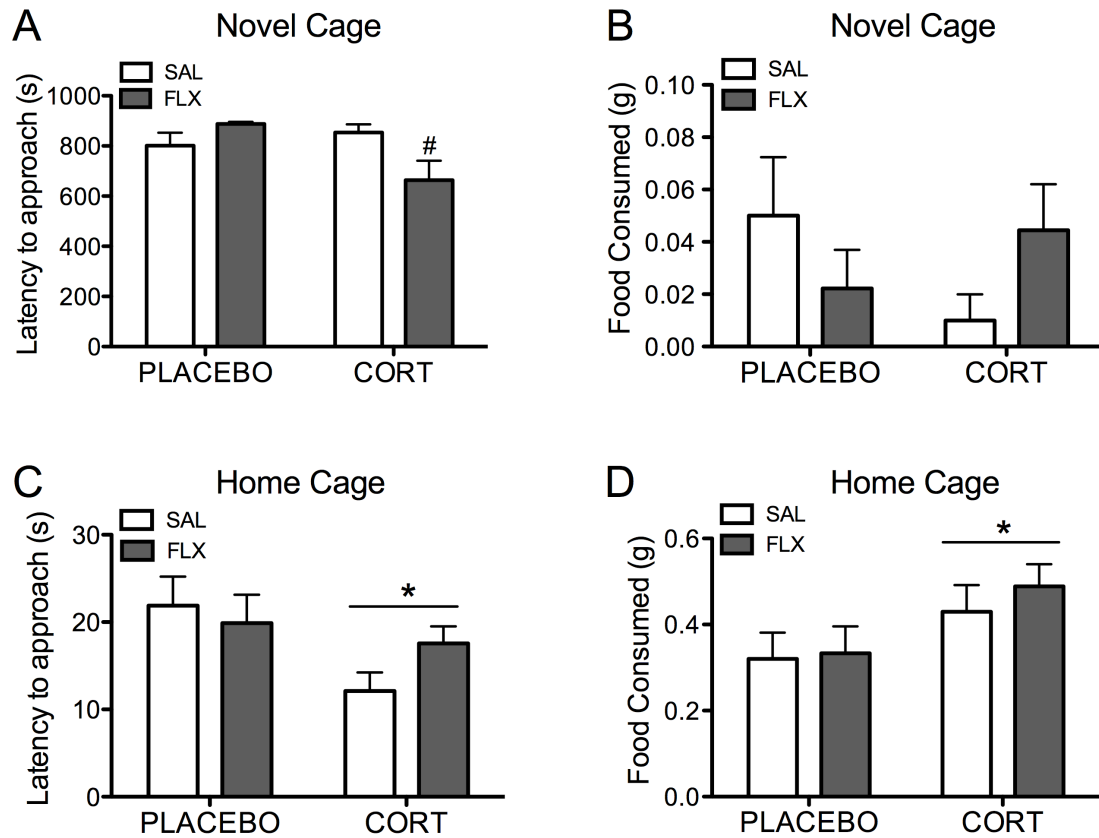
Effects of 10 mg CORT pellet and fluoxetine treatment in the novelty induced hypophagia test. SAL = Saline, FLX = Fluoxetine, CORT = Corticosterone. A) Fluoxetine significantly reduced approach latency in the novel arena in animals exposed to CORT, but not placebo. B) There was no effect of treatment on amount consumed. C) In the home cage, CORT exposure significantly reduced latency to approach and D) increased the amount of food consumed (n = 8-10 per group). Data is depicted as mean ± SEM. #p < 0.05 within CORT treated groups, *p < 0.05 between placebo and CORT treated groups.

FIGURE 4.3
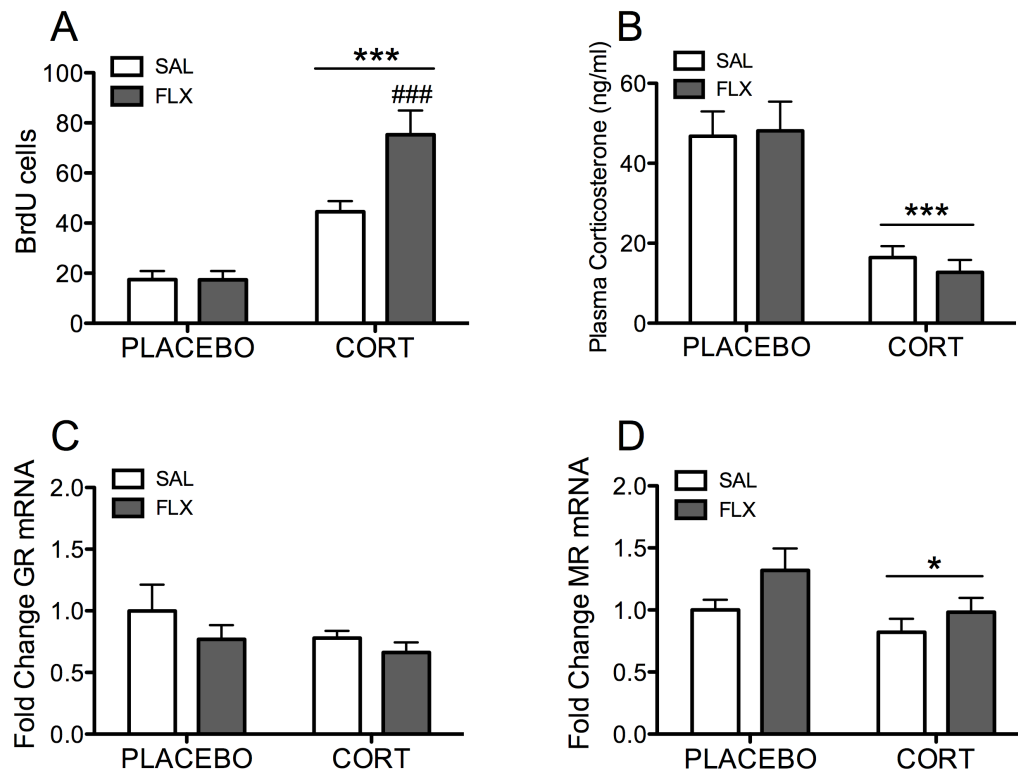
Effects of 10 mg CORT pellet and fluoxetine treatment on hippocampal cell proliferation, CORT plasma levels, and hippocampal corticosteroid receptor expression. SAL = Saline, FLX = Fluoxetine, CORT = Corticosterone. A) Values are expressed as the number of BrdU-positive cells per 10,000 7-AAD events. Intact animals exhibited a significant increase in hippocampal cell proliferation after treatment with CORT. This effect was further augmented in CORT-exposed animals treated with fluoxetine. Fluoxetine had no effect in placebo treated animals (n = 14-18 per group). B) CORT treatment significantly reduced plasma CORT levels. Fluoxetine had no additive effect on CORT levels (n = 15-19 per group). C) CORT had no effect on hippocampal GR mRNA expression but (D) reduced hippocampal MR mRNA expression (n = 15-19 per group). Data is depicted as mean ± SEM. ###$p < 0.001$ within CORT treated groups, ***$p < 0.001$, *$p < 0.05$ between placebo and CORT treated groups.

FIGURE 4. 4
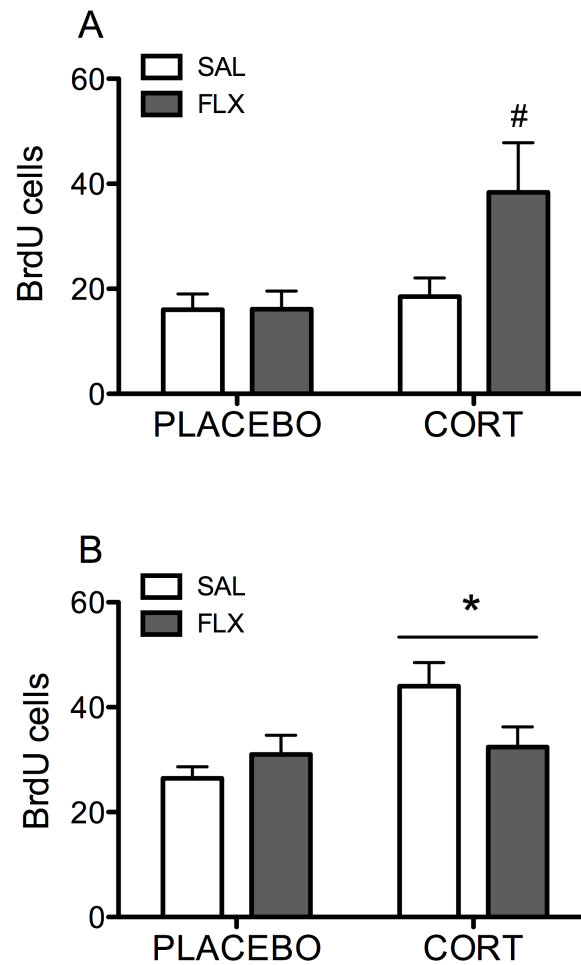
Effects of adrenalectomy or low dose CORT exposure on hippocampal cell proliferation. SAL = Saline, FLX = Fluoxetine, CORT = Corticosterone. A) Values are expressed as the number of BrdU-positive cells per 10,000 7-AAD events.   In adrenalectomized animals exposed to 10 mg CORT pellets, fluoxetine produced an increase in proliferation. (n = 7-9 per group. B). In intact animals exposed to 2.5 mg CORT pellets, hippocampal cell proliferation was increased, but not further augmented by fluoxetine (n = 9-10 per group). Data is depicted as mean ± SEM. #p < 0.05 within CORT treated groups, *p < 0.05 between placebo and CORT treated groups.

FIGURE 4.5

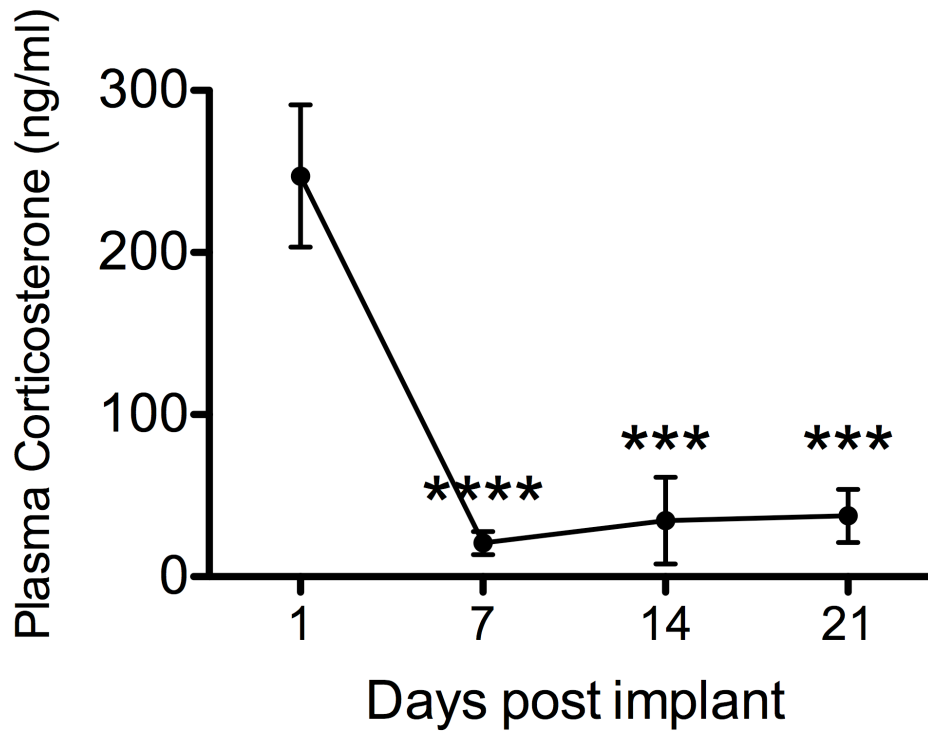

Effects of CORT pellet on plasma CORT levels over time. Adrenalectomized animals implanted with 10 mg CORT pellets displayed significantly reduced CORT plasma levels after 7 days of treatment. (n = 5-6 per group). Data is depicted as mean ± SEM. Absolute mean values are: 247.2 ± 43.92, 20.8 ± 7.02, 34.7 ± 24.44 and 37.6 ± 16.32 ng/ml. ****$p < 0.0001$, ***$p < 0.001$ compared to day 1.

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CHAPTER 5: GENERAL DISCUSSION

Summary of findings

Efforts to elucidate the pathophysiological underpinnings of depression have been hindered by the heterogeneous classification of the disease. The Research Domain Criteria (RDoC) initiative (Cuthbert, 2014) spearheaded by the National Institute of Mental Health has called for the deconstruction of psychiatric syndromes into key components that can be mapped onto physiological processes. Deficits in motivational and hedonic processing are a hallmark symptom of depression. Moreover, they are more prevalent in individuals with a history of excessive stress exposure during childhood or early development. These observations support exposure to stress as a critical mediator in the development of depressive phenotypes. Because of the importance of the nexus between stress and reward, the goal of this thesis research was to further examine the intersection of brain stress and reward circuitry in the manifestation and treatment of motivational deficits that contribute to behavioral phenotypes related to depression.

The research presented in this thesis first characterized the neural substrates underlying conditioned approach behavior for palatable food, revealing a critical role for nucleus accumbens (NAc) dopamine (DA) transmission in the acquisition and maintenance of motivational output for a palatable food reward in mice. These findings were further expanded to show that exposure to acute stress, as modeled in the novelty-induced hypophagia (NIH) paradigm, diminished approach behavior and blunted the DA response to palatable food. Pretreatment with the mixed opioid drug buprenorphine prevented the neurochemical and behavioral effects of the stressor, thus identifying the

brain opioid system as a potential pharmacological target for mitigating the aversive effects of stress on incentive salience for natural rewards. This hypothesis was further explored by investigating the role of individual opioid receptors in mediating restoration of conditioned approach behavior in the novelty-induced hypophagia test, leading to the novel finding that selective antagonism of the mu opioid receptor is sufficient to alleviate suppressed approach behavior. Lastly, an exogenous corticosterone (CORT) administration paradigm was employed to examine the interaction between stress hormones and antidepressant treatment on approach behavior in the NIH paradigm. Remarkably, CORT treatment potentiated the behavioral effects of the selective serotonin reuptake inhibitor fluoxetine in a mouse strain that is otherwise non-responsive to this antidepressant treatment. Altogether, these findings expand our understanding of how stress impacts neurobiological processes involved in the expression of goal-directed behaviors. Simultaneously, these findings identify novel pharmacological targets and strategies for the treatment of depressive phenotypes characterized by anhedonia.

NAc DA as a neural underpinning of incentive salience

Multiple lines of evidence support the involvement of the mesocorticolimbic DA system, specifically the nucleus accumbens (NAc), in the processing of the reward value of a stimulus. Animals will operantly respond for NAc infusions of agents that increase extracellular DA (Hoebel et al., 1989; Phillips et al., 1994; Carlezon et al., 1995) and exhibit conditioned place preference for environments where they received microinjections of DA agonists into the NAc, (Carr and White, 1983, 1986). However, the exact role of DA in reward processing *per se* remains controversial. Early studies posited that DA regulates the hedonic impact of a stimulus (Wise, 1985; Koob and Le Moal, 1997), though that hypothesis has since been proven unlikely (Berridge et al., 1989).

Recent empirical evidence supports a role for subcortical DA transmission in reinforcement learning and attribution of incentive salience, or "wanting", towards effort-worthy goals (Berridge, 2012). Interestingly, exposure to unconditioned novel stimuli in rats transiently elevates NAc DA and triggers investigatory activity (Rebec et al., 1997; Legault and Wise, 2001). Similarly, consumption of a novel food, but not a familiar food, produces robust increases in NAc DA release in the rat (Bassareo and Di Chiara, 1997; Gambarana et al., 2003). Thus, some have argued that NAc DA promotes exploratory behaviors that aid in the formation of new motivational connections between salient stimuli and environmental cues (Ikemoto and Panksepp, 1999). In contrast, the findings reported in this thesis research propose an alternative role for NAc DA in the processing of unconditioned versus conditioned stimuli in mice.

The data presented in Chapter 2 established that repeated exposure to palatable food in a familiar feeding environment is necessary for the acquisition of stable conditioned approach behavior and the food-evoked DA response in the NAc. The majority of animals that were naïve to the food reward failed to approach or consume the food within the fifteen-minute time frame. Furthermore, in comparison to animals familiar with the food reward, naïve mice exhibited more signs of active avoidance, such as increased burying and freezing during food presentation. Lastly, animals that were familiar with the food, but received the food in an unconditioned feeding environment displayed attenuated NAc DA release and slightly increased (though not reaching significance) approach latencies compared to those receiving the food reward in a familiar feeding environment. Altogether, these findings highlight the importance of environmental context in the acquisition of conditioned approach behavior and neurochemical response to rewards in the mouse.

These studies also revealed important species differences in the role of the

mesolimbic DA system in the processing of predictive cues. In rats, anticipatory cues

reliably promote DA release in the NAc across laboratories, whereas reports of

increased DA release to actual consumption of the food reward have been variable

(Cenci et al., 1992; Salamone et al., 1994; Martel and Fantino, 1996; Hajnal and

Norgren, 2001). Consistent with the rat literature, mice exhibited a robust increase in

NAc DA efflux when presented with an empty food dish (predictive stimulus) after being

trained to expect the presentation of food. This finding is in accordance with classical

Pavlovian conditioning theory, which theorizes that a neutral stimulus associated with a

conditioned stimulus can become a conditioned stimulus in and of itself as it acquires the

ability to elicit a conditioned response. Interestingly, the time course for DA response to

the conditioned and predictive stimulus deviated. When presented with the food, animals

displayed elevated DA efflux that persisted throughout the period of food presentation

and after the food had been removed. In contrast, when presented with the empty food

dish, animals displayed a rapid rise in NAc efflux that quickly returned back to baseline.

Thus, DA transmission in the NAc potentially serves both as an anticipatory cue and

consummatory cue, with the latter possibly encoding reinforcement learning for

subsequent exposures.

Stress as an inhibitor of incentive salience

Approach-based behavioral adjustments to salient stimuli are considered one of the

more rudimentary and elemental components of goal-directed behavior (Lewin, 1935;

Schur and Ritvo, 1970; Elliot et al., 2006). Thus, perturbation of the neural mechanisms

that regulate the manifestation of incentive salience likely results in dysfunctional

behavioral output. Previous studies in rats have demonstrated that exposure to chronic

mild stress blunts NAc DA response to palatable food (Di Chiara and Tanda, 1997; Di

Chiara et al., 1999; Gambarana et al., 2003). The authors concluded that reduced DA responsiveness to the food reward denotes an anhedonic state. Yet, stressed rats showed similar approach latencies and food consumption to non-stressed animals. It is possible that these animals had become so overtrained to the food stimulus that DA transmission signaling the presence of an anticipatory cue was no longer necessary to initiate approach behavior. Indeed, it has been suggested that NAc DA transmission is not necessary for previously learned behaviors or habits but may be more important for new learning (Ikemoto and Panksepp, 1999). Nonetheless, a lack of notable change in behavior in these studies makes it difficult to interpret how diminished NAc DA response influences motivational salience. To address the question of whether disruption of NAc DA transmission impairs incentive salience we employed a hyponeophagia-based paradigm in which conditioned approach behavior for palatable food is suppressed by an acute environmental stressor. We observed that presentation of a bright light and novel scent during food exposure blunted NAc DA reactivity to the conditioned food stimulus and increased approach latency without affecting the amount of food consumed. The fact that stressed animals do eventually approach and consume comparable amounts of food to non-stressed animals lends support to the hypothesis that conditioned approach behaviors are not dependent on NAc DA transmission. However, the delay in initial approach likely reflects alterations in DA mediated incentive salience. For example, DA may amplify appetitive behavioral states to more readily engage motor systems involved in approach behavior. Thus, in the absence of DA response, animals are not as immediately energized towards salient stimuli. Alternatively, suppression of DA response may alter the valence of a stimulus so that it no longer elicits "wanting". Further studies are needed to dissociate these mechanisms.

It is important to note that both appetitive and aversive stimuli evoke activation of

NAc DA (Abercrombie et al., 1989; Salamone, 1994; Kalivas and Duffy, 1995). However, recent studies have demonstrated that distinct anatomical regions within the NAc are differentially modulated depending on the context of the stimuli. Lammel et al. (2011) demonstrated that rewarding experiences selectively modify excitatory synapses on dopaminergic cells projecting to the NAc medial shell, whereas aversive stimuli modify synapses on DA neurons projecting to the mPFC. Similarly, corticolimbic glutamate signals within the NAc generate appetitive or fearful behavior depending on rostrocaudal location with the medial shell (Faure et al., 2008). Thus, stress exposure during the presentation of food might selectively activate a distinct circuitry involved in processing of aversive stimuli while suppressing signaling pathways associated with motivational salience.

A role for the mu opioid receptor in mediating incentive salience and stress

One of the more striking findings of Chapter 2 is that pretreatment with buprenorphine (BPN), a mixed-opioid drug currently in development for the treatment of depression and anxiety, prevented the stress-induced suppression of NAc DA and restored approach latencies to that seen in non-stressed animals. These data complement previous studies conducted in our laboratory showing that acute BPN treatment reduces approach latencies in the NIH test (Falcon et al., 2015), and suggest a role for opioid systems in mitigating the aversive effects of stress on incentive salience for natural rewards. This hypothesis was explored further in Chapter 3 by probing the role of individual opioid receptors in mediating BPN's effect on approach behavior in the NIH test.

Buprenorphine is a partial agonist at the mu opioid receptor (MOR) and an antagonist at the kappa opioid receptor (KOR). Accumulating evidence has linked the KOR and its endogenous ligand dynorphin to the negative consequences of stress in

rodents. Acute administration of KOR agonists induces anxiogenic and prodepressive behaviors in rodents (Maisonneuve et al., 1994; Carlezon et al., 2006; McLaughlin et al., 2006), whereas treatment with KOR antagonists has been shown to elicit antidepressant life effects (Mague et al., 2003; Carr et al., 2010; Almatroudi et al., 2015; Falcon et al., 2015; Huang et al., 2016). Recent studies from our laboratory have confirmed the KOR as a key mediator of BPN's effects in tests with predictive validity for antidepressant efficacy, such as the forced swim test (FST) (Falcon et al., 2016). Therefore, we originally hypothesized that BPN's effects in the NIH test are primarily derived from antagonism at the KOR. However, the work presented in Chapter 3 proved this hypothesis to be incorrect. We observed that BPN's effects in the NIH test was conserved in KOR knockout animals, but abolished in MOR knockout mice. Moreover, administration of selective KOR antagonist nor-BNI in wildtype mice was ineffective in reducing approach latencies. The discrepancies between previous findings and those reported here are unclear. It is possible that inherent strain differences dictate response to KOR antagonists in this paradigm- the present work is the only of our knowledge to test the effects of the selective MOR antagonist cyprodime and the selective KOR antagonist nor-BNI in the NIH test using the C57BL/6 mouse strain. The discrepancies may also be due in part to differences in procedural definitions of "approach" in the NIH paradigm. For example, some groups might consider investigatory sniffing or licking as approach, whereas we took a more conservative determining of approach. To this end, KOR antagonism may reduce latency to investigate but not to consume. Alternatively, in our experiments KOR antagonism may have increased exploratory behavior rather than restoring incentive salience for the food reward. Indeed, KOR antagonists have previously been shown to increase exploratory behaviors in the elevated plus maze and open field (Knoll et al., 2007; Bruchas et al., 2009; Wiley et al., 2009; Wittmann et al.,

2009).

Chapter 3 of this thesis established a novel role for modulation of the MOR in restoring approach behavior in the NIH test. Given the role of MOR in mediating the reinforcing properties of rewards, we predicted that BPN's agonist action at MOR might underlie its behavioral effects. However, several points of evidence contradict this original hypothesis. Pharmacological blockade of MOR and KOR with the nonselective opioid receptor antagonist naltrexone did not abolish BPN's effects in the NIH test. Furthermore, administration of morphine alone was not effective in altering suppressed approach behavior. On the contrary, acute antagonism of MOR was sufficient to reduce approach latency in the NIH test. This observation suggests that MORs may facilitate prodepressive behaviors under stressful situations. Stimulation of MORs induces activation of the hypothalamic-pituitary-adrenal (HPA) axis (Kiritsy-Roy et al., 1986; Pechnick, 1993). Moreover, CRF significantly enhances the release of β-endorphin and dynorphin from the hypothalamus in vitro (Nikolarakis et al., 1986). In contrast, MOR knockout mice exhibit less stress-induced increases in plasma corticosterone concentrations and reduced anxiety and depressive-like behavior in the elevated plus maze and FST, respectively (Filliol et al., 2000; Ide et al., 2010). Consequently, antagonism of MORs during stress exposure may prevent the manifestation of affective behavior. Notably, studies in healthy human populations have indicated that low doses of BPN enhance attention for positive emotional cues and reduce sensitivity to fearful cues (Ipser et al., 2013). Moreover, a recent clinical trial testing the efficacy of buprenorphine combined with a MOR antagonist revealed significant antidepressant activity in treatment resistant MDD patients (Ehrich et al., 2015).

Antagonism of MORs during stress exposure may restore incentive behavior in the NIH paradigm via disinhibition of mesoaccumbens DA transmission in response to

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stress. Stress-induced activation of MORs in the ventral tegmental area (VTA) has been

shown to reduce DA transmission in the NAc through enhanced DA signaling in the

medial prefrontal cortex (mPFC) (Latagliata et al., 2014). This is consistent with previous

findings establishing that in response to an acute stressor, mice of the C57BL/6 inbred

strain exhibit a robust and rapid activation of mesocortical DA release that is

accompanied by inhibition of DA release in the accumbens (Ventura et al., 2002).

Recent studies indicate that activation of VTA DA neurons is differentially modulated by

input-specific circuits. Lammel et al. (2012) demonstrated that laterodorsal tegmentum

(LDTg) neurons predominantly synapse on DA neurons projecting to the NAc lateral

shell and produce conditioned place preference when activated, whereas neurons

originating from the lateral habenula (LHb) synapse primarily on DA neurons projecting

to the mPFC and produce conditioned place aversion. Thus, aversive experiences

appear to selectively activate DA neurons projecting to the mPFC. Acute exposure to

stress or administration of glucocorticoids also produces robust increases in extracellular

glutamate levels preferentially in the PFC (Moghaddam, 1993; Bagley and Moghaddam,

1997; Popoli et al., 2012). Stress-induced enhancement of basal glutamate transmission

is mediated by increased expression of postsynaptic NMDA and AMPA receptors (Yuen

et al., 2009; Yuen et al., 2011). Activation of D1 receptors in the mPFC increases

excitability of glutamatergic pyramidal cells (Tseng and O'Donnell, 2004), which have

been shown to project to the NAc (Carr et al., 1999). Optogenetic activation of

glutamatergic projections in the mPFC suppresses striatal response to DA and

diminishes the reinforcing effects of midbrain DA neuron stimulation (Ferenczi et al.,

2016). This effect may be mediated by stimulation of GABAergic medium spiny output

neurons in the NAc (Sesack and Pickel, 1992). Thus, MOR-induced potentiation of

mPFC dopamine activity in response to stress may lead to blunted mesoaccumbens DA

release in the mesolimbic pathways, and consequently, diminished incentive behavior in response to rewards. Alternatively, intra-VTA LHb axonal stimulation has been shown to inhibit VTA DA neurons projecting to the NAc via increased GABAergic signaling from the rostromedial tegmental area (RMTg) to the VTA (Lammel et al., 2012). Activation of MORs expressed on GABAergic neurons in the RMTg putatively increase VTA DA activity (Matsui and Williams, 2011). However, recent studies have revealed that GABAergic afferents to dopamine neurons exhibit projection specific sensitivity to opioids. Specifically, MOR activation induced inhibition of inhibitory postsynaptic currents (IPSCs) evoked from the RMTg but not VTA interneurons (Matsui et al., 2014). These findings suggest that opioid-dopamine interactions in the VTA are likely regulated by complex local circuitry. Indeed, there is evidence that local administration of MOR antagonists into the VTA can increase striatal dopamine concentrations (Devine et al., 1993). LHb neurons are known to be excited by the absence of an expected reward (Hikosaka, 2010), however, both central administration of CRF and exposure to acute stress increases c-fos activation in the habenula, indicating that this region is also activated during stress response (Imaki et al., 1993). Thus, presentation of aversive stimuli with rewarding stimuli may override activation of reward-processing pathways and subsequent DA release in the NAc by enhancing GABAergic signaling in the VTA. Accordingly, acute stress exposure has been shown to increase firing of GABAergic neurons within the VTA (Tan et al., 2012). Future studies are needed to fully elucidate the role of MORs within this circuitry.

FIGURE 5.1
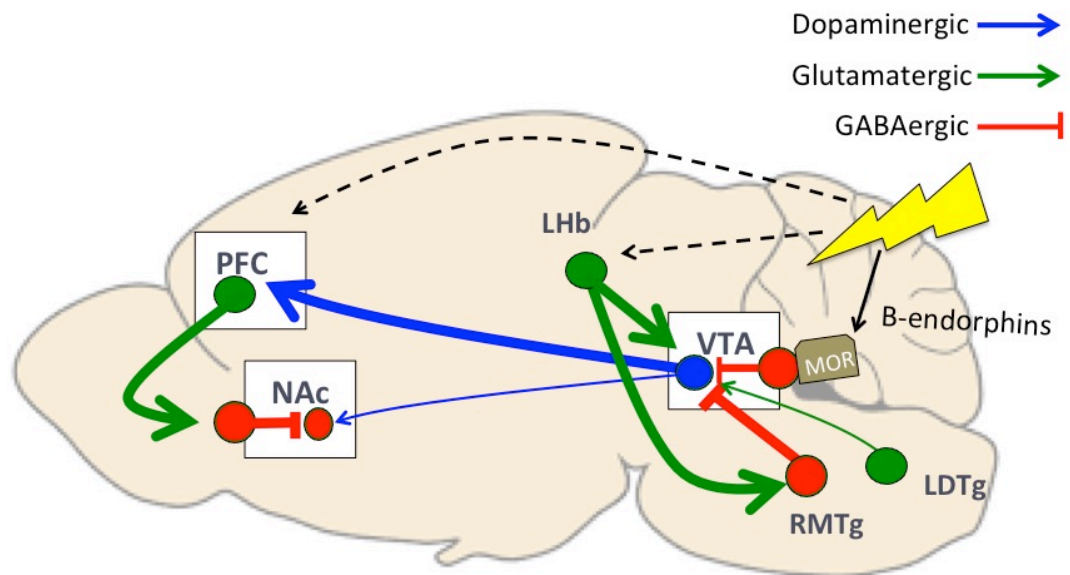
Hypothetical mechanism of action for MOR mediated suppression of NAc DA by stress. Exposure to stress results in the release of stress hormones (denoted by dashed line) and B-endorphins. Activation of VTA MORs preferentially enhance dopaminergic signaling in the PFC. Glutamatergic signals from the habenula selectively activate VTA neurons projecting to the PFC and inhibit VTA DA neurons projecting to the NAc via activation of GABAergic neurons in the RMTg. The PFC exerts top-down control of NAc DA activity through dense glutamatergic input to inhibitory GABA neurons that project to the NAc. Collectively, this circuitry results in reduced DA activity in the NAc and blunted incentive behavior towards rewarding stimuli.

Stress as a facilitator of antidepressant response

Whereas Chapters 2 and 3 of this thesis reflected on how stress impairs reward circuitry and associated motivational behaviors, Chapter 4 proposes a distinctive role for the HPA stress axis in sensitization of behavioral and neurogenic responses to selective serotonin reuptake inhibitors (SSRIs), the most commonly prescribed medication for the treatment of depression. Previous studies have reported the NIH test to be sensitive to the antidepressant effects of chronic, but not acute, treatment with SSRIs (Bodnoff et al., 1988; Bodnoff et al., 1989; Dulawa et al., 2004; Dulawa and Hen, 2005; Bechtholt et al., 2007; David et al., 2009). However, studies conducted in our laboratory (Balu and Lucki, 2009) and others (David et al., 2009) have revealed strain dependent responses to chronic fluoxetine treatment in the NIH test, showing C57BL/6 mice, in particular, to be unresponsive to fluoxetine's effects. The absence of behavioral response to fluoxetine in this mouse strain may parallel the phenomenon of treatment resistance seen in human depression (Nemeroff, 2007). We utilized the intrinsic SSRI-insensitivity characteristic of C57BL/6 mice to investigate strategies for enhancing behavioral sensitivity to this class of antidepressants in the NIH paradigm.

Hypercortisolism and dexamethasone insensitivity are among the most consistent pathophysiological findings in depression (Pariante and Miller, 2001; Pariante, 2006; Anacker et al., 2011). Elevated basal cortisol levels are thought to arise from dysregulation of inhibitory feedback signals to the HPA axis and contribute to affective behavior. The deficit of negative cortisol feedback is restored in depressed patients after successful therapeutic treatment with antidepressant drugs (Holsboer et al., 1982). Accordingly, animals genetically modified to exhibit reduced forebrain corticosteroid

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receptor expression demonstrate elevated CORT levels, hypersensitivity to stressors, and depressive-like behavior (Ridder et al., 2005; Berger et al., 2006).

The studies in Chapter 4 employed an endogenous CORT exposure paradigm to examine the influence of dysregulated HPA axis activity on antidepressant response to chronic fluoxetine treatment in the NIH test. Notably, our chosen method of CORT exposure (subcutaneous pellet) was ineffective in maintaining persistently elevated CORT levels, and instead released a large bolus of CORT that rapidly diminished over the thee week treatment period. Nonetheless, we observed a marked behavioral response to fluoxetine treatment in the NIH test in animals previously exposed to CORT. Glucocorticoids have been reported to increase the salience of pleasurable or compulsive activities. Thus, CORT exposure may prime neurochemical pathways associated with motivational processing (Dallman et al., 2003). However, animals exposed to CORT and treated with vehicle did not exhibit reduced approach latencies in the NIH, suggesting a synergistic interaction between CORT-mediated sensitization and serotonergic signaling in this paradigm. A potential mechanism for this synergistic effect may lie in modulation of 5-HT receptor activity. Exposure to chronic mild stress has been shown to desensitize 5-HT_{1A} autoreceptors in the dorsal raphe (Lanfumey et al., 1999). Activation of 5-HT_{1A} autoreceptors inhibits serotonin release and subsequent postsynaptic 5-HT_{1A} receptor activity (Hensler et al., 2007; Lanfumey et al., 2008). Thus, reduced presence of $5HT_{1A}$ autoreceptors after CORT exposure may enhance the therapeutic effects of chronic treatment with fluoxetine by boosting increased extracellular serotonin levels in forebrain regions. In accordance with this, a small clinical study conducted in the late 1990s found that dexamethasone therapy significantly enhanced antidepressant response to SSRIs in treatment resistant patients (Dinan et al., 1997).

In Chapter 4 we also showed that reduced novel arena approach latency in CORT and fluoxetine combination treated animals was associated with a robust increase in hippocampal neurogenesis. Stress exposure is more commonly reported to reduce hippocampal cell proliferation in rodents (Cameron and Gould, 1994; Wong and Herbert, 2004; Bilsland et al., 2006; Murray et al., 2008; Brummelte and Galea, 2010). However, other groups have reported augmented cell proliferation in animals exposed to CORT and treated with fluoxetine (David et al., 2009). Several groups have reported that neurogenesis-dependent mechanisms are responsible for the behavioral response to fluoxetine in the NIH test (Santarelli et al., 2003; Sahay and Hen, 2007; Surget et al., 2008; Wang et al., 2008; David et al., 2009). In humans, reduced hippocampal volume is associated with untreated recurrent depression (Sheline et al., 1996; Bremner et al., 2000; Colla et al., 2007) but not treated depression (Sheline et al., 2003), suggesting that successful antidepressant treatment can exert critical neuroprotective effects against chronic stress. Whether reduced hippocampal volume in humans reflects reduced cell proliferation or other morphological processes is controversial. Nonetheless, general hippocampal dysfunction is likely to play a role in motivational processes, seeing as hippocampal synaptic input to the NAc has been shown to mediate the cognitive functions necessary for initiating goal-directed behavior (Goto and Grace, 2005).

Concluding remarks

This dissertation work has employed a unique and innovative approach to elucidating mechanisms underlying conditioned approach behavior for natural rewards, an RDoC construct relevant to the human condition. The focus on a single behavioral paradigm enabled the methodical assessment of neurochemical and neuropharmacological

mechanisms specific to stress-induced changes in incentive behavior, thus delivering new insight into the interactions between brain stress and reward circuitry. The findings presented here further expand our understanding of the neural correlates of motivation and propose novel targets for the treatment depression and other psychiatric disorders characterized by of anhedonia.

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