Corticotropin-Releasing Factor (CRF) Modulation of Cognition and Frontostriatal Neural Function

By

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Abstract

The prefrontal cortex (PFC) and downstream striatal targets play a pivotal role in higher cognitive processes that guide flexible, goal-directed behavior. Dysfunction of the PFC and extended frontostriatal circuitry is associated with many psychiatric disorders, including attention deficit hyperactivity disorder (ADHD), schizophrenia, and post-traumatic stress disorder. The majority of drugs used to treat these disorders target catecholamine neurotransmitters. However, these drugs bear significant limitations, including less than full efficacy, and in the case of ADHD and psychostimulants, potential for abuse. Thus, identification of novel, non-catecholamine drug targets may advance the treatment of PFC-dependent cognitive dysfunction. The neuropeptide, corticotropin-releasing factor (CRF) and its receptors are prominent in the PFC, yet the cognitive actions of CRF signaling in the PFC are entirely unknown.

The goal of this thesis is to examine the cognitive and neurophysiological actions of CRF neurotransmission in the PFC, and determine if CRF represents a potential drug target in the treatment of ADHD. Specifically, these studies addressed three major questions: 1) Do PFC CRF receptors and neurons modulate PFC-dependent cognition measured in a test of spatial working memory? 2) What are the effects of PFC CRF neurotransmission on neural encoding of working memory across the frontostriatal circuit? 3) And lastly, does CRF signaling in the PFC broadly modulate PFC-dependent cognitive processes?

These studies demonstrate that endogenous activity of PFC CRF neurons impair working memory performance via activation of local CRF receptors coupled to the intracellular protein kinase A (PKA) cascade. These actions are associated with robust alterations in task-related neural representations in the PFC and dorsomedial striatum. PFC CRF neuronal activity also

impairs sustained attention, another PFC-dependent cognitive process, yet these actions involve CRF receptors outside of the PFC. Importantly, systemic CRF antagonists improve performance in both working memory and sustained attention tasks, similar to FDA-approved treatments for ADHD. Collectively, these studies are the first to demonstrate that CRF acts in the PFC to impair cognition and frontostriatal neural function, and suggest that CRF antagonists may represent a novel therapeutic for treatment of ADHD and other PFC-related cognitive disorders.

Chapter 1

Introduction

Introduction

The prefrontal cortex (PFC) is a key region involved in higher cognitive processes that guide goal-directed behavior, particularly under distracting and/or ambiguous conditions (Miller and Cohen, 2001). These actions involve topographically organized projections to the striatum (Voorn et al., 2004; Haber et al., 2006). PFC-dependent cognitive dysfunction is associated with a variety of mental health disorders such as attention deficit hyperactivity disorder (ADHD), post-traumatic stress disorder (PTSD), schizophrenia, and addiction (Barrett et al., 1996; O'Carroll, 2000; De Bellis et al., 2005; Castellanos et al., 2006; Keefe and Harvey, 2012). For example, individuals with ADHD exhibit abnormalities in functional activation of the frontostriatal circuit which are correlated with symptom severity (Schneider et al., 2010). Beyond psychiatric dysfunction, stress-related impairment in PFC-dependent cognition is estimated to contribute to over half of all workplace accidents (Barrios-Choplin et al., 1997). Therefore, a deeper understanding of the neurobiology of PFC-dependent cognition may lead to new insight and novel treatment strategies for PFC-related cognitive dysfunction.

Extensive evidence demonstrates that catecholamines are potent modulators of PFC-dependent cognitive processes (Arnsten and Li, 2004). Consistent with this, the majority of clinically efficacious drugs used in the treatment of ADHD and other PFC cognitive disorders target catecholamines (Berridge and Arnsten, 2015). However, there are limitations to currently available treatments for PFC-related cognitive dysfunction. For example, most pharmacological treatments lack full efficacy across all patients (Faraone and Buitelaar, 2010). Moreover, although psychostimulants are the most effective treatment for ADHD, these drugs bear significant potential for abuse and addiction (Compton and Volkow, 2006; White et al., 2006; Setlik et al., 2009). Thus, there is a need for improved pharmacological treatments for PFC-

related cognitive dysfunction. However, the development of novel treatments is limited by a scarcity of non-catecholamine targets.

Therefore, it is of significant interest that corticotropin-releasing factor (CRF) neurons, axons and receptors are prominent in the PFC (Swanson et al., 1983; Merchenthaler, 1984; Charlton et al., 1987; Lewis et al., 1989; Potter et al., 1994; Sanchez et al., 1999). Research spanning over three decades has demonstrated that CRF modulates physiological, endocrine, and behavioral responses in stress (Dunn and Berridge, 1990; Bale and Vale, 2004). In addition, clinical studies implicate CRF in a variety of psychopathologies associated with PFC cognitive dysfunction including PTSD, addiction, schizophrenia, and depression (Nemeroff et al., 1988; Arató et al., 1989; Craig et al., 1992; Lindström, 1996; Bremner et al., 1997; Heim et al., 1997; Sautter et al., 2003; Merali et al., 2004a; Hauger et al., 2009). Surprisingly, despite decades of intensive study of CRF, there is virtually no information regarding the cognitive actions of CRF within the PFC. Therefore, to address this gap, the goal of this dissertation is to investigate the cognitive and neurophysiological actions of CRF neurotransmission in the PFC. Information gained in these studies will provide new insight into the neurobiology of PFC-dependent cognition and may lead to novel approaches to treat disorders of PFC dysfunction.

Neurophysiology of PFC-dependent Cognition

The PFC has long been known to play a pivotal role in higher cognitive and behavioral processes that guide flexible, goal-directed behavior (Fuster, 2015). Seminal studies demonstrated that anatomical and pharmacological lesions of the PFC impair performance in cognitive tasks requiring working memory, planning, cognitive flexibility, and response inhibition in the face of competing and/or distracting stimuli (Tizard, 1958; Passingham, 1975;

Funahashi et al., 1993). Historically, the neurobiology of PFC-dependent cognition has primarily been studied using delayed-response tasks of working memory in which task-relevant information is retained over a short delay to guide subsequent behavior (Goldman-Rakic, 1995). In addition, working memory deficits are ubiquitous in disorders including ADHD, schizophrenia, and depression (Manoach et al., 2000; Martinussen et al., 2005; Joormann and Gotlib, 2008). Therefore, a better understanding of the mechanisms that support working memory can further our understanding of PFC neurobiology in both healthy and diseased states.

Response Properties of PFC Neurons during Working Memory

Early electrophysiological studies in non-human primates demonstrated that subpopulations of dorsolateral PFC neurons display elevated discharge rates during the delay interval of delayed-response tests of working memory (Fuster and Alexander, 1971; Kojima and Goldman-Rakic, 1982). More recent studies have also observed delay-related activity in the PFC of rodents (Batuev et al., 1990; Jung et al., 1998; Devilbiss et al., 2012) and humans (functional imaging studies; Courtney et al., 1997; Sakai et al., 2002). Originally, delay-related activity was posited to reflect the storage of information in working memory (Fuster and Alexander, 1971; Kojima and Goldman-Rakic, 1982). However, research over the past two decades demonstrates that delay-related activity in the PFC is more likely to reflect attentional processes, understanding of rules, and/or action plans (Curtis and D'Esposito, 2003; Postle, 2006; Messinger et al., 2009). Meanwhile, the storage of information held in working memory has been linked to other cortical regions. For example, many additional regions exhibit delay-related activity, including premotor, parietal, cingulate, and temporal association cortical areas (Riley and Constantinidis, 2016), and subcortical structures such as striatum and mediodorsal thalamic

nucleus (Schultz and Romo, 1988; Watanabe and Funahashi, 2004). As such, it is now hypothesized that the PFC maintains higher-level abstract representations about rules, categories, or goals, while other higher-order sensory cortical areas hold task-relevant, domain-specific information in working memory (D'Esposito and Postle, 2015).

In addition to the delay, subpopulations of PFC neurons also respond (i.e. 'are tuned') to a diversity of working memory task-relevant stimuli and rules that guide attention and decision-making. For example, primate and rodent PFC neurons display sensitivity to decision-related outcomes (e.g. rewards and errors) and to conditioned stimuli that predict outcomes in working memory tasks (Niki and Watanabe, 1979; Watanabe, 1989, 1990, 1992; Batuev et al., 1990; Sakurai and Takahashi, 2006). In primates, delay-related activity in the dorsolateral PFC is uniquely related to the direction of the saccade made by the animal, indicating that PFC neurons can encode associations between environmental cues and corresponding actions (Asaad et al., 1998). In addition, PFC neurons can respond differently to the same stimulus in a context-specific manner (Jung et al., 1998; White and Wise, 1999; Warden and Miller, 2010).

Moreover, PFC neurons can simultaneously respond to multiple working memory task events (e.g. multitasking; Cromer et al., 2010; Rigotti et al., 2013). For example, subpopulations of delay-tuned neurons in the primate PFC also respond to rewards (Watanabe, 1996). Consistent with this, recent studies in our laboratory demonstrated that approximately one-third of PFC pyramidal neurons strongly tuned to delay were also tuned to reward in working memory-tested rats. In addition, half of all recorded reward-tuned PFC neurons displayed elevated spiking rates during the choice phase of the task (Devilbiss et al., 2017). Taken together, these observations demonstrate that PFC neurons exhibit heterogeneous response properties, adapt flexibly to

changing contextual demands, and encode multiple task-relevant stimuli to guide goal-oriented behaviors.

Mechanisms Underlying Sustained Delay Activity in the PFC

A central question in this field of research is how PFC cell assemblies maintain sustained neuronal activity in the absence of sensory inputs. One mechanism underlying sustained delay-related activity of PFC neurons is proposed to involve self-excitatory (reverberatory) activity within PFC microcircuits (Wang, 2001; Ramos and Arnsten, 2007; Wang et al., 2013). Based on anatomical modeling and limited *in vitro* observations, self-excitatory activity is posited to arise, at least in part, from recurrent PFC pyramidal cell circuits within and/or between layers II/III and V (Wang, 2001; Ramos and Arnsten, 2007; Papoutsi et al., 2013; Wang et al., 2013). The strength of sustained activity is modulated by glutamate, γ-aminobutyric acid (GABA), and catecholamine neurotransmitters (see below), all of which recruit intracellular pathways that regulate cell excitability (Arnsten and Jin, 2014). Moreover, studies in our laboratory have used a conditional intensity model that explicitly measures the influence of past spiking activity to ongoing task-related activity (Devilbiss et al., 2012). These studies provided the first empirical evidence that prior spiking activity of putative PFC pyramidal neurons contributes to delay- and reward-related spiking activity in working memory-tested rats. Collectively, these observations suggest that reverberatory activity contributes to PFC neuronal encoding of working memory task events.

Catecholamine Modulation of PFC-dependent Cognition

Working Memory

The PFC is innervated by dopaminergic and noradrenergic fibers originating in brainstem and midbrain nuclei which modulate arousal, sensory processing, and salience of external stimuli (Lindvall et al., 1978; Lewis et al., 1988; Lewis and Morrison, 1989). Extensive research demonstrates that the catecholamines, dopamine (DA) and norepinephrine (NE), are important modulators of PFC-dependent cognitive function (Berridge and Arnsten, 2015). For instance, the effects of catecholamine depletion in the PFC are as detrimental to PFC-dependent cognition as PFC lesions (Brozoski et al., 1979; Simon et al., 1980; Mair et al., 1985; Bubser and Schmidt, 1990). Catecholamines exert inverted-U shaped modulatory effects on working memory, such that either too low or high levels of PFC catecholamines impair cognitive performance (Figure 1; Arnsten and Pliszka, 2011). These actions are mediated by different catecholamine receptor families within the PFC. For example, excessive signaling at DA D1, and to some extent, D2 receptors, is associated with impaired working memory (Cai and Arnsten, 1997; Druzin et al., 2000; Gibbs and D'Esposito, 2005). Moreover, noradrenergic α_2 receptors display higher affinity for NE than α_1 and β receptors, and are thus preferentially engaged at moderate rates of NE release associated with optimal working memory performance (Ramos and Arnsten, 2007).

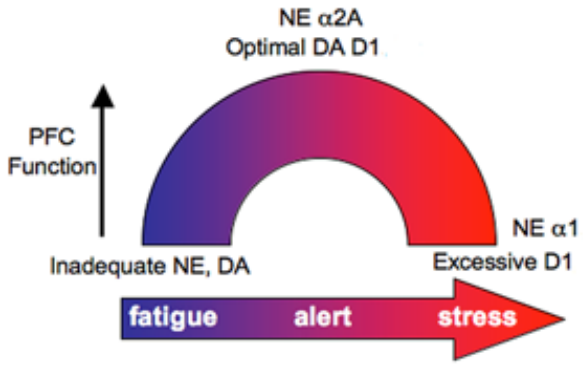

Figure 1. Inverted-U shaped catecholamine effects on PFC-dependent cognition. In a fatigued state, low catecholamine levels inadequately activate DA and NE receptors. In an alert, optimally-performing state, moderate levels of PFC catecholamines activate high affinity α_{2A} receptors and D1 receptors. In stress, high levels of catecholamines engage lower affinity α_1 receptors and excessively stimulate D1 receptors, leading to cognitive impairment.

Meanwhile, high-arousal conditions associated with high rates of NE release (e.g. stress, high-dose psychostimulants) lead to engagement of lower affinity α_1 receptors, resulting in impaired working memory (Figure 1; Birnbaum et al., 1999; Arnsten, 2009).

The inverted-U shaped modulatory actions of catecholamines on working memory are posited to arise from their effects on recurrent network activity in the PFC. A model proposed by Arnsten and colleagues suggests that during optimal working memory performance, self-excitatory pyramidal cell networks within PFC layer III display elevated discharge rates in response to task-relevant spatial cues (Figure 2). Meanwhile, cell assemblies representing irrelevant spatial information are inhibited by local GABAergic interneurons (Figure 2D; Arnsten, 2009). Moderate NE release leads to preferential stimulation of α_{2A} receptors which strengthen task-related activity of spatially-tuned PFC pyramidal neurons (Wang et al., 2007; Arnsten and Pliszka, 2011). These actions are driven by α_{2A} receptor-mediated suppression of cyclic adenosine monophosphate (cAMP) and closing of hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels within dendritic spines, which increases post-synaptic excitatory currents and strengthens task-related activity (Figure 2A; Paspalas et al., 2013). Interestingly, these actions are posited to underlie the clinical efficacy of the α_{2A} receptor agonist, guanfacine, in the treatment of ADHD (Biederman et al., 2008). In contrast, when PFC NE release is high (e.g. during stress), recruitment of lower-affinity α_1 receptors within the soma activates protein kinase C (PKC) and Ca^{2+} signaling, and opens small conductance Ca^{2+}-activated K^+ channels (Figure 2G). This reduces cell excitability and suppresses activity of PFC pyramidal cell assemblies representing task-relevant spatial information (Birnbaum, 2004).

With regard to DA, it is proposed that during optimal working memory performance, moderate D1 receptor activity leads to cAMP-mediated opening of HCN channels within

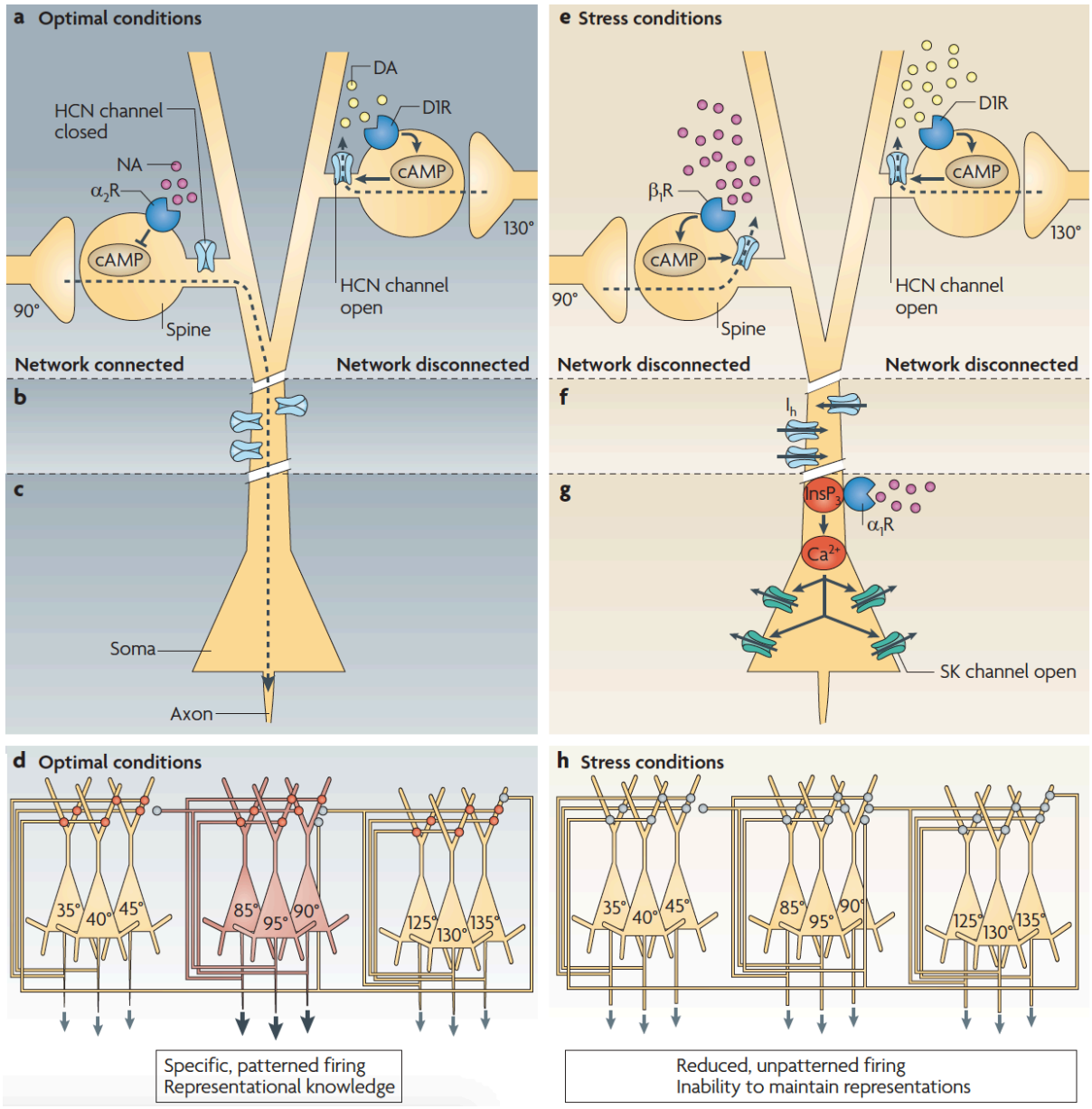

Figure 2. PFC intracellular pathway modulation of working memory. ***A***) Under conditions of optimal working memory performance, preferential engagement of α_{2A} postsynaptic receptors suppresses cAMP production and closes HCN channels within dendritic spines of spatially-tuned layer III PFC pyramidal neurons (90°). Meanwhile, DA D1 receptor signaling within dendritic spines of pyramidal neurons representing spatial information at 130° weakens these connections. ***B***) HCN channels on dendritic shafts are closed, allowing appropriate excitatory information to be received by the cell body and (***C***) efficiently represented by PFC microcircuits relaying spatial information at 90° (***D***). ***E***) Under high levels of PFC catecholamines (e.g. stress), elevated cAMP production and (***F***) subsequent activation of HCN channels within spines and dendritic shafts weakens inputs to pyramidal neurons representing information at 90°. ***G***) Engagement of α_1 receptors and PKC signaling (via Gq) within the soma further weakens microcircuit connections representing spatial information at 90°, eroding task-relevant representation of spatial information at 90° and impairing working memory performance. From Arnsten, 2009.

dendritic spines that receive noisy/irrelevant network inputs, thereby reducing PFC responses to 'noise' (Arnsten et al., 2010). In contrast, excessive D1 receptor activity (e.g. during stress) broadly increases cAMP signaling, non-selectively suppressing responses to all synaptic inputs and eroding neuronal firing to the 'signal' as well as the 'noise' (Figure 2H; Vijayraghavan et al., 2007; Arnsten et al., 2012).

Combined, these observations demonstrate that moderate rates of PFC catecholamine release associated with optimal working memory involve concurrent stimulation of α_{2A} and D1 receptors, which *strengthen* PFC networks representing task-relevant inputs while *weakening* task-irrelevant inputs. In contrast, high levels of PFC catecholamines lead to α_1 receptor activation and excessive D1 receptor signaling, broadly suppress task-relevant and irrelevant inputs, and impair working memory.

Attentional Processes

Aside from working memory, the PFC supports a variety of cognitive processes that are modulated by different circuit and receptor mechanisms. Evidence indicates that NE exerts receptor-specific modulatory actions across PFC-dependent cognitive processes. For example, while PFC α_1 receptors impair working memory performance, these receptors facilitate both flexible (attentional set shifting) and focused/sustained attention (Arnsten et al., 1999; Lapiz and Morilak, 2006; Berridge et al., 2012). Consistent with this, clinically relevant doses of methylphenidate maximally enhance working memory performance, while doses 4-fold higher than this maximally improve sustained attention in an α_1 receptor-dependent manner (Berridge and Spencer, 2016). Collectively, these observations demonstrate that NE signaling in the PFC differentially modulates distinct PFC-dependent cognitive processes.

Frontostriatal Circuit Modulation of Cognition

The PFC does not act in isolation. PFC projections to downstream striatal targets play a critical role in cognitive and affective processes historically described as 'PFC-dependent' (Voorn et al., 2004; Chudasama and Robbins, 2006). The rat medial PFC is subdivided into three distinct dorsoventral subfields that send topographically-organized projections to the striatum: the dorsal anterior cingulate (dAcg) cortex, the prelimbic (PL) cortex, and the infralimbic (IL) cortex. Anatomical and behavioral evidence demonstrates the dAcg and dorsal PL comprise a dorsomedial PFC (dmPFC) functional unit, while the ventral PL and IL form a ventromedial PFC (vmPFC) functional unit (Ragozzino et al., 1998; Hoover and Vertes, 2007; Kesner and Churchwell, 2011). Furthermore, the dmPFC projects more heavily to the dorsomedial striatum (dmSTR), while the vmPFC preferentially innervates the ventromedial striatum (vmSTR). Each of these pathways represent functionally distinct frontostriatal circuits (Voorn et al., 2004; Mailly et al., 2013). Consistent with this, evidence indicates the dmSTR and vmSTR are as necessary as the PFC for higher cognitive processes, including working memory (Spencer et al., 2012) and sustained attention (Spencer et al., 2014). In addition to a dorsoventral functional topography, there also exists a rostrocaudal topography within the rat medial PFC. For example, while catecholamines act in the rostral dmPFC to modulate working memory, they act within the caudal dmPFC to modulate sensorimotor gating (Zahrt et al., 1997; Arnsten et al., 1999; Alsene et al., 2011). Limited anatomical evidence suggests there is also a rostrocaudal organization within frontostriatal projections, such that the rostral PL PFC sends denser projections to rostral portions of the nucleus accumbens, while the caudal PL PFC is more heavily connected to the dorsal nucleus accumbens and caudal dorsal STR (Gorelova and Yang, 1996).

The STR integrates inputs from both cognitive and motivational circuits to initiate goal-directed action plans in reinforcement-based learning (Haber et al., 2006). Consistent with this, neurons in the dorsal STR respond to spatial and reward-based cues in spatial navigation tasks (Schultz et al., 1997; Schmitzer-Torbert and Redish, 2004). In addition, changes in spiking patterns of STR neurons correlate with changes in performance during habit learning (Alexander and DeLong, 1985; Aosaki et al., 1995; Barnes et al., 2005). Although the role of the STR in working memory is less well understood, evidence from functional imaging and electrophysiological studies demonstrates the dorsal STR displays sustained delay-related activity in spatial working memory tasks (Schultz and Romo, 1988; Levy et al., 1997; Kawagoe et al., 1998; Postle and D'Esposito, 1999). In rodents, dmSTR neurons exhibit sequential peaks of activity that span the entire delay period in a delayed non-match to position working memory task. Interestingly, dmSTR neurons participating in this sequential activation appear to encode the sample stimulus most robustly at the onset of the delay rather than during their peak activation in the delay period (Akhlaghpour et al., 2016). Taken together, these observations demonstrate the STR encodes task-relevant information during learning and working memory.

Beyond spiking activity of individual neurons, oscillations in local field potentials (LFPs) are driven by synchronized changes in extracellular currents of local neuronal populations and are posited to facilitate communication among neuronal networks (Başar et al., 2001; Buzsaki, 2006; Uhlhaas et al., 2010; Benchenane et al., 2011). LFP oscillations within different frequency ranges have been associated with various cognitive and working memory processes. For example, delay-related gamma oscillations (30-90 Hz) have been associated with maintenance of items held in working memory in human electroencephalography (EEG) studies (for review, see Roux and Uhlhaas, 2014). Moreover, frontal cortical theta oscillatory power (3-10 Hz) increases

during successful, but not unsuccessful completion of working memory tasks in humans and monkeys[1] (Itthipuripat et al., 2013; Hsieh and Ranganath, 2014). Consistent with this, in rodent studies, successful trial-by-trial working memory performance is associated with increased entrainment of PFC spikes to the local theta rhythm (4-10 Hz; Hyman et al., 2010). Within the dmSTR of rats navigating a working memory task, theta power increases when animals leave the start gate and diminishes at the decision point, while gamma (30-60 Hz) power increases at goal completion (Tort et al., 2008). However, the degree to which task-related LFP oscillatory activity in the STR is necessary for successful working memory performance is unknown.

Coherence between neuronal networks is a form of functional connectivity that may serve as a binding mechanism to organize functionally connected neuronal ensembles (Womelsdorf et al., 2007). In rats, theta-range coherence between the PFC and hippocampus is enhanced during correct, but not incorrect responses in a working memory task (Jones and Wilson, 2005). In human imaging studies, increases in frontostriatal functional connectivity are associated with improved performance in working memory tasks (Wallace et al., 2011). Moreover, the strength of frontostriatal connectivity correlates with improved performance in human subjects performing cognitive flexibility and decision making tasks, supporting the hypothesis that functional connectivity within the frontostriatal circuit is important for PFC-dependent cognition (Nagano-Saito et al., 2008; Forstmann et al., 2010).

Frontostriatal Circuit Function in Stress and Disease

Stress impairs frontostriatal-dependent cognition in humans, non-human primates, and rodents (Arnsten and Goldman-Rakic, 1998; Holmes and Wellman, 2009; Liston et al., 2009;

[1] Note there exist species- and study-specific differences in the way frequency bands are defined, see Buzsáki et al., 2013.

Devilbiss et al., 2012). Recent studies in our laboratory demonstrated that stress-related impairment in working memory is associated with a suppression of spiking activity of dmPFC neuronal subpopulations strongly tuned to key working memory task events in rats (Devilbiss et al., 2017). Meanwhile, stress increased task-related activity of the larger population of dmPFC neurons displaying low levels of activity during the delay, reward or error events. This is consistent with observations demonstrating that stress-related impairment in working memory is dependent on noradrenergic α_1 receptors, and that α_1 receptor activation suppresses delay-related activity within the primate dorsolateral PFC (Birnbaum et al., 1999; Birnbaum, 2004). Furthermore, preliminary observations suggest stress also suppresses delay-related activity of dmSTR medium spiny neurons in working memory-tested rats (Devilbiss et al., unpublished observations). Thus, stress-related cognitive impairment is associated with a profound decrease in the signal-to-noise ratio of task-related activity across the frontostriatal circuit.

In human functional imaging studies, acute psychosocial stress produces deficits in PFC-dependent cognition that are associated with reduced frontopariental functional connectivity (i.e. reduced covariance of BOLD signals; Liston et al., 2009). Meanwhile, stress *increases* functional connectivity between frontal cortical areas and limbic structures such as the amygdala, midbrain, and hypothalamus. Additionally, stress-induced increases in connectivity between these regions are positively correlated with the stress response (i.e. levels of cortisol and norepinephrine metabolites; Hermans et al., 2011). Taken together, these results suggest that during stress, PFC networks fail to exert proper top-down control over stress-related information processing in subcortical circuits.

In summary, frontostriatal circuit dysfunction is associated with a variety of mental health disorders, including ADHD and schizophrenia (Uhlhaas and Singer, 2006; Cubillo et al.,

2010; Liston et al., 2011). It is important to note that the degree to which abnormal neurophysiological and functional connectivity patterns cause or simply correlate with pathophysiological brain states is not known. Therefore, a better understanding of the mechanisms that modulate information flow through the frontostriatal circuit may lead to improved treatment of disorders associated with frontostriatal circuit dysfunction.

Neurobiology of CRF

A large body of research demonstrates that CRF coordinates physiological, endocrine, and behavioral responses during stress. CRF is a 41-amino acid peptide that binds two receptor subtypes: CRF_1 and CRF_2 (Perrin et al., 1995; Bale and Vale, 2004). Both receptor subtypes are seven transmembrane, G-protein coupled receptors predominantly linked to the G_s-cAMP-protein kinase A (PKA) intracellular cascade. Indeed, the behavioral actions of CRF_1 signaling in the PFC have been most closely associated with PKA signaling (Meng et al., 2011; Miguel et al., 2014). However, *in vitro* assays demonstrate that cortical CRF_1 receptors in rats can also couple to G_q, G_i, and G_o, albeit at much lower affinities (Grammatopoulos et al., 2001). Consistent with this, CRF_1-dependent activation of dopamine neurons in the ventral tegmental area involves protein kinase C (PKC), not PKA signaling (Wanat et al., 2008).

Animal studies have demonstrated that CRF modulates behavioral and physiological responses during stress (Dunn and Berridge, 1990; Bale and Vale, 2004). Limited evidence also suggests a role for subcortical CRF in anticipatory and appetitive behaviors (Merali et al., 2004b; Peciña et al., 2006). These responses are mediated by CRF-synthesizing neurons in hypothalamic and extrahypothalamic structures including the paraventricular nucleus (PVN) of the hypothalamus, central nucleus of the amygdala, bed nucleus of the stria terminalis (BNST), and

various brainstem nuclei (Swanson et al., 1983). In addition, CRF-containing cell bodies are present in cortical regions, particularly the PFC (Swanson et al., 1983; Charlton et al., 1987; Sakanaka et al., 1987). In the rat, CRF_1 mRNA and binding sites are present in all layers of the entire dorsoventral and rostrocaudal aspects of the PFC (De Souza et al., 1985; Potter et al., 1994; Lovenberg et al., 1995). Early reports indicated that CRF_2 is not expressed in the rat neocortex (Lovenberg et al., 1995). However, subsequent studies identified very low levels of CRF_2 mRNA in the neocortex (Kostich et al., 1998; Van Pett et al., 2000). Nonetheless, CRF binds CRF_1 with a tenfold greater affinity compared to CRF_2, and thus CRF_1 is presumed to be the primary functional receptor subtype in the PFC (Perrin et al., 1995).

The distribution of CRF receptors within the rodent and primate brain varies markedly. In the rat, all CRF binding sites in the pituitary and cortex are CRF_1, whereas in humans and non-human primates both CRF_1 and CRF_2 are found in these regions (Chalmers et al., 1995; Kostich et al., 1998; Van Pett et al., 2000). Moreover, in the rat, CRF_2 is generally limited to subcortical structures where they co-occur with CRF_1 in regions including the BNST and amygdaloid nuclei. On the other hand, CRF_2 is present almost exclusively in the BNST in non-human primates (Sanchez et al., 1999). Therefore, the differential distribution of CRF_1 and CRF_2 receptors in primates and rodents may result in species differences in the actions of CRF within these circuits.

Limited evidence suggests CRF signaling in the PFC modulates affective and endocrine processes. First, acute stress exposure activates the PFC CRF system, as evidenced by increases in CRF expression in response to restraint stress (Meng et al., 2011). Consistent with this, CRF-expressing neurons in the PFC become activated during alcohol withdrawal in rats (George et al., 2012). Furthermore, intra-PFC infusions of CRF produce anxiogenic effects while intra-PFC

infusion of a non-selective CRF antagonist attenuates stressor-induced activation of the hypothalamic-pituitary-adrenal axis (Jaferi and Bhatnagar, 2007).

Clinical studies have implicated CRF and CRF receptor abnormalities in disorders associated with PFC-dependent cognitive dysfunction. For example, levels of CRF in the PFC and cerebrospinal fluid are greater in depressed suicide victims, whereas prefrontal CRF receptor levels are lower relative to healthy controls (Nemeroff et al., 1988; Arató et al., 1989; Hucks et al., 1997; Merali et al., 2004a). These and other observations support the hypothesis that CRF hypersecretion and dysregulation of cortical CRF systems is associated with pathophysiological states (Craig et al., 1992). However, despite 30+ years of research on the neurobiology of CRF and the PFC, there is virtually no information regarding the *cognitive actions* of CRF signaling in the PFC.

Aims

To date, the majority of work on the neurobiology of PFC-dependent cognition has focused on catecholamines and catecholamine-related signaling pathways. This work has provided significant insight into the neurobiology supporting PFC-dependent cognitive function. However, catecholamines are not the only modulators of PFC function. Although the PFC contains a high density of CRF and CRF receptors, and the neurobiology of CRF has been intensively studied for decades, the cognitive actions of CRF signaling in the PFC are largely unknown. This represents a significant gap in our understanding of both CRF and the PFC. Thus, the goal of this thesis is to test the hypothesis that CRF acts within the PFC to modulate cognition and frontostriatal neural function.

Specifically, this dissertation has three major goals:

1) Determine the cognitive actions of CRF receptor signaling in the PFC as measured in a PFC-dependent test of working memory (Chapter 2).

2) Examine the effects of PFC CRF neuronal activity on working memory and neural encoding of working memory task events across the frontostriatal circuit (Chapter 3).

3) Investigate the broader cognitive actions of PFC CRF neurons and receptors using a PFC-dependent signal detection test of sustained attention (Chapter 4).

Chapter 2

Working Memory Impairing Actions of Corticotropin-Releasing Factor (CRF) Neurotransmission in the Prefrontal Cortex

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Abstract

The prefrontal cortex (PFC) regulates cognitive processes critical for goal-directed behavior. PFC cognitive dysfunction is implicated in multiple psychopathologies, including attention deficit hyperactivity disorder (ADHD). Although it has long been known that corticotropin-releasing factor (CRF) and CRF receptors are prominent in the PFC, the cognitive effects of CRF action within the PFC are poorly understood. The current studies examined whether CRF receptor activation in the PFC modulates cognitive function in rats as measured in a delayed response task of spatial working memory. CRF dose-dependently impaired working memory performance when administered either intracerebroventricularly (ICV) or directly into the PFC. The working memory actions of CRF in the PFC were topographically-organized, with impairment observed only following CRF infusions into the caudal dorsomedial PFC (dmPFC). Additional studies examined whether *endogenous* CRF modulates working memory. Both ICV and intra-dmPFC administration of the non-selective CRF antagonist, D-Phe-CRF, dose-dependently improved working memory performance. To better assess the translational potential of CRF antagonists, we examined the cognitive effects of systemic administration of the CRF_1 receptor selective antagonist, NBI 35965. Similar procognitive actions were observed in these studies.

These results are the first to demonstrate that CRF acts in the PFC to regulate PFC-dependent cognition. Importantly, the ability of CRF antagonists to improve working memory is identical to that seen with all approved treatments for ADHD. These observations suggest that CRF antagonists may represent a novel approach for the treatment of ADHD and other disorders associated with dysregulated prefrontal cognitive function.

Introduction

The prefrontal cortex (PFC) plays a pivotal role in higher cognitive processes required for flexible goal-directed behavior (Miller and Cohen, 2001). PFC cognitive dysfunction is associated with a variety of behavioral disorders, including depression, schizophrenia and attention-deficit hyperactivity disorder (ADHD; Millan et al., 2012). The majority of clinically efficacious drugs used in the treatment of prefrontal cognitive dysfunction target catecholamines, consistent with extensive evidence demonstrating an important role of these transmitters in the regulation of PFC-dependent cognition (Berridge and Arnsten, 2015). However, there is a serious need for improved treatments for PFC-related cognitive dysfunction. For example, currently available treatments lack full efficacy across the broader population of patients. Moreover, while psychostimulants are highly effective in a large proportion of ADHD patients, these drugs possess significant potential for misuse/abuse (Setlik et al., 2009). Unfortunately, the development of novel pharmacological treatments for PFC-dependent cognitive dysfunction is limited by a scarcity of alternative targets.

The neuropeptide, corticotropin-releasing factor (CRF), is prominent in the PFC (Swanson et al., 1983) and early correlative observations suggested a potential role of PFC CRF in PFC-related psychopathology (Nemeroff et al., 1988). However, despite extensive research into the neurobiology of CRF over the ensuing decades, the functional significance of CRF receptor signaling in the PFC is poorly understood. In the rat, CRF receptors are observed throughout the medial PFC (De Souza et al., 1985; Lovenberg et al., 1995; Van Pett et al., 2000) and limited observations indicate that CRF receptor activation in this region exerts behaviorally and physiologically relevant actions (Jaferi and Bhatnagar, 2007; Miguel et al., 2014). However, the cognitive effects of CRF in the PFC are currently unknown.

To address this issue, we first examined the effects of intracerebroventricular (ICV) and intra-PFC infusion of CRF on performance in a delayed response task of spatial working memory in rats. This task has been used extensively to study catecholamine modulation of PFC-dependent higher cognitive function and has been demonstrated to possess strong translational relevance (Arnsten and Pliszka, 2011; Berridge et al., 2012; Spencer et al., 2012). In particular, the pharmacology of performance in this task mirrors the pharmacology of ADHD, with all approved ADHD treatments improving task performance (Berridge and Arnsten, 2015). Our results demonstrate that CRF receptor activation, both globally in the brain and selectively within the PFC, elicits a dose-dependent impairment in working memory. The cognitive actions of CRF receptor activation in the PFC were topographically organized, with cognitive impairment only observed following CRF infusions into the caudal portion of the dorsomedial PFC (dmPFC). These findings are consistent with evidence demonstrating that, in general, the rat medial PFC is topographically organized along the dorsoventral axis, with more dorsal portions attributed to cognitive-like functions, while ventral aspects of the PFC are more closely associated with affect and motivation (Heidbreder and Groenewegen, 2003; Voorn et al., 2004; Gabbott et al., 2005). To assess the cognitive actions of *endogenous* CRF within the PFC, we examined the working memory effects of intra-PFC infusion of a non-selective CRF antagonist (D-Phe-CRF). CRF receptor blockade in the caudal dmPFC elicited a dose-dependent improvement in working memory. Similar actions were observed with both ICV D-Phe-CRF and systemic treatment with a CRF_1 receptor selective antagonist (NBI 35965).

Collectively, these studies demonstrate that CRF receptor signaling in the PFC plays a prominent role in higher cognitive function. Moreover, given all FDA-approved treatments for ADHD enhance working memory (Berridge and Arnsten, 2015), these results suggest the

possibility that CRF receptor antagonists may represent a novel treatment strategy for ADHD and other disorders associated with PFC-dependent cognitive dysfunction.

Methods and Materials

Animals

Male Sprague-Dawley rats (280-400 grams; Charles River, Wilmington, Massachusetts) were pair-housed in clear polycarbonate cages on a 13/11-hour light/dark cycle. Animals were fed *ad libitum* for the first 4-7 days after arrival. Subsequently, the amount of food was titrated for each animal (15 to 17 grams of chow/day) to maintain motivation for food reward while avoiding weight loss. Rats were handled extensively before behavioral testing commenced. Training/testing was conducted between 0800 and 1600 hours (5-6 days/week). All facilities and procedures were in accordance with the guidelines regarding animal use and care put forth by the National Institutes of Health of the United States and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

Pre-surgery Behavioral Training

T-maze training and testing was conducted in rooms devoid of external spatial cues and/or in a dark room lit with a red light as previously described (Berridge et al., 2012). Briefly, animals were trained to enter the arm of a T-maze not chosen on the previous trial to receive a food reward (one chocolate chip or sucrose pellet per trial). Between trials, rats were placed in a start box at the base of the maze and prevented from exiting by a removable gate. Sessions consisted of 20 trials (one session per day).

Surgery

After completion of maze training, rats were anesthetized with isoflurane (1%-1.5%) and stainless steel cannulae (26-gauge) were surgically implanted bilaterally over one of the following subregions: rostral (A +3.6 to 3.0; L ± 0.8; V -0.2 mm from dura and bregma) or caudal (A +2.8 to +2.3) PFC. Dorsal (V -2.5 to -3 mm) and ventral (V -4 mm) aspects of the medial PFC were targeted via different needle projection length in different groups of animals (see below). For ICV infusions, cannulae were unilaterally aimed at the lateral ventricles (A -0.8, L ± 1.5, V -2.0; hemisphere counterbalanced). Only one region was targeted per animal. Given anatomical and behavioral evidence implicating the dmPFC in higher cognitive/behavioral processes, care was taken to maintain the structural integrity of the dmPFC, with cannulae lowered no more than 200 µm below the dura. Cannulae were secured to the skull with stainless steel screws and acrylic cement (Plastics One, Roanoke, Virginia). Stainless steel stylets prevented occlusion of cannulae and were replaced as needed.

Drugs

CRF (human/rat, Bachem, Torrance, CA) was dissolved in buffered artificial extracellular fluid (AECF; 147 mmol/L NaCl, 1.3 mmol/L CaCl, 0.9 mmol/L MgCl, 2.5 mmol/L KCl; pH = 7.4). The CRF antagonists, D-Phe-CRF **(**D-Phe^{12},Nle^{21,38},α-Me-Leu^{37})-CRF (12-41); human/rat, Bachem, Torrance, CA) and NBI 35965 (Tocris, Bristol, United Kingdom) were dissolved in 0.9% saline.

Infusions and Working Memory Testing

Following surgery, rats resumed T-maze testing until performance reached pre-surgery levels. Following this, a short delay of 10 seconds between each trial was introduced. Baseline performance levels of 80-92% and 70-85% were required for CRF and CRF antagonist studies, respectively. In this task, performance improves with repeated testing. Therefore, delays were increased when performance exceeded the desired range (Zahrt et al., 1997; Berridge et al., 2012; Spencer et al., 2012). To ensure task dependence on the PFC, animals requiring delays greater than 60 seconds were excluded from further study. For all studies, the number of accurate trials was recorded per session. Run-time was also calculated, defined as: (total run duration) – (total delay duration).

Prior to testing, animals were given two mock infusions, consisting of an initial needle insertion followed by a second needle insertion with vehicle infusion 48 hours later. This allowed animals to acclimate to the gentle handling associated with infusions and to minimize detrimental behavioral effects of tissue damage related to the initial needle insertion.

Bilateral intra-PFC infusions (500 nl) were made with 33-gauge needles. For the dmPFC, needles projected 2.5-3.0 mm. For ventromedial PFC (vmPFC), needles projected 4.0 mm. Intra-PFC infusions were performed using a microprocessor-controlled pump (Harvard Apparatus, South Natick, MA) at a rate of 250 nl/minute for 2 minutes (500 nl total volume). ICV infusions were made through 33-gauge needles projecting 2.0 mm past the cannula at a rate of 1 µl/minute for 2 minutes (2 µl total). Needles were kept in place for 2 minutes following the infusion, after which the stylets were replaced.

To limit tissue damage, the number of infusions was limited to 4 for intra-tissue infusions and 6 for ICV infusions (excluding mock infusions). Our prior experience demonstrates that this results in minimal tissue damage. In general, animals received only one type of treatment (CRF

vs. D-Phe-CRF vs. NBI 35965). The one exception to this was for ICV treated animals that were tested with CRF and D-Phe-CRF (on different days). This latter approach permitted aligning treatment with baseline performance to minimize animal usage (higher baselines for CRF treatment; lower baselines for antagonist treatment). This was feasible given the greater limit on the number of infusions relative to intra-tissue infusions (see above).

On the day of testing, rats were transported to the testing room in their home cage, infused with CRF or D-Phe-CRF and returned to their home cage for 15 minutes prior to testing. NBI 35965 was injected subcutaneously (1 ml/kg) 60 minutes before testing (Million et al., 2003). For all treatments, doses were counterbalanced.

Treatments were administered when stable baseline performance was observed (2 consecutive days in which performance accuracy did not differ by more than 10%). Given performance improves over time, it is essential to confirm that post-treatment performance is in the same range of pre-treatment baseline performance. This ensures that any change in performance on a treatment day reflects the treatment and not time-dependent changes in performance. For this reason, rats were tested at the same delay on the first 2 post-treatment days. Data were included in analyses only if performance was stable (pre- vs. post-infusion performance difference <10%). To facilitate data collection, only the first post-treatment day was used to determine stable performance for studies involving the systemic CRF_1 antagonist, NBI 35965.

Given the limits on delays and infusion number, not every animal received each dose of a given treatment.

Feeding Analyses

In the only study in which drug treatment affected run-time (ICV CRF), we additionally examined whether this might reflect changes in motivation for food reward. For these studies, testing occurred in the T-maze testing room in a 23x43x20 cm clear Plexiglas cage that animals were habituated to for 2 days prior to the start of testing (20 min/day). During acclimation, animals were allowed to explore the cage and consume 20 sugar pellets scattered throughout the cage (same quantity as in T-maze testing). During testing, animals were placed in the testing cage and the latency to initiate and time to complete consumption of all sugar pellets within a 20-minute period was recorded by an experimenter in the room. Animals received 2 µl infusions (1 µl /minute) of vehicle and 0.1, 0.2, and 1 µg CRF 15 minutes before testing in a within-subjects design. All treatments were counterbalanced.

Histological Analyses

Rats were deeply anesthetized with isoflurane and transcardially perfused with 10% wt/vol formaldehyde and brains stored in formaldehyde for at least 24 hours before sectioning. Injector needle placement was verified in 40 µm thick coronal sections stained with Neutral Red dye (Sigma-Aldrich, St. Louis, MO).

Statistical Analyses

Data from a given experiment were included only when histological analyses verified accurate placement of injectors, minimal PFC damage, and stable performance before and after treatment. Given limits on delay and number of infusions and the requirement for stable performance across pre- and post-treatment testing days, it was not possible for all T-maze animals to receive all doses of a given treatment. For this reason, treatment effects could not be

analyzed using between- or within-subjects analyses of variance (ANOVA). Therefore, we estimated a linear mixed-effects model using the lmer package in R to analyze effect of treatments on performance accuracy and run-time. To assess the effects of ICV CRF on feeding, a one-way repeated measures ANOVA was used. When statistical significance was indicated, comparisons between drug dose vs. vehicle were determined using Bonferroni corrected t-tests.

Results

Effects of CRF receptor activation on working memory performance

Initial studies examined whether ICV administered CRF impairs working memory performance. For these studies, animals were treated with vehicle (n=9), 0.1 µg (n=7), 0.2 µg (n=8), or 1 µg (n=8) CRF. Doses were based on previous studies demonstrating behavioral/cognitive actions of ICV CRF (Dunn and Berridge, 1990; Snyder et al., 2011; Cole et al., 2016). As shown in Figure 1 and Table S1, ICV CRF elicited dose-dependent impairments in working memory performance ($F_{1,28.3}$ = 32.8; P < 0.001), with significant impairment observed at all doses. Run-time was only significantly affected (increased) at the highest dose (Table S1; $F_{1,19.3}$ = 28.2; P < 0.001). This effect on run-time does not appear to reflect decreased motivation for

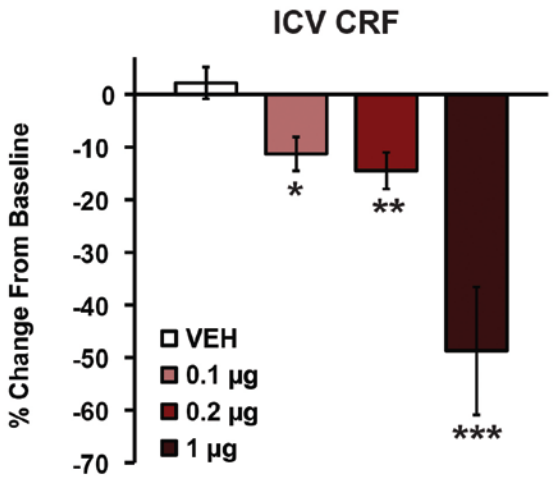

Figure 1. Intracerebroventricular (ICV) CRF impairs working memory. Shown are the effects of ICV administered vehicle (n=9) and varying doses of CRF (0.1 µg CRF, n=7; 0.2 µg CRF, n=8; 1 µg CRF, n=8) on working memory performance as measured by percentage change in correct trials (accuracy) from baseline. Results represent mean ± SEM percentage change in correct trials (accuracy) from baseline. * P < 0.05, ** P < 0.01, ***P < 0.001 vs. vehicle.

or ability to consume (due to competing behaviors) sugar, as CRF had no effect on latency to initiate ($F_{3,24}$ = 0.67; P = 1.0) or time to consume ($F_{3,24}$ = 0.56; P = 1.0) sugar (n=9; Table S2).

Additional studies examined the working memory effects of CRF receptor activation within the PFC. Given the topographic organization of the rat PFC reviewed above, we initially examined the effects of bilateral 100 ng CRF infusions into the dmPFC. Multiple studies demonstrate that this dose is sufficient to elicit significant behavioral and physiological actions when infused into the PFC and other brain regions (Jaferi and Bhatnagar, 2007). As shown in Figure 2 and Table S1, we observed a CRF-induced impairment in working memory that was topographically organized across the rostrocaudal axis. Specifically, CRF impaired performance when infused into the caudal dmPFC (+2.8 mm, n=6 to +2.4 mm,

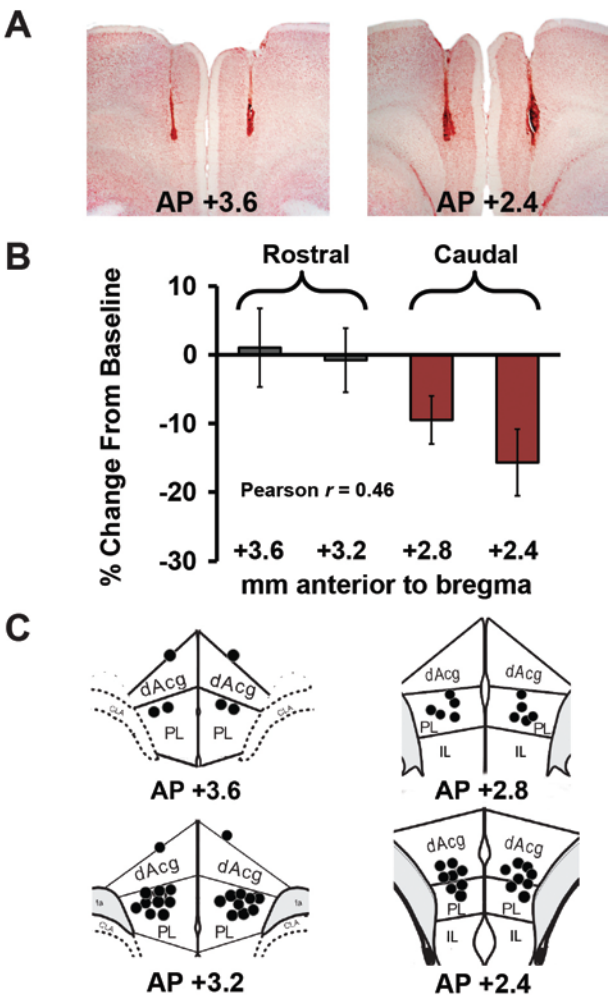

Figure 2. CRF acts in the dmPFC to elicit topographically organized impairments in working memory performance. ***A***) Representative micrograph depicting the main body of infusion sites into the rostral (left) and caudal (right) dmPFC. ***B)*** Working memory effects of 100 ng/hemisphere CRF infused into varying rostrocaudal subfields of the dmPFC (AP +3.6, n=6; +3.2, n=13; +2.8, n=6; +2.4, n=8). There was a significant correlation between infusion needle placement and CRF-induced working memory impairment (Pearson r = 0.46, P < 0.01). These results identify functionally distinct rostral and caudal portions of the dmPFC that were targeted in subsequent studies. Results represent mean ± SEM percentage change in accuracy relative to baseline. ***C)*** Schematic depiction of rostral (left) and caudal (right) dmPFC infusion sites.

n=8 anterior to bregma), but not more rostrally (+3.6, n=6 to +3.2 mm, n=13). Consistent with this, correlation analyses indicated a significant relationship between the placement of infusion needles within the dmPFC and the magnitude of CRF-induced performance impairment (Pearson r = 0.46, P = 0.008).

We then conducted a detailed dose-response analysis of the working memory effects of CRF when infused into the caudal dmPFC (AP +2.8 to +2.4; vehicle, n=10; 25 ng, n=6; 50 ng, n=8; 100 ng, n=15; 250 ng, n=8). CRF elicited a robust dose-dependent impairment in working memory performance, with significant impairment observed at the 100 ng and 250 ng doses (Figure 3A, Table S1; $F_{1,30.4}$ = 10.5, P = 0.003). Given 250 ng CRF elicited a greater impairment in task performance than the 100 ng

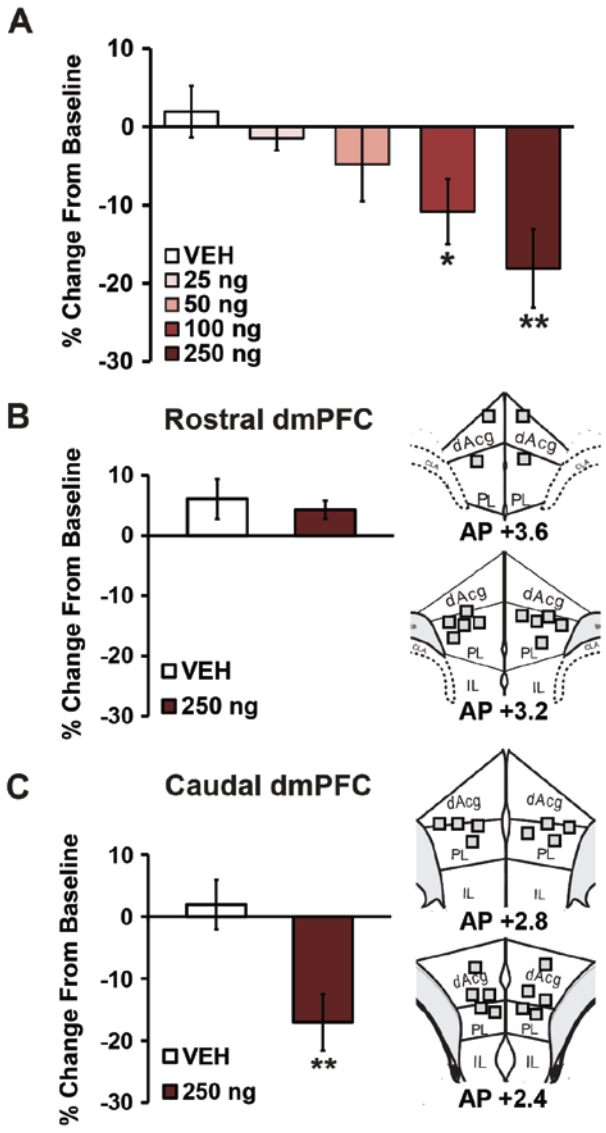

Figure 3. CRF in the caudal dmPFC dose-dependently impairs working memory. ***A***) Shown are the effects of vehicle (n=10) and varying doses of CRF infused bilaterally into the caudal dmPFC (25 ng CRF, n=6; 50 ng, n=8; 100 ng, n=15; 250 ng, n=8). ***B***) *Left:* Given 250 ng CRF elicited a more robust impairment than 100 ng CRF in the caudal dmPFC, the effects of 250 ng CRF infusions into the rostral dmPFC were also examined (n=7). Within this region, this dose had no significant effects on working memory performance relative to vehicle (n=10). *Right:* Schematic depiction of infusion sites shown to the left. ***C***) *Left:* Effects of 250 ng CRF in the caudal dmPFC on performance. *Right:* Schematics of infusion sites shown to the left. Results represent mean ± SEM percentage change in accuracy vs. baseline. *P < 0.05, **P < 0.01 vs. vehicle.

dose, we additionally examined the effects of 250 ng CRF infusions into the rostral dmPFC (n=7) to confirm insensitivity of this portion of the dmPFC to the working memory effects of CRF. In contrast to that seen in the caudal dmPFC, 250 ng CRF did not significantly affect performance when infused into the rostral dmPFC (Figure 3B vs. 3C; $F_{1,16} = 0.19$, $P = 0.7$). As shown in Table S1, intra-PFC infusions of CRF had no effect on run-times in the maze (rostral dmPFC: $F_{1,31} = 0.11$; $P = 0.74$; caudal dmPFC: $F_{1,16.2} = 0.0007$; $P = 0.99$).

While the dmPFC is most strongly implicated in higher cognitive function, both anatomical and functional studies argue for a role of the vmPFC in working memory/cognition (Heidbreder and Groenewegen, 2003). Thus, additional studies examined the working memory effects of intra-vmPFC infusions of CRF (100 ng, 250 ng). As shown in Figure 4 and Table S1, CRF had no effect on working memory performance when infused into either the rostral vmPFC (vehicle, n=10; 100 ng, n=9; 250 ng, n=6; $F_{1,16.3} = 3.7$, $P = 0.08$; Figure 4A) or caudal vmPFC (vehicle, n=10; 100 ng, n=6; 250 ng, n=6; $F_{1,19.2} = 0.28$; $P = 0.59$;

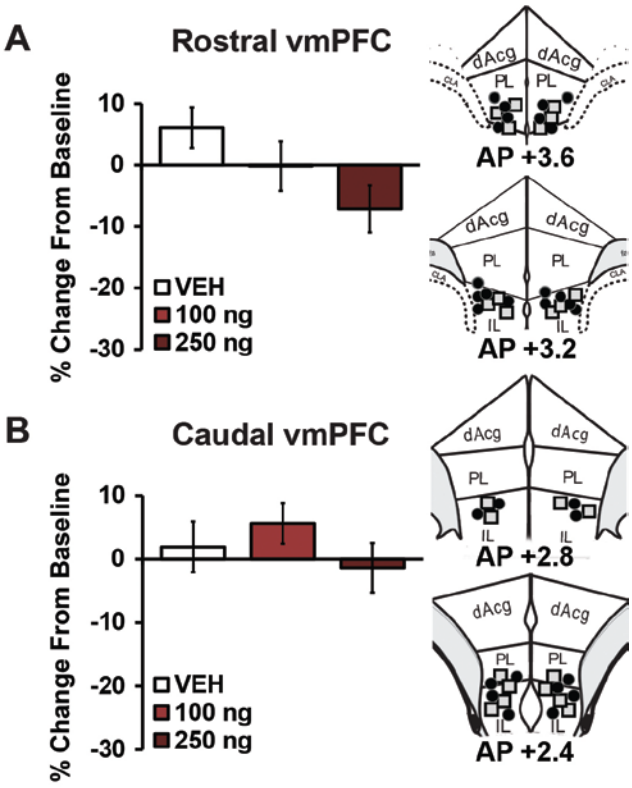

Figure 4. CRF does not act in the vmPFC to modulate working memory performance. ***A***) *Left Panel*. Working memory effects of bilateral vehicle (n=10) or 100 ng (n=9) or 250 ng (n=6) CRF infusions into the rostral vmPFC on task performance. *Right*. Schematics of infusion sites: 100 ng CRF, black circles; 250 ng CRF, gray squares. ***B***) *Left*. Effects of bilateral vehicle (n=10) or 100 ng (n=6) or 250 ng (n=6) CRF infusions into the caudal vmPFC on task performance. *Right*. Schematics of infusion sites: 100 ng CRF, black circles; 250 ng CRF, gray squares. Results represent mean ± SEM percentage change in correct trials (accuracy) relative to baseline performance.

Figure 4B). Similarly, CRF infusions into both rostral and caudal subfields of the vmPFC had no effect on run-time (Table S1; rostral vmPFC: $F_{1,7.9} = 1.5$; $P = 0.3$; caudal vmPFC: $F_{1,7.4} = 3.7$; $P = 0.09$).

Role of endogenous CRF actions within the PFC in working memory

The above-described results indicate that CRF receptor activation, both globally and within the caudal dmPFC, impairs working memory. Additional studies examined the actions of *endogenous* CRF signaling in the PFC on working memory using bilateral intra-caudal dmPFC infusions of the non-selective CRF antagonist, D-Phe-CRF (vehicle, n=9; 50 ng, n=7; 200 ng, n=9). D-Phe-CRF elicited a dose-dependent improvement in working memory performance relative to vehicle (Figure 5A and Table S1; $F_{1,20.5} =$, $P = 0.02$) while lacking significant effects on run-time ($F_{1,20.8} = 0.01$, $P = 0.94$; Table S1).

The procognitive effects of intra-PFC CRF receptor blockade in this task are similar to that seen with all FDA-approved treatments for ADHD (Berridge and Arnsten, 2015). To better assess the potential clinical utility of CRF antagonists, we examined whether CRF antagonist distribution more globally in the brain improves working memory performance. Specifically, we tested the effects of vehicle (n=7) and varying doses of ICV administered D-Phe-CRF (2 µg, n=8; 4 µg, n=6; 10 µg, n=8). As shown in Figure 5B and Table S1, ICV infusion of this CRF antagonist elicited a dose-dependent improvement in working memory performance ($F_{1,11.7} = 28.4$, $P < 0.001$) in the absence of significant changes in run-time ($F_{1,10.8} = 2.3$; $P = 0.2$; Table S1). At the highest dose tested, the magnitude of working memory improvement was comparable to that seen with systemic administration of clinically-relevant doses of the ADHD medication, methylphenidate (Ritalin; Berridge et al., 2006).

Finally, to assess the cognitive effects of selective CRF_1 receptor antagonism, additional studies examined the working memory effects of systemic administration of the CRF_1 receptor-selective antagonist, NBI 35965 (Million et al., 2003). As shown in Figure 5C and Table S1, systemic treatment with this antagonist (2.5 mg/kg, n=6; 5 mg/kg, n=8; 10 mg/kg, n=7) also elicited a dose-dependent improvement in working memory performance relative to vehicle (n=14) that is comparable to that seen with methylphenidate (Berridge et al., 2012). Systemic administration of this antagonist had no effect on run-time ($F_{1,24.9}$ = 0.05; P = 0.8; Table S1).

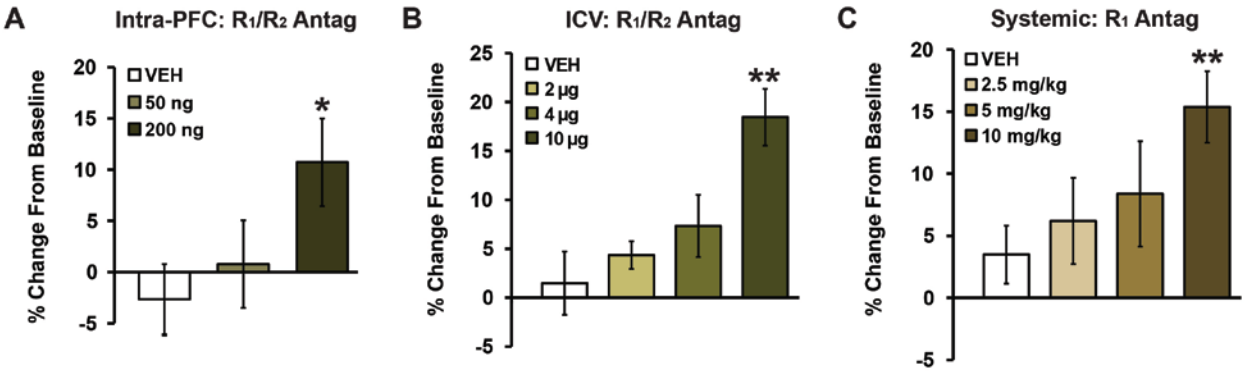

Figure 5. Cognition-enhancing effects of CRF receptor blockade. Shown are the working memory effects of ***A***) bilateral intra-caudal dmPFC infusions of the non-selective $CRF_{1/2}$ receptor antagonist, D-Phe-CRF (vehicle, n=9; 50 ng, n=7; 200 ng, n=9) and ***B***) ICV infusion of D-Phe-CRF (vehicle, n=7; 2 µg, n=8; 4 µg, n=6; 10 µg, n=8) on working memory performance. For both routes of administration, this CRF antagonist dose-dependently improved task performance. (c) Systemic administration of the CRF_1 receptor selective antagonist, NBI 35965, similarly improved working memory performance in a dose-dependent manner (vehicle, n=14; 2.5 mg/kg, n=6; 5 mg/kg, n=8; 10 mg/kg, n=7). Results represent mean ± SEM percentage change in accuracy relative to baseline. *P < 0.05, **P < 0.01 vs. vehicle.

Discussion

These studies are the first to show that CRF acts in the PFC to impair higher cognitive function, as measured in a delayed response task of working memory. The cognitive effects of CRF receptor activation were topographically organized, such that working memory impairment was only observed following CRF infusions into the caudal dmPFC. The cognition-enhancing actions of CRF antagonists observed in these studies demonstrate a cognitive role of *endogenous* CRF and mimic that seen with all FDA-approved drugs for ADHD (Berridge et al., 2012; Spencer et al., 2012; Berridge and Arnsten, 2015). Combined, these observations demonstrate a prominent role of CRF in the PFC in the regulation of higher cognitive function and suggest CRF may represent a novel pharmacological target for the treatment of ADHD or other forms of PFC-dependent cognitive dysfunction.

The restriction of the working memory actions of CRF to the dmPFC is consistent with the well-documented dorsoventrally-organized functional topography of the rodent medial PFC. Specifically, the dmPFC is more closely associated with higher cognitive function while the vmPFC is more closely associated with affect and motivation (Heidbreder and Groenewegen, 2003; Voorn et al., 2004; Gabbott et al., 2005). However, our results also highlight a less-well studied rostrocaudally-organized topography. The restriction of the working memory effects of CRF to the *caudal* dmPFC is identical to that observed for noradrenergic modulation of sensorimotor gating (Alsene et al., 2011). In contrast, catecholamine-dependent modulation of working memory in rodents is more closely linked to the rostral dmPFC (Zahrt et al., 1997; Arnsten et al., 1999). The neural bases for the rostrocaudal topography observed in the current study are unclear. CRF receptors are distributed throughout the dorsoventral and rostrocaudal medial PFC (Van Pett et al., 2000). Thus, preferential sensitivity of the caudal dmPFC to CRF

does not reflect a differential distribution of CRF receptors. Instead, this topography may reflect functional differences in PFC efferent connectivity within corticostriatal and corticothalamic circuits. For example, the rostral prelimbic PFC sends denser projections to the rostral ventral striatum and innervates more medial regions of the dorsal striatum relative to the caudal prelimbic PFC. In addition, the caudal prelimbic PFC is more strongly connected to the caudal ventral striatum and sends denser projections to the mediodorsal thalamic nucleus relative to the rostral prelimbic PFC (Sesack et al., 1989; Gorelova and Yang, 1996).

Currently, the cellular mechanisms underlying the cognition-impairing actions of CRF receptor activation in the PFC are unknown. In the rodent, the PFC predominantly expresses CRF_1 receptors while in the primate both CRF_1 and CRF_2 receptor subtypes are present in the PFC (Sanchez et al., 1999; Van Pett et al., 2000). Limited evidence indicates that both CRF_1 and CRF_2 receptors can activate protein kinase A (PKA) and phosphatidylinositol-protein kinase C (PI/PKC) pathways (Tan et al., 2004; Miguel et al., 2014). Evidence demonstrates that both PKA and PI/PKC signaling modulate working memory via regulation of PFC pyramidal neuron activity (Arnsten, 2009). Moreover, evidence indicates that at least in rodents, cortical pyramidal neurons express CRF_1 receptors (Gallopin et al., 2006). Thus, CRF-dependent modulation of working memory may involve activation of both PKA and PI/PKC cascades within PFC pyramidal neurons.

To date, the sources of PFC CRF have not been definitively identified. CRF-containing cell bodies are present in most layers of the rat PFC and at least some of these cell bodies are GABAergic interneurons (Mohila, 2004; Helmeke et al., 2008). However, whether interneurons comprise the entire population of CRF cell bodies within the PFC is unclear. Additionally, CRF fibers within the PFC may arise from regions outside the PFC. For example, CRF neurons are

found in regions known to project to the PFC, such as the bed nucleus of the stria terminalis (Swanson et al., 1983; Hoover and Vertes, 2007).

In the present study, distribution of CRF and CRF antagonists globally in the brain (e.g. ICV, systemic) appeared to elicit larger cognitive effects relative to intra-PFC administration. This likely reflects the fact that CRF activates systems outside the PFC, many of which are known to modulate PFC function, including catecholamines (Valentino et al., 1983; Dunn and Berridge, 1987; Arnsten et al., 1999; Lapiz and Morilak, 2006) and glucocorticoids (Roozendaal, 2004; Barsegyan et al., 2010).

CRF plays a pivotal role in regulating behavioral responding during stress (Dunn and Berridge, 1990; Bale and Vale, 2004). However, the cognition-enhancing actions of CRF antagonists we observed unlikely reflect solely an anti-stress effect. First, our animals were highly habituated to testing over many weeks. Additionally, our subjects displayed relatively high performance accuracy, while stress is well documented to impair working memory performance (Arnsten, 2009; Devilbiss et al., 2012). Moreover, evidence from humans and rodents demonstrates that stress impairs behavioral and PFC neuronal responses to rewards (Bogdan and Pizzagalli, 2006; Ossewaarde et al., 2011), while doses of CRF used in these studies had no effect on motivation to obtain sugar reward and perform the task. The fact that, in general, run-time was not altered indicates that CRF-induced performance impairment did not reflect the presence of a competing behavior, including stress-related behavior (e.g. grooming, motor activation). Collectively, these observations suggest that CRF signaling in the PFC modulates higher cognitive function under non-stressful conditions associated with alert, high-motivation goal-directed behavior. Thus, at least in the PFC, CRF may modulate arousal-

dependent cognitive processes that include, but may not be limited to, stress. Whether PFC CRF contributes to stress-related cognitive impairment is an important question of future studies.

Finally, evidence indicates that CRF exerts differential actions in males vs. females, with females displaying greater sensitivity to certain behavioral and cellular actions of CRF during stress (Valentino et al., 2012). The current study used male animals to 1) better compare the actions of CRF with the known modulatory actions of PFC catecholamines on PFC-dependent working memory and 2) to better align with a large literature documenting cognition-enhancing actions of ADHD medications. In recent studies, ICV CRF elicited larger impairments in some, but not all, aspects of sustained attention performance in females relative to males (Cole et al., 2016). Collectively, the available evidence suggests that CRF signaling in the PFC likely impairs working memory in females to a similar or greater degree than males, though this is an important topic for future studies.

Summary

These studies demonstrate that CRF exerts topographically-organized cognitive effects within the rat medial PFC, with receptor activation impairing and receptor blockade improving working memory. Cognition enhancing actions were similarly observed when CRF antagonists were administered ICV and systemically. The ability of CRF antagonists to improve working memory performance mimics that seen with all approved drugs for the treatment of ADHD. Thus, CRF antagonists may be a useful tool for treating PFC-dependent cognitive dysfunction associated with various disorders, including ADHD.

Acknowledgements

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Supplementary Results

Supplementary Table 1. Average Delays, Accuracy (Pre-baseline, Treatment) and Run-Times

Experiment	Group	Avg Delay (sec)	Avg Pre-baseline (% correct)	Avg Treatment (% correct)	Run-time (min)
ICV CRF	VEH (n=9)	23 ± 5.0	82.5 ± 1.7	84.0 ± 1.9	4.9 ± 0.9
	0.1 µg (n=7)	11.4 ± 1.4	87.5 ± 1.0	77.9 ± 2.6*	5.0 ± 2.1
	0.2 µg (n=8)	15.6 ± 2.4	86.7 ± 1.0	73.9 ± 2.3**	8.9 ± 2.1
	1 µg (n=8)	15 ± 1.9	83.8 ±1.4	43.1 ± 10.4***	13.8 ± 2.0**
CRF: Intra-rostral dmPFC	VEH (n=10)	19 ± 5.5	82.3 ± 1.5	87.0 ± 2.3	4.7 ± 1.2
	100 ng (n=17)	17.3 ± 5.0	83.0 ± 1.9	82.0 ± 2.4	4.2 ± 0.3
	250 ng (n=7)	30.0 ± 7.6	82.9 ± 2.1	86.4 ± 2.6	4.3 ± 0.3
CRF: Intra-caudal dmPFC	VEH (n=10)	21 ± 5.3	82.5 ± 1.6	84.0 ± 3.5	4.8 ± 1.1
	25 ng (n=6)	16.7 ± 3.3	81.3 ± 1.3	80.0 ± 1.3	3.7 ± 0.6
	50 ng (n=8)	18.8 ± 6.1	84.7 ± 1.5	80.6 ± 4.2	3.9 ± 0.5
	100 ng (n=15)	14.7 ± 2.2	82.3 ± 1.6	71.5 ± 3.9 *	3.5 ± 0.1
	250 ng (n=8)	15.6 ± 2.4	85.6 ± 1.5	70.0 ± 4.0 **	3.9 ± 0.2
CRF: Intra-rostral vmPFC	VEH (n=10)	19.0 ± 5.5	82.3 ± 1.5	87.0 ± 2.3	4.7 ± 1.2
	100 ng (n=9)	13.3 ± 1.7	83.9 ± 1.4	82.8 ± 3.3	3.7 ± 0.3
	250 ng (n=6)	13.3 ± 2.0	81.7 ± 1.9	75.8 ± 3.5	3.4 ± 0.4
CRF: Intra-caudal vmPFC	VEH (n=10)	21 ± 5.3	82.5 ± 1.6	84.0 ± 3.5	4.8 ± 1.2
	100 ng (n=6)	16.7 ± 4.2	81.7 ± 2.9	85.8 ± 0.8	4.0 ± 0.3
	250 ng (n=6)	21.7 ± 4.0	81.3 ± 2.5	80.0 ± 3.4	4.2 ± 0.3
Intra-PFC CRF Antagonist	VEH (n=9)	21.0 ± 5.3	82.5 ± 1.6	84.0 ± 3.5	5.0 ± 0.9
	50 ng (n=7)	35.7 ± 11.3	79.4 ± 0.4	80.0 ± 3.7	3.2 ± 0.3
	200 ng (n=9)	22.5 ± 3.1	75.3 ± 1.5	83.1 ± 2.5*	4.1 ± 0.5
ICV CRF Antagonist	VEH (n=7)	18.6 ± 4.0	83.2 ± 1.9	84.3 ± 2.5	4.3 ± 0.5
	2 µg (n=8)	25.0 ± 8.5	76.9 ± 1.6	79.4 ± 2.0	4.3 ± 0.7
	4 µg (n=6)	22.5 ± 7.4	78.3 ± 1.2	84.2 ± 3.3	3.1 ± 0.5
	10 µg (n=8)	15.0 ± 2.9	75.0 ± 1.0	86.3 ± 1.3**	4.4 ± 0.7
Systemic CRF Antagonist	VEH (n=14)	20.7 ± 3.0	84.0 ± 1.3	86.7 ± 1.6	3.3 ± 0.2
	2.5 mg/kg (n=6)	32.0 ± 7.4	81.0 ± 0.6	86.0 ± 3.7	3.2 ± 0.2
	5 mg/kg (n=8)	23.8 ± 3.2	79.7 ± 1.3	86.3 ± 3.2	4.0 ± 0.6
	10 mg/kg (n=7)	22.9 ± 4.2	77.5 ± 1.7	89.3 ± 2.3**	3.3 ± 0.1

Shown are group sizes, average delays, accuracy (pre-treatment baseline and treatment) and run-times for all treatment conditions. Values represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P <$ 0.001vs. vehicle.

Supplementary Table 2. Effects of ICV CRF on Latency to Initiate and Time to Consume Food Reward

	Latency to Initiate (sec)	Total Time to Feed (sec)
VEH	0.67 ± 0.4	39.2 ± 9.0
0.1 µg	1.6 ± 0.9	31.7 ± 5.5
0.2 µg	1.9 ±0.8	32.6 ± 4.6
1 µg	1.4 ± 0.7	50.8 ± 21.0

Shown are mean ± SEM values (seconds) for latency to initiate and time to consume 20 sugar pellets in a 20 minute testing session for each ICV vehicle and CRF treatment group. The number of pellets and testing duration were similar to those associated with working memory testing. There were no significant effects of any treatment on these measures of food reward consumption.

Chapter 3

Prefrontal Corticotropin-Releasing Factor (CRF) Neurons Modulate Working Memory and Frontostriatal Circuit Function via Local Receptor Signaling

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Abstract

The dorsomedial frontostriatal circuit supports higher cognitive processes that guide goal-directed behavior. Recent evidence demonstrates that activation of corticotropin-releasing factor (CRF) receptors in the caudal dorsomedial prefrontal cortex (dmPFC) impairs, while blockade of these receptors improves working memory. The current studies examined whether local CRF-synthesizing neurons are a source of CRF to the caudal dmPFC. Chemogenetic activation of CRF neurons in this region impaired working memory performance. These actions were dependent on local protein kinase A (PKA)-coupled CRF receptors. Conversely, suppression of caudal dmPFC neurons improved working memory. Electrophysiological recordings demonstrated that the cognition-impairing effects of caudal dmPFC CRF neurons were associated with a robust suppression of delay- and reward-related activity of putative dmPFC pyramidal neurons and a pronounced reduction in the population size of delay-tuned neurons in both the dmPFC and dorsomedial striatum. Combined, these results demonstrate that CRF neurotransmission within the PFC impairs higher cognitive function and degrades task-related activity within the dorsomedial frontostriatal circuit.

Introduction

The prefrontal cortex (PFC) supports a diversity of 'executive' cognitive processes that guide goal-directed behavior (Fuster, 2015). Many psychiatric disorders, including attention deficit hyperactivity disorder (ADHD), are associated with dysregulation of PFC-dependent cognition (Cubillo et al., 2010; Liston et al., 2011). The majority of drugs used to treat these disorders target catecholamine neurotransmitters. However, these drugs have limitations. In the case of ADHD, although psychostimulants are the most effective treatment, they possess significant potential for abuse (Compton and Volkow, 2006; Setlik et al., 2009; Berridge and Arnsten, 2015). Therefore, there is a need for improved pharmacological treatments for PFC-dependent cognitive dysfunction. We recently demonstrated that activation of CRF receptors in the caudal, but not rostral, dorsomedial PFC (dmPFC) disrupts spatial working memory in rats. Conversely, CRF receptor blockade in this region improved working memory, similar to all FDA-approved treatments for ADHD (Hupalo and Berridge, 2016). Collectively, these observations indicate PFC CRF receptors impact higher cognitive function and may represent a novel pharmacological target for the treatment of ADHD.

Currently, the source of CRF for the cognition-modulatory PFC CRF receptors is unknown. One possible source are CRF-synthesizing neurons prominent within the PFC (Olschowka et al., 1982; Swanson et al., 1983; Merchenthaler, 1984; Charlton et al., 1987; Lewis et al., 1989). To examine the cognitive actions of these neurons, we developed a chemogenetic approach to bidirectionally manipulate PFC CRF neurons in wild-type rats tested in a delayed-response task of spatial working memory. Chemogenetic activation of CRF neurons in the caudal, but not rostral, dmPFC impaired, while suppression of these neurons improved, working

memory performance. The cognition-impairing effects of PFC CRF neuronal activation were reversed with local infusions of either a CRF antagonist or a protein kinase A (PKA) inhibitor.

The dmPFC extends projections to the dorsomedial striatum (dmSTR), forming frontostriatal circuits that support higher cognition (Voorn et al., 2004; Spencer et al., 2012; Mailly et al., 2013). Subpopulations of PFC pyramidal neurons encode a diversity of information required for successful goal attainment, as measured in tests of working memory (Curtis and D'Esposito, 2003; Histed et al., 2009; Rigotti et al., 2013). In contrast, the striatal neural coding mechanisms that support working memory are less well understood. To better understand the effects of CRF neurotransmission in the caudal dmPFC on the coding properties of frontostriatal neurons, we combined ensemble single-unit recordings with chemogenetic activation of PFC CRF neurons in cognitively-tested animals. The cognition-impairing actions of PFC CRF neuronal activation were associated with a suppression of delay- and reward-related activity of putative dmPFC pyramidal neurons. This was accompanied by a robust reduction in the population size of dmPFC neurons strongly tuned to delay, but not reward. Within the dmSTR, PFC CRF neuronal activation similarly decreased the population size of medium spiny neurons (MSNs) strongly tuned to delay and elicited weaker reductions in delay- and reward-related activity. Together, these studies provide the first evidence that PFC CRF neuronal activity potently disrupts higher cognitive function and degrades neuronal coding within dorsomedial frontostriatal circuitry.

Methods and Materials

Animals

Male Sprague-Dawley rats (300-500 grams; Charles River, Wilmington, Massachusetts) were pair-housed in polycarbonate cages with environmental enrichment (Nylabone chews, Bio-Serv, Frenchtown, New Jersey) on a 13/11 hr light/dark cycle. Animals used for electrophysiological recordings were singly-housed with environmental enrichment. Animals were fed *ad libitum* for the first 4-7 days. Subsequently, the amount of food was titrated for each animal (15-17 grams of chow/day) to maintain motivation for food reward while avoiding weight loss. Training/testing was conducted between 0800-1600 hrs 5-6 days/week. All facilities and procedures were in accordance with the guidelines put forth by the National Institutes of Health of the United States and approved by an Institutional Animal Care and Use Committee.

Pre-surgery Working Memory Training

T-maze training and testing was conducted in rooms devoid of external spatial cues and/or in a dark room lit with a red light, as previously described (Berridge et al., 2012b; Hupalo and Berridge, 2016). Briefly, animals were trained to enter the arm of a T-maze not chosen on the previous trial to receive a food reward (one sucrose pellet/trial, 45 mg pellets, Dustless Precision Pellets; Bio-Serv, Frenchtown, New Jersey). Between trials, rats were placed in a start box at the base of the maze and prevented from exiting by a removable gate (Figure 3A). Sessions typically consisted of 20 trials (one session/day). For electrophysiology studies, sessions consisted of two sets of 30 trials.

Surgery

Viral Infusions. Rats were anesthetized with isoflurane (1%-1.5%) and a cocktail of two viruses was infused into the rostral (A+4.0; L±0.8; V-2.0) or caudal (A+2.6; L±0.8; V-3.0) dmPFC: AAV8-CRF-Cre (titer $6.5X10^{13}$ gc/mL; Vector Biolabs, Malvern, PA) + AAV8-hSyn-DIO-hM3Dq-mCherry (titer $4.6X10^{12}$ gc/mL; Addgene, Cambridge, MA) or AAV8-hSyn-DIO-hM4Di-mCherry (titer $4X10^{12}$ gc/mL) or AAV8-hSyn-DIO-mCherry (control; titer $3.1X10^{12}$

gc/mL). The viral cocktail was mixed at a 1:2 ratio of CRF-Cre to DIO-mCherry. 1.6 µL of this cocktail was infused into each dmPFC hemisphere at a rate of 0.25 µL/min. Injector needles were left in place for 3 min before removal.

Cannulae. A subset of animals receiving caudal dmPFC CRF-hM3Dq infusions had stainless steel cannulae (26 gauge) implanted bilaterally over this region (A+3.0 to +2.4; L±0.8; V-0.2). Stainless steel stylets prevented occlusion of cannulae and were replaced as needed. Another group of animals received CRF-hM3Dq infusions in the caudal dmPFC and was bilaterally cannulated targeting the medial septum (MS; A+0.5; L±0.9, 4° angle mediolaterally; V-0.5).

Chronic Electrode Implantation. Five animals receiving CRF-hM3Dq and three animals receiving control virus in the caudal dmPFC were additionally implanted with chronic recording electrodes targeting the dmPFC and dmSTR. A linear electrode array (8 electrodes/array, 50 µm stainless-steel wires, ~200 µm separation; NB Labs, Dennison, TX) was stereotaxically implanted into layer V of the dmPFC, oriented in the rostrocaudal direction (A +3.6 to +2.2, L ± 0.75; V -3.0), as described previously (Devilbiss et al., 2012, 2017). Subsequently, an 8-electrode bundle was unilaterally implanted into the dmSTR (A +0.45; L ±3.5 with 11.5° angle; V -3.6; NB Labs, Dennison, TX).

Cannulae and electrodes were secured in place with skull screws and dental acrylic.

Drugs

CRF (human/rat, Bachem, Torrance, CA) and the PKA inhibitor, Rp-cAMPs (Tocris, United Kingdom) were dissolved in buffered artificial extracellular fluid (AECF; 147mmol/L NaCl, 1.3mmol/L CaCl, 0.9mmol/L MgCl, 2.5mmol/L KCl; pH=7.4). D-Phe-CRF **(**D-Phe^{12},Nle$^{21.38}$,α-Me-Leu^{37})-CRF (12-41); human/rat, (Bachem, Torrance, CA) and clozapine-*N*-oxide (CNO; NIMH Chemical Supply and Synthesis Program) were dissolved in 0.9% saline.

Working Memory Testing and Treatments

Following surgery, rats resumed training until pre-surgery performance levels were attained, after which a delay was introduced between trials. Baseline performance levels of 80–92% or 70–82% were required to detect working memory impairing and improving effects, respectively. Given performance improves with repeated testing, delays were increased when performance exceeded the desired range (range = 10-30 sec). Animals receiving intra-tissue infusions received a mock injection and/or intra-tissue infusion prior to the first treatment.

Systemic and intra-tissue CNO. For systemic CNO treatment, animals were injected subcutaneously (1 mL/kg) 45 min before testing. Bilateral intra-PFC (3 mm projection) and intra-MS (5.5 mm projection) CNO infusions were performed with 33 gauge needles using a microprocessor-controlled pump (Harvard Apparatus, South Natick, MA) at a rate of 0.25 and 0.125 µL/min for 2 min, respectively. Needles were kept in place for 2 min following infusions. Smaller infusion volumes were used for the MS to minimize drug flow up the needle tract and into the lateral ventricles. Prior studies demonstrate this volume allows selective targeting of this region with multiple pharmacological manipulations (Berridge et al., 2012a).

Intra-PFC D-Phe-CRF or Rp-cAMPs combined with systemic CNO. Animals first received intra-PFC infusion of D-Phe-CRF, Rp-cAMPs, or vehicle followed by a subcutaneous CNO or vehicle injection (1 mL/kg). Animals were tested 30 min later.

Histology and Immunohistochemistry

Following testing, animals were anesthetized and transcardially perfused with 250 mL chilled heparinized saline (1 unit heparin/mL 0.9% saline) followed by 450 mL of 4% paraformaldehyde in 0.01 M phosphate buffer (PB; pH 7.4). For electrophysiology studies, cathodal 15 µA current was first passed through every electrode for 10 sec prior to perfusion. Brains were removed, stored in paraformaldehyde overnight, and taken through graded sucrose

solutions (20-30% sucrose in 0.1 M PBS, pH 7.4). Electrode placement was verified in 40 µm thick coronal sections stained with Neutral Red dye (Sigma-Aldrich, St. Louis, MO).

For immunohistochemical processing of 1) CRF and 2) Fos, sections were rinsed with 0.01 M PBS and incubated in a blocking agent containing 5% normal donkey serum in 0.01 M PBS + 0.1% Triton-X 100 (PBS-X) at room temperature for 45 min. Subsequently, the blocking agent was removed and sections were incubated in primary guinea pig anti-CRF antibody (1:4,000; cat# t-5007; Peninsula Labs, San Carlos, CA) or rabbit anti-fos (1:3,000; cat# abe457, EMD Millipore, Billerica, MA) in the blocking agent overnight at 4ºC. Sections were washed and incubated in secondary 1) donkey anti-guinea pig AF488 or 2) donkey anti-rabbit AF405 for 1.5 hrs at room temperature (Jackson Immunoresearch, West Grove, PA). Secondary-only controls were used in all instances. Sections were mounted and coverslipped using ProLong Diamond Antifade Mountant (ThermoFisher Scientific, Madison, WI). The primary antibodies used to target CRF and Fos have been extensively validated using pre-absorption and other controls (for guinea pig anti-CRF, see Das et al., 2007; Rajbhandari et al., 2015; for rabbit anti-Fos, see Scharner et al., 2016).

Placements, Cell Counts, and Photomicrographs

Brain sections from animals receiving viral infusions were examined using an Olympus BX51 light and reflected fluorescence microscope. The spread of virus was mapped throughout the rostrocaudal and dorsoventral extent of the PFC to ensure that the majority of viral expression was restricted to one quadrant (rostral vs. caudal dmPFC). For animals receiving intra-PFC viral infusions and intra-PFC/MS cannulae, alternating sections were Nissl stained to confirm injector needle placement while intervening sections were used to measure viral spread. Data were included only when histological analyses verified accurate placement of the transgene reporter protein (mCherry) and minimal tissue damage.

In a subset of animals, single- (CRF-ir/Fos-ir or mCherry) and double-labeled (CRF-ir/Fos-ir+mCherry) neurons were counted in 600x images using a Nikon A1R+ confocal microscope. For each section, 3-4 images were obtained from the main body of viral expression of each hemisphere. Images were obtained from 3-5 sections/animal (4 total animals). Cells were only counted if there was a clear nucleus. The percentage of double-labeled mCherry neurons was averaged. Cells were considered double-labeled only when fluorescence was clearly observed for mCherry (red signal, cytoplasm) and CRF-ir (green signal, cytoplasm) or Fos-ir (UV/blue signal, nucleus) within the same cell.

In Vitro Recordings in CRF-hM3Dq Transfected PFC Neurons

Coronal PFC slices were prepared as previously described (Guo and Rainnie, 2010). Four weeks following intra-PFC infusion of CRF-Cre + DIO-hM3Dq-mCherry, animals were deeply anesthetized with isoflurane and brains placed in oxygenated ice-cold artificial cerebrospinal fluid (ACSF). A Leica VTS-1000 vibratome (Leica Microsystems, Bannockburn, IL) was used to obtain 350 µm slices, incubated in a holding chamber containing oxygenated ACSF at 32ºC for one hr. Slices were transferred to a submersion chamber attached to the stage of a Leica STP6000 microscope and constantly perfused with oxygenated ACSF. PFC CRF neurons were visualized using differential interference contrast (DIC) microscopy, infrared illumination (IR), and an IR sensitive Hamamatsu CCD camera. Whole cell patch clamp was obtained using a MultiClamp 700B amplifier, Axon digidata 1550 AD/DA interface, and pClamp 10.4 software. The patch recording solution had a composition: 130 mM K-gluconate, 2 mM KCl, 10 mM HEPES, 3 mM MgCl2, 2 mM K-ATP, 0.2 mM NaGTP, and 5 mM phosphocreatine, titrated to pH 7.3 with KOH, and 290 mOsm. Recording pipettes were pulled from borosilicate glass (4-6 MΩ resistance). Membrane potential was held at -60 mV and membrane properties of each neuron were determined by running pre-established current clamp protocols. CNO was applied

by gravity perfusion after a stable baseline recording was achieved and membrane potential changes subsequently monitored.

Electrophysiological Recordings in Working Memory Tested Rats

Animals were transported to the recording room and tethered to a 32-channel commutator and a multichannel electrophysiology acquisition processor (MAP, Plexon, Dallas, TX). Putative single units in the dmPFC and dmSTR were isolated using template-matching algorithms to discriminate action potentials exhibiting at least a 3:1 signal-to-noise ratio. The following criteria ensure that sorted waveforms arose from single neurons: 1) variability of peak waveform voltage, 2) variability of waveform slope, 3) scattergram distribution in the first three principal components, and 4) refractory period (Figure 3C). For all recording sessions, neural activity was simultaneously amplified, discriminated, and time stamped as previously described (Devilbiss et al., 2012, 2017).

After spike sorting, a baseline recording session of 30 trials (10 sec delays) was conducted. Afterward, animals remained attached to the recording tether and were returned to their home cage placed above the T-maze while neuronal activity was monitored. One hr later, animals were injected with 3 mg/kg CNO or saline. 45 min later, animals were tested for an additional 30 trial test session. Video recordings (80 frames/sec) and IR beam grids were used to track and time-stamp the animal's location in the T-maze (Figure 3A-C). When animals fully entered a T-maze arm, the breaking of an IR beam (pink quadrant in Figure 3A) elicited either a high- or low frequency 0.5 sec tone on correct and incorrect trials, respectively (correct vs. error signals). Finally, the moment the animal bit on the sucrose pellet (reward) was time-stamped from video.

Neuron Identification

For the dmPFC, wide-spiking (WS), putative glutamatergic, vs. narrow-spiking (NS), putative inhibitory cell types were differentiated by quantifying the peak-to-peak latency of the extracellular action potential waveform, as previously described (WS > 200 μs; NS, 100–200 μs; Mitchell et al., 2007; Devilbiss et al., 2017). Evidence indicates that most cortical pyramidal neurons have broad action potentials, comprising 70-80% of all cortical neurons, while neurons with narrow action potentials and faster firing rates are typically interneurons (Povysheva et al., 2006). In this study, we identified only a limited number of NS neurons and thus did not analyze this subpopulation further. For the dmSTR, medium spiny neurons (MSNs) and fast-spiking (FS) interneurons were identified using the peak-to-valley duration (MSN > 250 μs; FS < 200 μs) and firing rate (MSN < 8 Hz) of extracellular action potential waveforms, as previously described (Berke et al., 2004; Kim et al., 2007; Stalnaker et al., 2012).

Distinct subpopulations of dmPFC and dmSTR neurons displayed excitatory responses to specific T-maze task events, including delay, reward, correct signal, and error signal (Figure 3E-F). Given the relatively high baseline task accuracy (84.6 ± 1.6%), it was not possible to analyze error responsivity of dmPFC or dmSTR neurons. The selective response or 'event-tuning' of a neuron was determined during baseline recording sessions from the z-score of a neuron's spiking activity during a particular task interval versus its overall spiking activity during the recording session. Z-scores >0.025 for the delay period and >0.18 for all other task events identified groups of neurons displaying strong tuning to task events, similar to exemplar responses described by other laboratories (Fuster and Alexander, 1971; Batuev et al., 1990; Horst and Laubach, 2013). Lower z-score thresholds were used for the delay interval given it was ~10-fold longer in duration than other task intervals. Units displaying z-scores between -0.18 and 0.08 (-0.045–0.01 for the delay) were considered untuned to task events. Units with z-scores ranging between the cutoffs for strongly tuned and untuned neurons were excluded from analyses.

Consistent with prior studies in working memory-tested rats (Hyman et al., 2010; Devilbiss et al., 2017), we observed no significant differences in baseline mean spiking rates for

left vs. right or correct vs. incorrect trials among strongly tuned and untuned neurons in either region. Therefore, these task events/conditions were grouped together in subsequent analyses. To account for any differences in baseline spiking rates among neurons and experimental cohorts, and to accurately compare the effects of CNO on task-related responses, mean spike rates are presented as a percent change from the baseline session.

Statistical Analyses

Cognitive Testing. We used a repeated measures analysis of variance (ANOVA) to analyze the effect of drug treatments on performance accuracy. When statistical significance was indicated, comparisons between drug dose and vehicle were determined using Bonferroni corrected t-tests.

Single Unit Analyses. The mean spike rate during each task interval was determined on a trial-by-trial basis using PETH analysis. For each neuron, the percent change in mean spike rate from baseline was determined for each task event and averaged for each neuron category (strongly tuned, untuned). Given we observed no significant changes in firing rate from baseline in saline-treated hM3Dq animals or CNO-treated viral controls, changes in firing rate were analyzed using group (hM3Dq-CNO, hM3Dq-SAL, control-CNO) as a between-subject variable in a one-way ANOVA. Changes in population sizes of strongly tuned neurons were calculated using a chi-square test. Statistically significant differences between the three groups were determined using Bonferroni corrected t-tests.

Results

Chemogenetic targeting of CRF neurons

We expressed ‘excitatory’ (hM3Dq) or ‘inhibitory’ (hM4Di) DREADDs selectively in CRF neurons in the caudal or rostral dmPFC (Figure 1A; see Methods). An additional group of animals received a control virus lacking the DREADD transgene (CRF-Cre + DIO-mCherry).

Robust reporter protein (mCherry) expression was observed within 3 weeks (Figure 1B). Viral expression was limited to a radius of approximately 500 µm, filling the majority of either the caudal or rostral dmPFC with minimal spread into adjacent dmPFC hemifields (Figure 2A,C). The viral cocktail transfected CRF neurons efficiently: mCherry-expressing neurons colocalized with 94 ± 5% of CRF-immunoreactive (CRF-ir) cells (n=354 cells from 4 animals; Figure 1C). Importantly, mCherry-positive cells lacking CRF-ir were never observed.

To assess whether the DREADD agonist, CNO, activates hM3Dq-expressing CRF neurons in the PFC, we first examined Fos-ir in hM3Dq (n=4) or control virus animals (n=3)

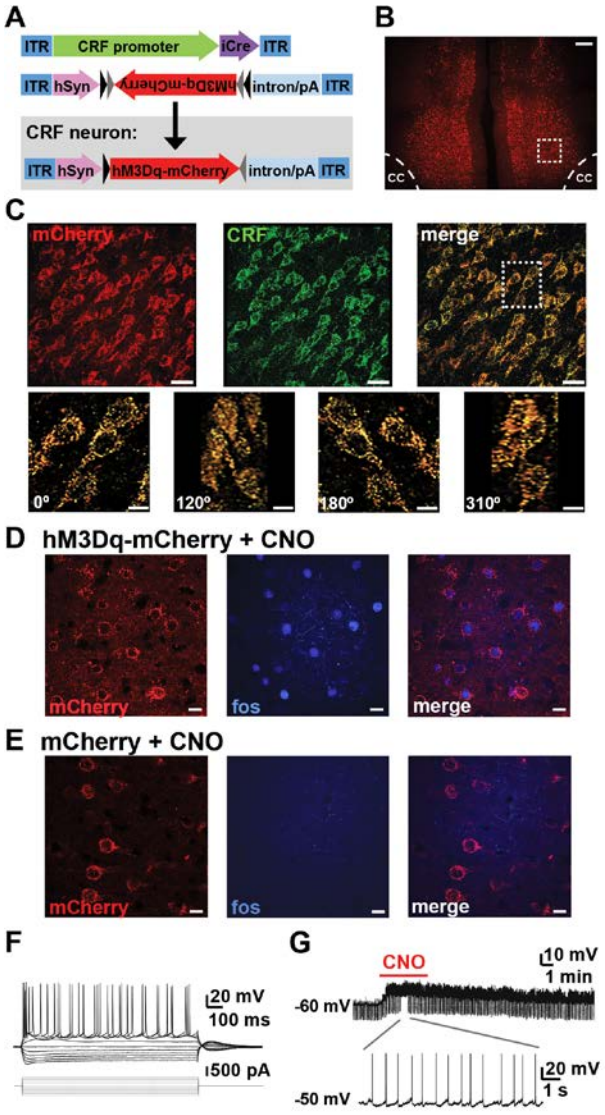

Figure 1. Validation of chemogenetic approach used to manipulate PFC CRF neurons. ***A)*** Schematic depicting dual viral system to activate (CRF-Cre + hSyn-DIO-hM3Dq-mCherry) or inhibit (CRF-Cre + hSyn-DIO-hM4Di-mCherry) CRF neurons in the PFC (control; CRF-Cre + hSyn-DIO-mCherry). ***B)*** Photomicrograph depicting mCherry expression in the caudal dmPFC; scale bar = 200 µm; cc: corpus callosum. ***C)*** *Top*, Collapsed 30 µm z-stack from inset in ***B***, demonstrating mCherry colocalization with CRF-ir cells; scale bar = 30 µm. *Bottom*, inset from above rotated at various angles; scale bar = 10 µm. ***D)*** CNO elicits an excitatory influence in PFC CRF neurons as demonstrated by colocalization of Fos-ir (UV/blue) in mCherry neurons in CNO-injected CRF-hM3Dq animals; scale bar = 10 µm. ***E)*** Absence of Fos-ir in the PFC of CNO-injected animals expressing CRF-mCherry control virus; scale bar = 10 µm. ***F)*** A representative current-clamp recording showing the voltage response of an hM3Dq-mCherry expressing PFC neuron (upper trace) to transient current injection steps (lower trace). ***G)*** In the same neuron, bath application of CNO (10 µM) induced a prolonged membrane depolarization and spontaneous action potential firing.

treated with 3 mg/kg CNO (the highest dose used in subsequent behavioral studies, see below). In hM3Dq animals, CNO elicited Fos-ir in 79 ± 0.6% of mCherry neurons (n=347; Figure 1D). In contrast, no Fos-ir was observed in mCherry neurons of control virus-treated animals (Figure 1E). Additionally, limited in vitro recordings examined the effects of bath application of CNO (10 μM) in mCherry-expressing PFC neurons of animals treated with the hM3Dq virus (n=8, 1 animal). CNO induced a membrane depolarization in all isolated mCherry neurons (8.9 ± 0.9 mV; Figure 1G). The peak depolarization was observed within 2 min and the effect was sustained after wash-out of CNO with vehicle. In 4/8 neurons, CNO led to spontaneous firing (Figure 1G). Combined, these results demonstrate this viral vector systems permits selective manipulation of PFC CRF neurons.

Working Memory Actions of PFC CRF Neurons

Chemogenetic activation of CRF neurons (CRF-Cre + DIO-hM3Dq, n=7) in the caudal dmPFC robustly and dose-dependently impaired working memory performance relative to vehicle ($F_{1,6} = 28.6$, $P = 0.002$) or CNO-treated viral controls (CRF-Cre + DIO-mCherry; n=7, $F_{1,13} = 35.0$, $P < 0.001$; Figure 2B). This performance impairment occurred in the absence of significant changes in run time, indicating it was not accompanied by an increase in competing behaviors (vehicle: 3.6 ± 1.0 min; 3 mg/kg CNO: 3.8 ± 0.5 min, $F_{1,6} = 1.1$, P = 0.4; viral controls: 3.9 ± 1.0 min, $F_{2,18} = 1.5$, $P = 0.3$). In contrast, chemogenetic activation of CRF neurons in the rostral dmPFC (n=6) had no significant effects on performance relative to vehicle ($F_{1,5} = 0.9$, $P = 0.39$) or CNO-treated viral controls (n=7; $F_{1,12} = 1.0$, $P = 0.3$; Figure 2D). Expression of mCherry was largely not observed outside the PFC. The one exception to this was in approximately 30% of animals receiving viral infusions in the caudal (but not rostral) dmPFC,

retrograde mCherry labeling of cell bodies was observed in the MS (Figure 2F), a region known to project to the PFC (Senut et al., 1989). To determine if activation of MS CRF neurons contributes to the cognition-impairing effects of systemic CNO, a subset of animals was infused with CNO directly into the MS or caudal dmPFC. While CNO infusions (0.5 mM) into the caudal dmPFC robustly impaired working memory performance (n=7, $F_{1,6} = 22.4$, $P = 0.003$),

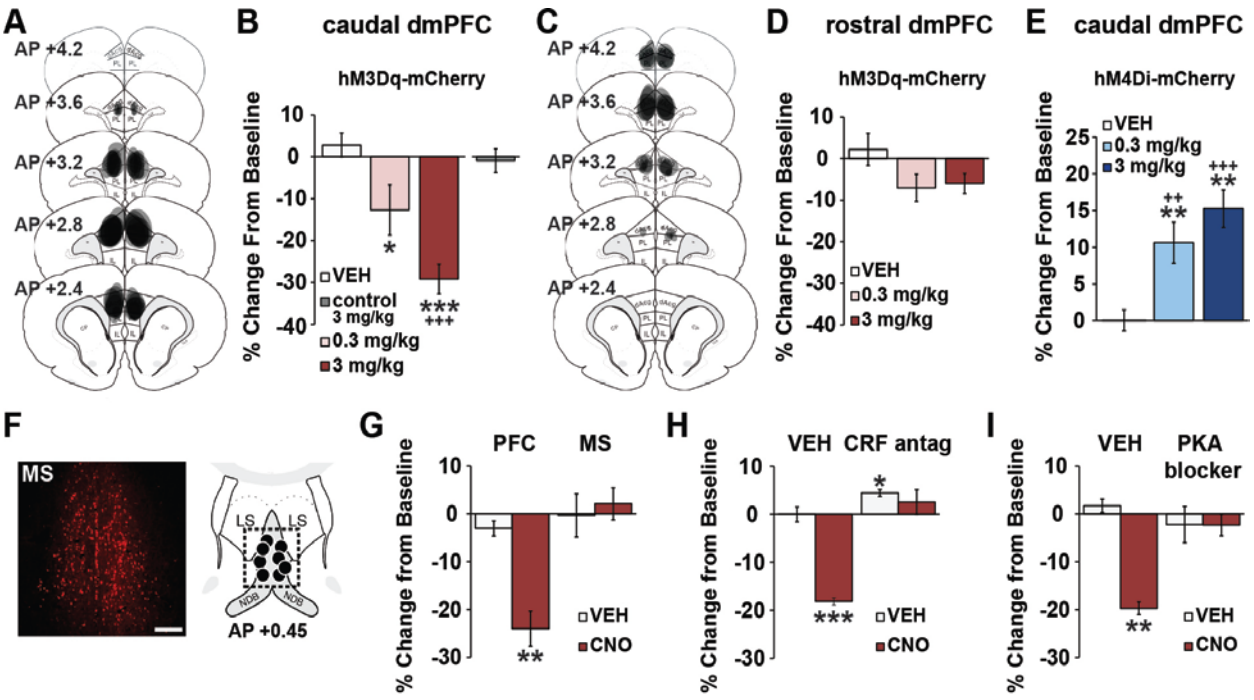

Figure 2. CRF neurons in the caudal dmPFC impair working memory via local CRF receptors coupled to PKA. ***A)*** Schematics depicting hM3Dq viral spread in the caudal dmPFC (AP +3.2 to +2.2) for animals shown in B. ***B)*** CNO-induced activation of CRF neurons in the caudal dmPFC impairs task performance relative to vehicle (n=7). CNO has no working memory effects in viral control animals (n=7; 'control 3 mg/kg'). ***C)*** hM3Dq viral spread in the rostral dmPFC (AP +4.2 to +3.2). ***D)*** Chemogenetic activation of CRF neurons in the rostral dmPFC has no significant effects on task performance relative to vehicle (n=6) and viral controls (n=7). ***E)*** Chemogenetic suppression of CRF neurons in the caudal dmPFC improves task performance relative to vehicle (n=8) or CNO-treated viral controls (n=7). ***F)*** *Left*, retrograde mCherry cell body labeling observed in the MS in ~30% of animals. *Right*, schematics representing intra-MS infusion sites (n=4). ***G)*** When infused into the PFC, 0.5 mM CNO robustly impaired task performance (n=7), while having no effects on performance when infused into the MS (n=4). ***H)*** Intra-PFC infusion of the non-selective CRF antagonist, D-Phe-CRF (100 ng/hemisphere) blocked the working memory-impairing effects of chemogenetic activation of caudal dmPFC CRF neurons (3 mg/kg CNO; n=9). ***I)*** Intra-PFC infusion of the PKA inhibitor, Rp-cAMPs (20 nM/hemisphere) also blocked the working memory-impairing effects of 3 mg/kg CNO (n=5). Results represent mean ± SEM percent change in accuracy relative to baseline. $*P < 0.05$, $**/^{++}P < 0.01$, $***/^{+++}P < 0.001$. *vs. vehicle; $^{+}$vs. viral controls.

intra-MS CNO infusions had no significant effects on task performance (n=4, $F_{1,3} = 0.8$, $P = 0.4$; Figure 2G).

In contrast to that observed with activation of CRF neurons, chemogenetic inhibition of caudal dmPFC CRF neurons (CRF-Cre + DIO-hM4Di) improved working memory performance relative to vehicle (n=8, $F_{1,7} = 15.6$, $P = 0.006$) or CNO-treated viral controls (n=7, $F_{1,14} = 20.9$, $P < 0.001$; Figure 2E), while having no significant effects on maze run time (vehicle: 2.8 ± 0.3 min; 3 mg/kg CNO: 2.7 ± 0.3 min, $F_{1,7} = 0.5$, $P = 0.6$; viral controls: 3.9 ± 0.8 min, $F_{2,18} = 2.1$, $P = 0.1$).

It is important to note that although CNO can be back-metabolized into physiologically active components (Gomez et al., 2017; Mahler and Aston-Jones, 2018; Manvich et al., 2018), CNO treatment in viral controls or animals expressing hM3Dq DREADDs in the rostral dmPFC lacked significant behavioral or neurophysiological effects (see below).

Circuit and Intracellular Signaling Mechanisms Underlying the Cognitive Actions of PFC CRF Neurons

To assess if CRF neurons in the caudal dmPFC release CRF locally to impair task performance, animals expressing CRF-hM3Dq in this region received local infusions of either vehicle or the CRF antagonist, D-Phe-CRF (100 ng/hemisphere) prior to systemic CNO (3 mg/kg; n=9). In animals pretreated with intra-PFC vehicle, CNO elicited a robust impairment in working memory ($F_{1,7} = 49.5$, $P < 0.001$; Figure 2H). This impairment was prevented by local D-Phe-CRF infusions (CNO x antagonist interaction, $F_{1,7} = 31.4$, $P < 0.001$; Figure 2H).

CRF_1 receptors are the dominant subtype in the rodent PFC and have been demonstrated to couple to the Gs-PKA intracellular signaling cascade (Chalmers et al., 1995; Miguel et al.,

2014). To examine whether the cognition-impairing actions of PFC CRF neurons are dependent on local PKA signaling, animals received bilateral infusions of vehicle or the PKA inhibitor, Rp-cAMPs (20 nM), into the caudal dmPFC prior to systemic CNO (3 mg/kg CNO; n=5). This dose of Rp-cAMPs alone had no significant effects on task performance ($F_{1,4} = 1.4$, $P = 0.3$). In vehicle pre-treated animals, CNO impaired task performance ($F_{1,4} = 29.4$, $P = 0.005$), while Rp-cAMPs pretreatment completely blocked this effect (Figure 2I; CNO x Rp-cAMPs interaction; $F_{1,4} = 27.2$, $P = 0.01$).

Effects of PFC CRF Neuronal Activation on Working Memory Task-Related Activity Across the Frontostriatal Circuit

Baseline Neuronal Response Properties. We isolated a total of 420 dmPFC neurons (largely confined to the dorsal aspect of the prelimbic PFC), 96% classified as WS and 4% as NS. Due to the low number of NS dmPFC neurons isolated, these neurons were not analyzed further. A variety of task events were tracked, including delay, reward, and correct or error signals (Figure 3A). Under baseline conditions, 25% of dmPFC WS putative pyramidal neurons were strongly tuned to the delay interval, while 10% were strongly tuned to reward (Figure 3E, Table S1). dmPFC WS neurons were less sensitive to the correct signal tone, displaying relatively weak activation that was rarely time-locked to tone onset and offset (Figure 3E), and thus were not analyzed further. A larger proportion of dmPFC WS neurons were insensitive (‘untuned’) to task events (delay, 39%; reward, 53%; Table S1).

Within the dmSTR, we isolated a total of 255 neurons, 88% classified as MSN, 9% as FS, and 3% as unclassified. Given the relatively small number of FS neurons, only MSNs were included in subsequent analyses. dmSTR neurons also displayed strong delay- (30%) and

reward-related (16%) activity (Table S2). In contrast to the dmPFC, a robust subpopulation of MSNs exhibited prominent and punctate excitatory responses to the correct signal tone (21%; Figure 3F, Table S2).

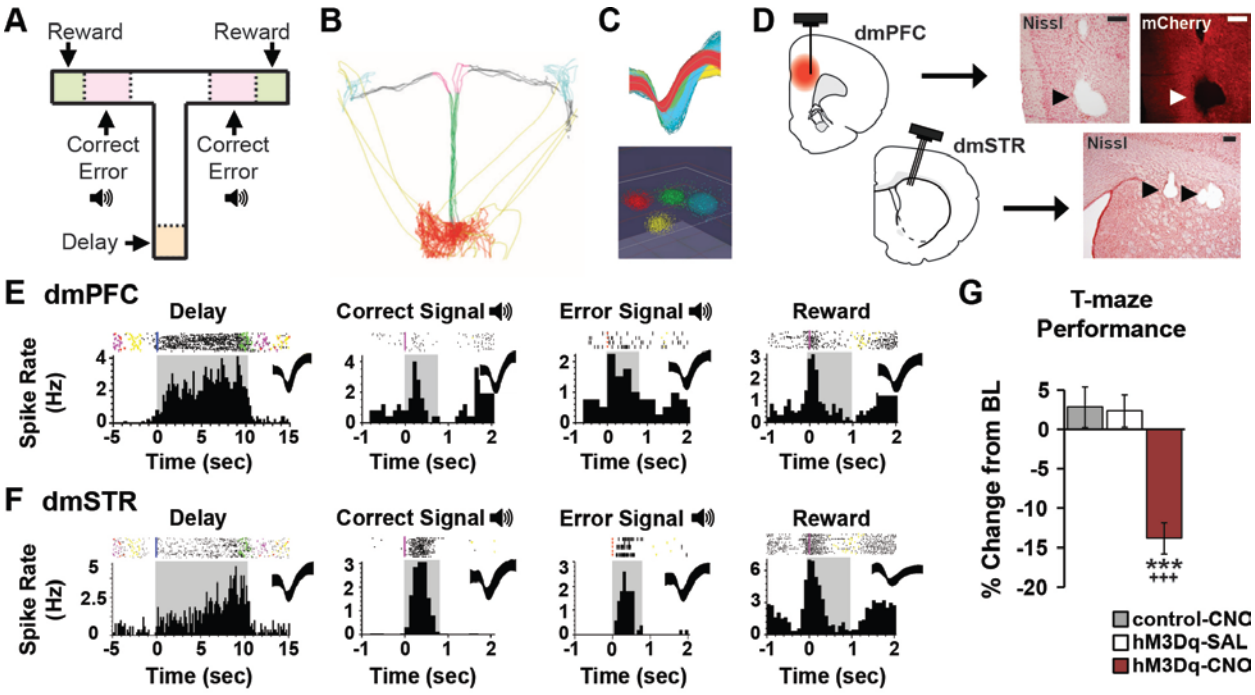

Figure 3. Frontostriatal recordings in working memory-tested rats. ***A)*** T-maze schematic illustrating task events, including delay, two distinct auditory tones serving as outcome-related signals on correct vs. error trials, and sugar reward. ***B)*** Animal position is tracked using video recordings and infrared beams. ***C)*** *Top*, action potential waveforms of 4 discriminated WS dmPFC neurons. *Bottom*, waveforms from these units exhibit separable clusters in principal component space. ***D)*** *Left*, schematics depicting CRF-hM3Dq expression in the caudal dmPFC and electrode placements in the dmPFC and dmSTR. *Right*, 4X photomicrographs depicting placement of one electrode in layer V of the dmPFC, and three electrodes in the dmSTR; scale bar = 250 µm. ***E)*** Exemplar rasters (top) and PETHs (bottom) of WS dmPFC neurons exhibiting punctate excitatory responses ('strongly-tuned') during T-maze events. Shaded areas of PETHs represent duration of task interval. Spiking rates was calculated for 100 ms time bins. ***F)*** Exemplar PETHs of putative MSNs within the dmSTR displaying strong task tuning. ***G)*** In recorded animals, CNO impaired task performance in the hM3Dq group (5 animals, 7 sessions), but not in saline (4 animals, 10 sessions) or CNO-treated viral controls (3 animals, 10 sessions). ***/^{+++}P < 0.001; *hM3Dq-CNO vs. control-CNO, $^{+}$hM3Dq-CNO vs. hM3Dq-SAL.

Effects of Caudal dmPFC CRF Neuronal Activation on Task-Related Activity in the dmPFC. Saline treatment of hM3Dq animals or CNO treatment (3 mg/kg) of viral controls had no significant effects on working memory performance (hM3Dq-SAL: +2%, $P = 0.14$; 10 sessions/4 animals; control-CNO: +3%, $P = 0.16$, 10 sessions/3 animals; Figure 3G). In hM3Dq animals, CNO significantly impaired task performance (-14%; 7 sessions/5 animals, $P = 0.0001$; Figure 3G). This was associated with a robust suppression of task-related activity of dmPFC WS neurons strongly tuned to delay ($F_{2,100} = 22.3$, $P < 0.001$, n=24, 54% reduction) relative to either saline or CNO-treated viral controls (hM3Dq-SAL: n=25, -0.6%; control-CNO: n=54, +0.1%; Figure 4A, Table S3). Activation of PFC CRF neurons also significantly diminished the population size of strongly tuned delay neurons by 75% relative to hM3Dq-SAL (-8%) and control-CNO (-30%) groups ($\chi2 = 6.3$, $P = 0.04$; Figure 4A). PFC CRF neuronal activation also significantly reduced reward-related activity of WS neurons ($F_{2,39} = 3.1$, $P = 0.05$, n=16, -29.1%) relative to saline (n=15, -0.9%) and viral controls (n=11, +2.6%), while not significantly affecting the population size of strongly tuned reward neurons ($\chi2 = 0.1$, $P = 0.9$; hM3Dq-CNO, -19%; hM3Dq-SAL, -13%; control-CNO, -27%; Figure 4A).

As shown in Figure 4B and Tables S2-3, PFC CRF neuronal activation had no significant effects on WS neurons that were untuned to either delay (spiking: $F_{2,157} = 1.1$, $P = 0.3$; population size: $\chi2 = 0.7$, $P = 0.7$) or reward (spiking: $F_{2,213} = 0.23$, $P = 0.8$; population size: $\chi2 = 2.04$, $P = 0.4$).

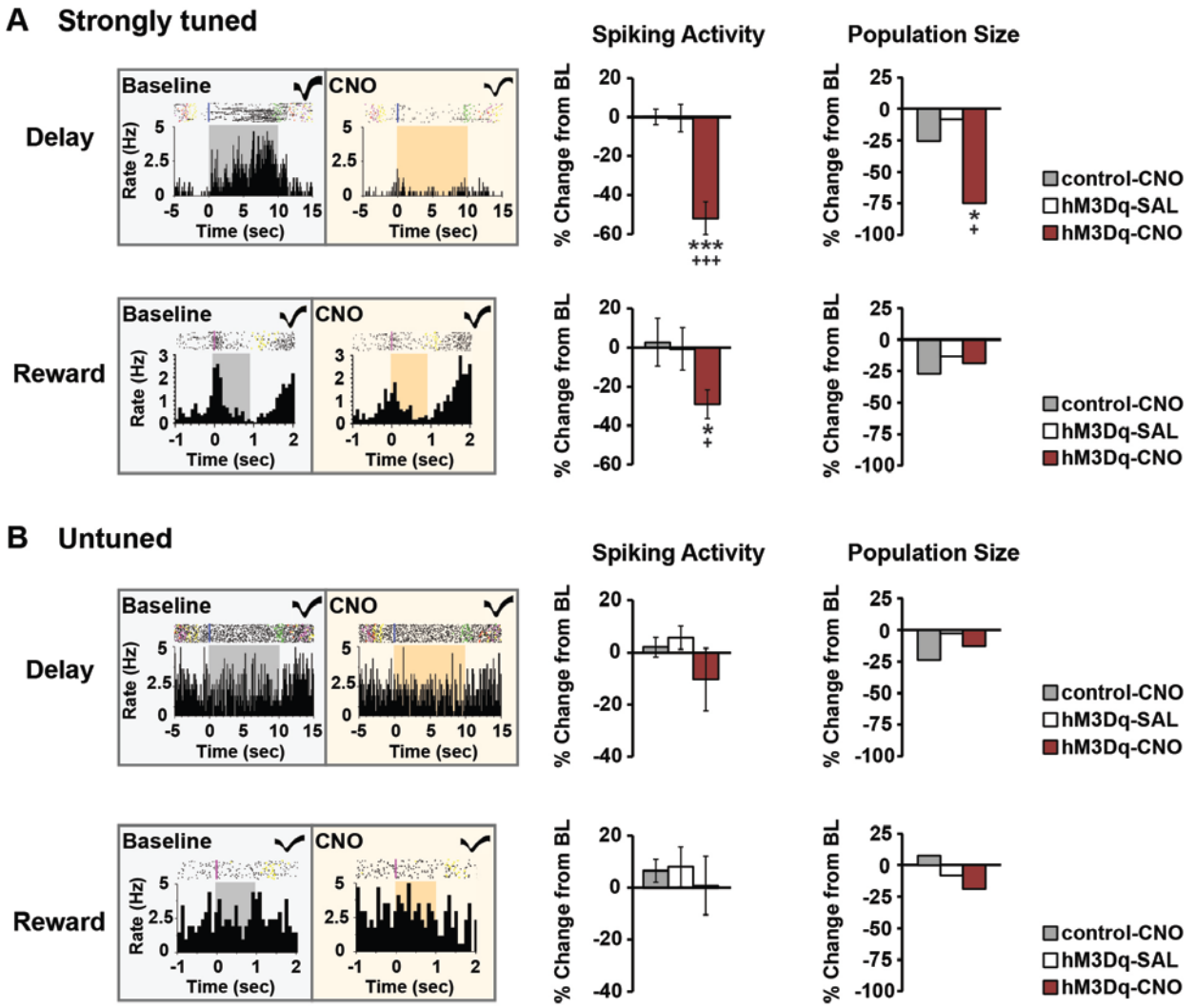

Figure 4. Chemogenetic activation of CRF neurons in the caudal dmPFC suppresses delay and reward-related activity of WS dmPFC neurons. ***A)*** *Left*, Exemplar PETHs demonstrating task-related activity of strongly tuned delay (top) and reward (bottom) WS neurons under baseline and CNO conditions (delay, 10 s; reward, 1 s). *Middle*, CNO-induced activation of dmPFC CRF neurons robustly suppressed task-related activity of strongly tuned delay (n=24) and reward (n=16) WS neurons relative to saline (delay, n=25; reward, n=15) and CNO-treated viral controls (delay, n=54; reward, n=11). *Right*, PFC CRF neuronal activation diminishes the population size of strongly tuned delay (top), but not reward (bottom) neurons. ***B)*** *Left*, Exemplar PETH of a WS neuron untuned to delay (top) and reward (bottom) under baseline and CNO conditions. CNO had no significant effects on task-related activity (middle) or population sizes (right) of these neurons in hM3Dq animals (delay, n=55; reward, n=65) relative to saline (delay, n=40; reward, n=59) and viral controls (delay, n=64; reward, n=92). */$^{+}$ P <0.05, ***/^{+++}P <0.001, *vs. control-CNO, $^{+}$vs. hM3Dq-SAL.

PFC CRF Neuronal Activation Elicits Weak Inhibitory Effects on Task-Related Activity in the dmSTR. Chemogenetic activation of PFC CRF neurons elicited a non-significant trend for

reduction in task-related activity of strongly tuned delay MSNs ($F_{2,65}$ = 1.3, P = 0.2, n=12, -18.4%) relative to saline (-3.6%, n=28) and CNO-treated viral controls (n=28, +1.0%; Figure 5A, Table S5). Nonetheless, PFC CRF neuronal activation robustly diminished the population size of strongly tuned MSNs (-75%) relative to vehicle (-7%) and CNO-treated viral controls (+14%; $\chi 2$ = 5.7, *P* = 0.05; Figure 5A). Similarly, there was a non-significant trend for a decrease in task-related activity of strongly tuned reward MSNs (-21%; $F_{2,33}$ = 1.2, *P* = 0.3, n=9) compared to saline (0%, n=20) and CNO-treated controls (+2.2%, n=19; Figure 5A, Table S5), while the population size of strongly tuned reward MSNs was unaffected ($\chi 2$ = 0.01, *P* = 0.9; Figure 5A). MSNs strongly tuned to the correct signal were insensitive to CNO or saline across all groups (spiking: $F_{2,45}$ = 0.03, *P* = 0.9; population size: $\chi 2$ = 0.6, *P* = 0.7), as were MSNs untuned to delay (spiking: $F_{2,89}$ = 0.5, *P* = 0.6; population size: $\chi 2$ = 0.6, *P* = 0.7), reward (spiking: $F_{2,158}$ = 0.06, *P* = 0.9; population size: $\chi 2$ = 0.9, *P* = 0.6), and correct signal (spiking: $F_{2,140}$ = 0.1, *P* = 0.9; population size: $\chi 2$ = 1.1, *P* = 0.5; Figure 5B, Table S5).

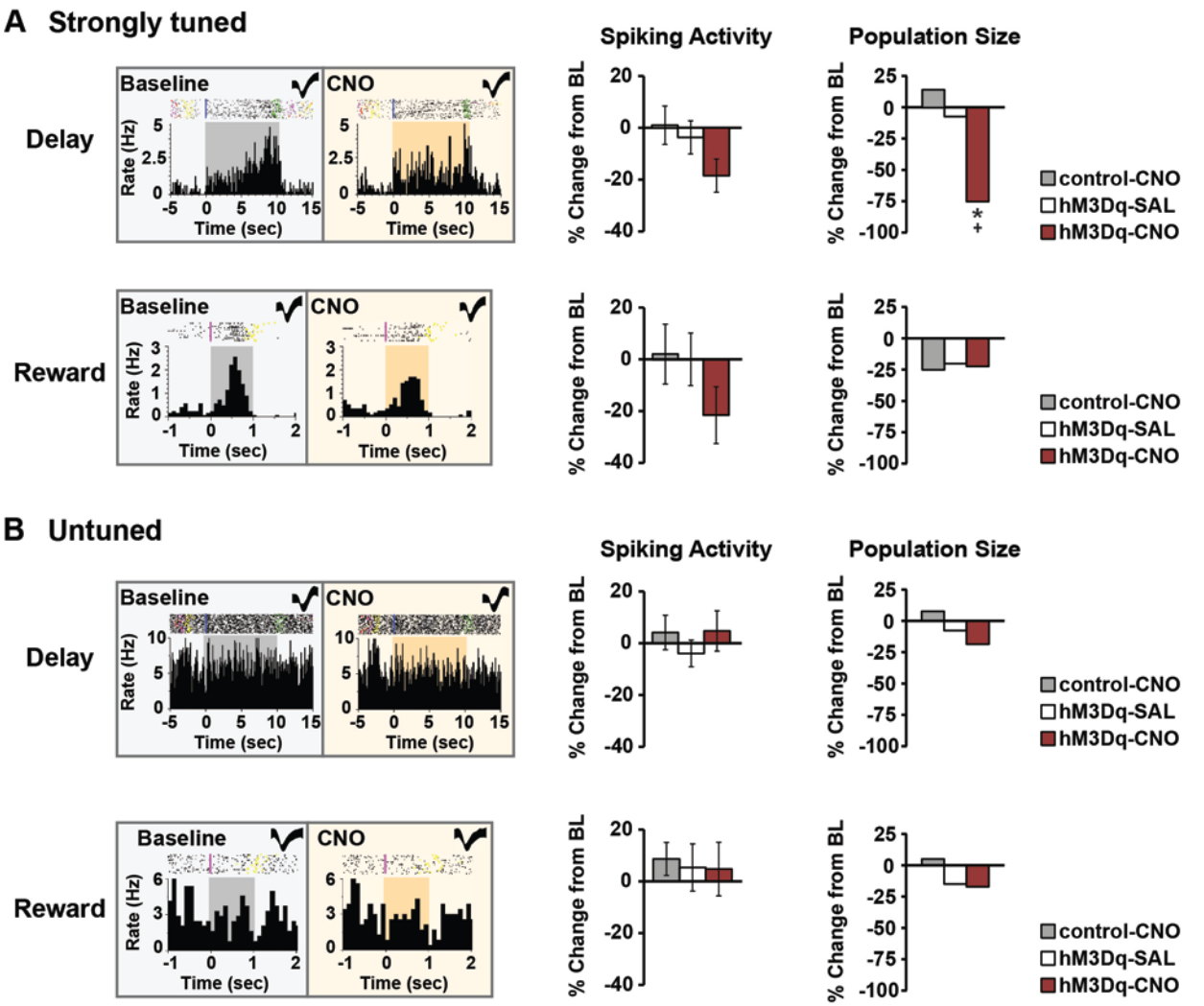

Figure 5. Chemogenetic activation of PFC CRF neurons diminishes the population size of strongly tuned delay MSNs, but has no significant effects on task-related activity in the dmSTR. ***A)*** *Left*, Exemplar PETHs demonstrating task-related activity of strongly tuned delay (top) and reward (bottom) MSN under baseline and CNO conditions. *Middle*, CNO elicited a trend for suppression of task-related activity of strongly tuned delay (n=12) and reward MSNs (n=9) in hM3Dq animals that was not observed with saline (delay, n=28; reward, n=15) or CNO-treated viral controls (delay, n=28; reward, n=12). *Right*, PFC CRF neuronal activation diminished the population size of strongly tuned delay (top), but not reward (bottom), MSNs. ***B)*** *Left*, Exemplar PETHs of delay- (top) and reward-untuned (bottom) MSNs. CNO elicited no significant effects on task-related activity (middle) or the population size (right) of untuned MSNs in hM3Dq animals (delay, n=27; reward, n=35), saline-treated hM3Dq animals (delay, n=26; reward, n=65), or CNO-treated viral controls (delay, n=39; reward, n=61).

Discussion

Both the PFC and CRF have long been implicated in a diversity of psychopathologies associated with cognitive impairment. Thus, it is surprising that the cognitive actions of CRF signaling in the PFC have been largely unexplored. The current studies are the first to demonstrate that CRF neurons in the caudal dmPFC are constitutively active under baseline testing conditions and act to impair working memory performance via local CRF release and activation of CRF receptors coupled to intracellular PKA signaling. Additionally, these studies demonstrate that when activated, caudal dmPFC CRF neurons robustly suppress delay- and reward-related activity in the PFC and elicit a collapse in the population size of delay-tuned, but not reward-tuned neurons. Downstream in the dmSTR, dmPFC CRF neuronal activation elicited a similar decrease in the population size of MSNs strongly tuned to delay, but not reward, while non-significantly reducing delay and reward-related activity in the dmSTR. Collectively, these observations provide novel insight into the neurobiology of frontostriatal-dependent cognition and further suggest that CRF neurotransmission may be a relevant target for the development of novel treatments for PFC-dependent cognitive dysfunction.

Topography of CRF Actions within the dmPFC

The restriction of the cognitive actions of CRF neurons to the caudal dmPFC is consistent with a well-described dorsoventral (Voorn et al., 2004; Gabbott et al., 2005) and rostrocaudal functional topography (Alsene et al., 2011; Hupalo and Berridge, 2016) within the rodent medial PFC. CRF neurons and receptors are present throughout the entire medial PFC (Swanson et al., 1983; De Souza et al., 1985). Thus, the topography associated with the cognitive actions of PFC CRF is unlikely to arise from differential neuronal/receptor distribution. Instead, this may reflect

a topographical organization in the projection patterns of PFC neurons (Sesack et al., 1989; Voorn et al., 2004).

PFC CRF Neurons Modulate Task-Related Coding Properties of Frontostriatal Neurons

Chemogenetic activation of caudal dmPFC CRF neurons robustly suppressed task-related activity of strongly tuned dmPFC neurons without altering the activity of untuned neurons. This indicates that PFC CRF neurons do not broadly increase inhibitory tone in dmPFC. PFC CRF neuronal activation also diminished the population size of neurons strongly tuned delay, but not reward, in both the dmPFC and dmSTR. However, within the dmSTR, PFC CRF neuronal activation elicited a weaker and non-significant suppression of delay- and reward-related activity. Combined, these observations indicate the cognition-impairing actions of caudal dmPFC CRF neurons involve a robust degradation in the ability of dmPFC neurons, and to a lesser extent dmSTR neurons, to represent key working memory task events. The weaker effects on task-related activity observed in the dmSTR suggest that task-related activity of MSNs is not solely driven by inputs from the caudal dmPFC. Indeed, the rostral portion of the dmPFC is known to project to the region of the dmSTR recorded from in the current studies (Spencer et al., 2012). Interestingly, correlation analyses indicated that chemogenetic activation of CRF neurons elicited weaker inhibitory effects on task-related activity at the rostral wires, where viral spread was also weaker (delay, $r2 = 0.15$, $P = 0.06$; reward, $r2 = 0.3$, $P = 0.03$; Figure S1). Thus, task-related activity within the dmSTR may, in part, depend on input from the rostral dmPFC.

There are multiple aspects of frontostriatal circuit function that can be explored, including both functional and effective connectivity. While we are currently exploring these

issues, this greatly exceeds the scope of the current studies which were designed to assess whether PFC CRF neurons exert an influence over frontostriatal cognition and neural function.

Translational Relevance of PFC CRF neurons

The cognition-enhancing actions of CRF neuronal suppression mimic that seen with all FDA-approved drugs for ADHD, suggesting CRF may represent a novel therapeutic target for the treatment of ADHD and/or other forms of cognitive dysfunction. Beyond ADHD, the cognitive and neurophysiological actions of PFC CRF neuronal activation are similar to those seen with stress (Birnbaum et al., 1999; Arnsten, 2009; Devilbiss et al., 2017). Moreover, limited evidence suggests that acute stress increases levels of CRF mRNA in the PFC (Meng et al., 2011). Combined, these observations suggest the possibility that stress-related cognitive impairment may involve increased CRF neurotransmission in the PFC. In the current studies, animals were highly habituated and motivated to engage in cognitive testing and displayed relatively high baseline performance accuracy. Thus, the cognition-enhancing actions of CRF neuronal suppression are unlikely to solely reflect an anti-stress effect. Instead, these results suggest that CRF neurons are active across a range of conditions associated with higher arousal and motivation states, both stressful and non-stressful (e.g. Merali et al., 2004). The degree to which PFC CRF contributes to stress-related cognitive impairment is an important question of future studies.

Summary

Collectively, these studies are the first to demonstrate potent modulatory actions of PFC CRF neurons on working memory and frontostriatal neural function. Beyond furthering our understanding of CRF, these studies provide a better understanding of the pharmacological and neurophysiological mechanisms that subserve frontostriatal circuit function. This information may be relevant to the development of novel treatment targets for ADHD and other forms of cognitive dysfunction.

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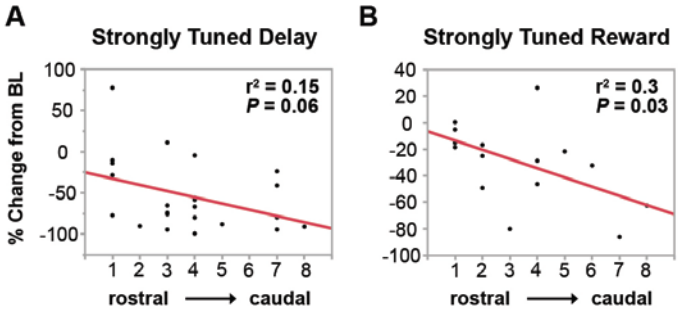
Supplementary Results



Supplementary Figure 1. Chemogenetic activation of CRF neurons in the caudal dmPFC elicits weaker inhibitory effects on strongly tuned WS delay (A) and reward (B) neurons isolated from rostral wires compared to caudal wires. 1 = most rostral (AP +3.6), 8 = most caudal electrodes (AP +2.2). All strongly tuned WS delay and reward neurons are the same as those presented in Figure 4A.

Supplementary Table 1. Population Sizes of WS Neurons in the dmPFC Strongly Tuned to Task Events.

Task Event	Neuron Category	% of Population
Delay	Strongly Tuned	25%
	Untuned	39%
Reward	Strongly Tuned	10%
	Untuned	53%

Values represent percent of total WS population (number of strongly tuned WS neurons/all isolated WS neurons * 100).

Supplementary Table 2. Population Sizes of Putative MSNs in the dmSTR Strongly Tuned to Task Events

Task Event	Neuron Category	% of Population
Delay	Strongly Tuned	30%
	Untuned	41%
Correct Signal	Strongly Tuned	21%
	Untuned	64%
Reward	Strongly Tuned	16%
	Untuned	72%

Values represent percent of total MSN population (number of task tuned MSNs/all isolated MSNs * 100).

S**upplementary Table 3.** Baseline (BL) and post-CNO Task-Related Firing Rates of dmPFC WS Neurons.

Task Interval	Neuron Category	Group	BL Task-Related Mean Firing Rate (Hz)	CNO Task-Related Mean Firing Rate (Hz)
Delay	Strongly Tuned	hM3Dq-CNO	1.97 ± 0.31	0.98 ± 0.31***/$^{+++}$
		hM3Dq-SAL	1.73 ± 0.35	1.77 ± 0.33
		control-CNO	3.07 ± 0.34	2.91 ± 0.31
	Untuned	hM3Dq-CNO	1.14 ± 0.12	0.76 ± 0.09
		hM3Dq-SAL	1.11 ± 0.12	1.10 ± 0.11
		control-CNO	1.61 ± 0.26	1.57 ± 0.24
Reward	Strongly Tuned	hM3Dq-CNO	2.26 ± 0.25	1.55 ± 0.22*/$^{+}$
		hM3Dq-SAL	2.95 ± 1.05	2.85 ± 1.10
		control-CNO	2.59 ± 0.36	2.73 ± 0.58
	Untuned	hM3Dq-CNO	0.98 ± 0.09	0.90 ± 0.24
		hM3Dq-SAL	1.02 ± 0.14	1.09 ± 0.17
		control-CNO	1.55 ± 0.17	1.64 ± 0.17

Results represent mean ± SEM values of spike rates. */$^{+}P < 0.05$, ***/$^{+++}P < 0.001$; *hM3Dq-CNO vs. control-CNO; $^{+}$hM3Dq-CNO vs. hM3Dq-SAL.

Supplementary Table 4. Chemogenetic Activation of CRF Neurons in the Caudal dmPFC Diminishes the Population Size of dmPFC WS Neurons Strongly Tuned to Delay

	Neuron Category	Group	BL Neuron Count	BL % of Population	CNO Neuron Count	CNO % of Population	Population Change
Delay	Strongly Tuned	hM3Dq-CNO	24	20%	6	5%	-75%*/$^{+}$
		hM3Dq-SAL	25	23%	23	21%	-8%
		control-CNO	54	30%	38	21%	-30%
	Untuned	hM3Dq-CNO	55	46%	48	40%	-13%
		hM3Dq-SAL	40	37%	39	36%	-3%
		control-CNO	64	36%	49	27%	-23%
Reward	Strongly Tuned	hM3Dq-CNO	16	13%	13	11%	-19%
		hM3Dq-SAL	15	14%	13	12%	-13%
		control-CNO	11	6%	8	4%	-27%
	Untuned	hM3Dq-CNO	65	55%	55	46%	-15%
		hM3Dq-SAL	59	55%	48	44%	-19%
		control-CNO	92	51%	101	56%	10%

Shown are numbers and percent of WS neurons in various tuning categories under baseline (BL) and CNO conditions in hM3Dq animals and viral controls.
*/$^{+}P < 0.05$, chi square test; *hM3Dq-CNO vs. control-CNO; $^{+}$hM3Dq-CNO vs. hM3Dq-SAL.

Supplementary Table 5. Baseline (BL) and post-CNO Task-Related Firing Rates of MSNs in the dmSTR.

Task Interval	Neuron Category	Group	BL Task-Related Mean Firing Rate (Hz)	CNO Task-Related Mean Firing Rate (Hz)
Delay	Strongly Tuned	hM3Dq-CNO	1.55 ± 0.50	1.29 ± 0.43
		hM3Dq-SAL	1.04 ± 0.18	0.98 ± 0.17
		control-CNO	2.72 ± 0.55	2.66 ± 0.52
	Untuned	hM3Dq-CNO	0.79 ± 0.16	0.79 ± 0.18
		hM3Dq-SAL	1.44 ± 0.41	1.31 ± 0.36
		control-CNO	1.13 ± 0.23	1.08 ± 0.25
Correct Signal	Strongly Tuned	hM3Dq-CNO	3.19 ± 1.54	2.92 ± 1.38
		hM3Dq-SAL	3.53 ± 0.77	3.21 ± 0.65
		control-CNO	3.32 ± 1.15	3.16 ± 1.17
	Untuned	hM3Dq-CNO	0.59 ± 0.16	0.76 ± 0.29
		hM3Dq-SAL	0.49 ± 0.08	0.48 ± 0.08
		control-CNO	0.85 ± 0.15	0.74 ± 0.12
Reward	Strongly Tuned	hM3Dq-CNO	1.76 ± 0.77	1.53 ± 0.73
		hM3Dq-SAL	2.07 ± 1.11	1.58 ± 0.69
		control-CNO	3.08 ± 1.05	2.67 ± 0.86
	Untuned	hM3Dq-CNO	0.68 ± 0.11	0.67 ± 0.12
		hM3Dq-SAL	0.52 ± 0.08	0.49 ± 0.07
		control-CNO	0.72 ± 0.13	0.76 ± 0.14

Results represent mean ± SEM values of firing rates.

Supplementary Table 6. Chemogenetic Activation of CRF Neurons in the Caudal dmPFC Diminishes the Population Size of Strongly Tuned MSNs.

	Neuron Category	Group	BL Neuron Count	BL % of Population	CNO Neuron Count	CNO % of Population	Population Change
Delay	Strongly Tuned	hM3Dq-CNO	12	24%	3	6%	-75%*/$^{+}$
		hM3Dq-SAL	28	32%	26	30%	-7%
		control-CNO	28	32%	32	37%	14%
	Untuned	hM3Dq-CNO	27	54%	22	44%	-19%
		hM3Dq-SAL	26	30%	24	28%	-8%
		control-CNO	39	45%	42	48%	8%
Correct Signal	Strongly Tuned	hM3Dq-CNO	9	18%	7	14%	-22%
		hM3Dq-SAL	20	18%	21	24%	5%
		control-CNO	19	22%	14	16%	-26%
	Untuned	hM3Dq-CNO	33	66%	38	76%	15%
		hM3Dq-SAL	54	62%	55	63%	2%
		control-CNO	56	64%	47	54%	-16%
Reward	Strongly Tuned	hM3Dq-CNO	9	18%	7	14%	-22%
		hM3Dq-SAL	15	17%	12	14%	-20%
		control-CNO	12	14%	9	10%	-25%
	Untuned	hM3Dq-CNO	35	70%	29	58%	-17%
		hM3Dq-SAL	65	75%	55	63%	-15%
		control-CNO	61	70%	64	74%	5%

Shown are numbers and percent of MSNs in various tuning categories under baseline (BL) and CNO conditions in hM3Dq animals and viral controls. */$^{+}$$P < 0.05$, chi square test; *hM3Dq-CNO vs. control-CNO; $^{+}$hM3Dq-CNO vs. hM3Dq-SAL.

Chapter 4

Corticotropin-Releasing Factor (CRF) Neurons in the Prefrontal Cortex Modulate Sustained Attention

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Abstract

The prefrontal cortex (PFC) supports a variety of cognitive processes critical for goal-oriented behavior. PFC dysfunction is associated with many psychiatric disorders, including attention deficit hyperactivity disorder (ADHD). We recently demonstrated that CRF neurons release CRF locally within the caudal dorsomedial PFC (dmPFC) to impair cognitive function as measured in a spatial working memory task in rats. To determine the broader cognitive actions of CRF in the PFC, the current studies examined the effects of CRF manipulations in a signal detection test of sustained attention.

Intracerebroventricular (ICV) CRF infusions impaired, while ICV or systemic administration of a CRF antagonist improved sustained attention. To determine whether CRF neurons in the PFC modulate sustained attention, we utilized a chemogenetic approach (DREADDs) to activate this neuronal population. Similar to that seen with working memory, chemogenetic activation of CRF neurons in the caudal, but not rostral, dmPFC dose-dependently impaired sustained attention. This action was blocked by pretreatment with a systemic, but not intra-PFC, CRF antagonist. Consistent with this, CRF infusions into various PFC subregions had no effects on sustained attention.

Collectively, these studies demonstrate that CRF neurons within the caudal dmPFC impair sustained attention via projections terminating outside the PFC. The cognition-enhancing actions of systemically administered CRF antagonists in both working memory and sustained attention tasks mirror the effects of ADHD drugs. Thus, CRF antagonists may represent a novel pharmacological approach for the treatment of ADHD and other PFC cognitive disorders.

Introduction

The prefrontal cortex (PFC) plays a pivotal role in higher cognitive processes that guide goal-directed behavior, particularly under distracting or ambiguous conditions (Fuster, 2015). PFC cognitive dysfunction is implicated in multiple psychopathologies, including attention deficit hyperactivity disorder (ADHD; Millan et al., 2012). The majority of drugs used to treat ADHD target catecholamine neurotransmitters (Berridge and Arnsten, 2015). However, these drugs have their limitations. For example, while psychostimulants are the most effective treatment for ADHD, they possess risk for abuse and addiction (Compton and Volkow, 2006). Thus, there is a need for new pharmacological treatments for PFC cognitive dysfunction.

Corticotropin-releasing factor (CRF) receptors and neurons are prominent in the PFC (Swanson et al., 1983; De Souza et al., 1985; Charlton et al., 1987; Lewis et al., 1989; Van Pett et al., 2000). In recent studies, we demonstrated that CRF neurons in the caudal dmPFC act on local CRF receptors to impair performance in a delayed response task of working memory (Hupalo et al., 2017). Moreover, CRF receptor blockade either globally in the brain or within the PFC *improved* working memory, similarly to all approved treatments for ADHD (Hupalo and Berridge, 2016).

Evidence indicates that a given neurotransmitter receptor can exert qualitatively different actions across distinct PFC-dependent processes (Berridge and Spencer, 2016). Therefore, to understand the broader cognitive effects of CRF neurotransmission in the PFC, it is important to examine the actions of CRF in multiple PFC-dependent cognitive processes. Toward this goal, the current studies examined the effects of global and intra-PFC manipulations of CRF on performance in an operant-based signal detection test of sustained attention (Bushnell, 1998; Berridge et al., 2006). This task closely mirrors tests of sustained attention in human subjects

(Sarter and McGaughy, 1998; Bushnell et al., 2003). In addition, performance in this task is highly dependent on an intact PFC (Rueckert and Grafman, 1996; Pezze et al., 2014; Spencer and Berridge, 2015). As previously described, ICV CRF elicited a robust and dose-dependent impairment in sustained attention (Cole et al., 2016). Conversely, ICV infusion of a non-selective CRF antagonist or systemic administration of a CRF_1 antagonist dose-dependently improved sustained attention performance.

To determine if PFC CRF neurons modulate sustained attention, subsequent studies utilized a dual adeno-associated virus (AAV) approach to express hM3Dq-coupled designer receptors (DREADDs) in PFC CRF neurons. Chemogenetic activation of CRF neurons within the caudal, but not rostral, dmPFC impaired sustained attention. Interestingly, this action was prevented with systemic, but not intra-PFC, administration of a CRF antagonist. Consistent with this, CRF infusions into various PFC subfields had no impact on task performance.

These studies provide the first evidence that CRF neurons within the caudal dmPFC modulate sustained attention. In contrast to working memory, the sustained attention effects of PFC CRF neurons involve projections outside the PFC. Collectively, these observations indicate that CRF receptors within the PFC do not uniformly modulate distinct PFC-dependent cognitive processes (Hupalo and Berridge, 2016). Given the procognitive actions of CRF antagonists in both working memory and sustained attention tasks closely resemble that seen with ADHD medications (Mehta et al., 2004; Berridge et al., 2012), these compounds may represent a novel pharmacological target for the treatment of ADHD and other PFC-dependent cognitive disorders.

Methods and Materials

Animals

Male Sprague-Dawley rats (300-500 grams; Charles River, Wilmington, Massachusetts) were pair-housed in clear polycarbonate cages on a 13/11-hour light/dark cycle. Animals were fed *ad libitum* for the first 4-7 days after arrival. Subsequently, the amount of food was titrated for each animal (15-17 grams of chow/day) to maintain motivation for food reward while avoiding weight loss. Training/testing was conducted between 0800 and 1600 hours 5-6 days/week. All facilities and procedures were in accordance with the guidelines regarding animal use and care put forth by the National Institutes of Health of the United States and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

Signal Detection Test of Sustained Attention

Animals were trained and tested in an operant-based signal detection test of sustained attention as previously described (Berridge et al., 2006; Berridge et al., 2012). Briefly, on half of all trials (selected at random, $p = .5$), an LED was illuminated ("signal trials"). The signal length was variable, randomly selected from the following list: .12, .24, .37, .49, .62, .74, and .99 sec., with replacement. On the other half of the trials, the LED remained dark, after which both levers were projected ("no-signal trials"). Following this, two levers were projected into the chamber and remained projected until a response was made and then retracted. On signal trials, a right lever press was scored as a "Hit" and reinforced with one sucrose pellet (45 mg pellets, Dustless Precision Pellets; Bio-Serv, Frenchtown, New Jersey), whereas a left lever press was scored as a "miss". On a no-signal trial, a left lever press was reinforced with one pellet ("correct rejection") while a right lever press was scored as a "false alarm." For all correct response trials, the house

lights were illuminated for 5-sec with reward presentation. For all incorrect trials, the levers were retracted and a 5-sec time-out period ensued (houselights off). Trials were considered omitted upon failure to respond within 5-sec of lever insertion, after which the levers were retracted followed by a 5-sec blackout. A variable intertrial interval of 14-sec (minimum 5-sec), on average, elapsed before the start of new trial. Single trials lasted about 20-sec and all sessions consisted of 100 trials. Animals were trained until proportion of correct trials reached 75%.

Dependent measures included the probability of a hit (number of hits/number of signal trials), probability of a false alarm (number of false alarms/number of no-signal trials), d' (a relative measure of stimulus detectability that takes into account both the probability of a hit and the probability of a false alarm Gescheider, 1985; Berridge et al., 2012), response latency (sec between lever projection and lever press response), and omissions (number of trials in which no response was made out of 100).

Surgery and Viral Infusions

ICV manipulations of CRF receptors. After completion of training, rats were anesthetized with isoflurane (1-1.5%) and stainless steel cannulae (26 gauge) were surgically implanted unilaterally aimed at the lateral ventricle (A -0.8, L ± 1.5, V -2.0; hemisphere counterbalanced). Cannulae were cemented and secured to the skull with stainless steel screws and acrylic cement (Plastics One, Roanoke, Virginia). Stainless steel stylets prevented occlusion of cannulae and were replaced as needed.

Intra-tissue manipulations of CRF neurons and receptors. Animals were anesthetized and a cocktail of two viruses was infused into either the rostral (A +4.4 to 3.4; L ± 0.8; V -2.2) or caudal (A +3.2 to +2.2; L ± 0.8; V -3.0) dmPFC: one driving CRF promoter-specific expression

of Cre-recombinase (AAV8-CRF-Cre; 6.5 X 10^{13} gc/mL; Vector Biolabs, Malvern, PA), and another driving Cre-dependent expression of hM3Dq-coupled DREADDs (AAV8-hSyn-DIO-hM3Dq-mCherry; 4 X 10^{12} gc/mL) or a control virus lacking the DREADD transgene (AAV8-hSyn-DIO-mCherry; 3.1 X 10^{12} gc/mL; Addgene, Cambridge, MA).

The viral cocktail was mixed at a 1:2 ratio of CRF-Cre to DIO-mCherry, and a total volume of 1.6 µL of this mixture was infused into select medial PFC subfields at a rate of 0.25 µL/min. Injector needles were left in place for 3-min before removal. Animals receiving viral infusions in the caudal dmPFC were also implanted with bilateral 26 gauge cannulae targeting either the caudal dmPFC (A +3.2 to +2.2; L ± 0.8; V -0.2) or medial septum (MS; A +0.5, L ± 0.9, V -0.5; 4° angle). Animals receiving viral infusions in the rostral dmPFC were also implanted with cannulae targeting this region (A +4.4 to +3.4; L ± 0.8; V -0.2). Cannulae were inserted no more than 200 µm below the dura to avoid damage to the PFC.

A separate cohort of animals received no viral infusions and was bilaterally cannulated targeting the rostral and caudal subfields of the dmPFC and ventromedial PFC (vmPFC). Dorsal and ventral aspects of the medial PFC were targeted via different needle projection length in different groups of animals (see below).

Drugs

CRF (human/rat, Bachem, Torrance, CA) was dissolved in buffered artificial extracellular fluid (AECF; 147 mmol/L NaCl, 1.3 mmol/L CaCl, 0.9 mmol/L MgCl, 2.5 mmol/L KCl; pH = 7.4). D-Phe-CRF **(**D-Phe^{12},Nle$^{21.38}$,α-Me-Leu^{37})-CRF (12-41); human/rat, Bachem, Torrance, CA), NBI 35965 (Tocris, United Kingdom), and CNO (Tocris, United Kingdom) were dissolved in 0.9% saline.

Treatments and Testing

Following surgery, rats resumed testing until performance reached pre-surgery levels. Animals receiving intra-tissue infusions were first given two mock infusions prior to testing, consisting of an initial needle insertion followed by a second needle insertion with vehicle infusion 48-hrs later. This allowed animals to acclimate to the gentle handling associated with infusions and to minimize potential adverse behavioral effects of tissue damage related to the initial needle insertion.

Infusions of CRF, D-Phe-CRF, and CNO. On the day of testing, rats were transported to the testing room in their home cage, infused with CRF, D-Phe-CRF, CNO or vehicle and returned to their home cage for 15-min prior to testing. All infusions were performed with 33-gauge stainless steel needles using a microprocessor-controlled pump (Harvard Apparatus, South Natick, MA). ICV infusions were made using needles projecting 2.0 mm past the cannula at a rate of 1 µL/min for 2-min (2 µL total). For the dmPFC, bilateral infusions were made with needles projecting 2.0 mm (rostral dmPFC) or 3.0 mm (caudal dmPFC). For bilateral infusions targeting the rostral and caudal vmPFC, needles projected 4.0 mm. All intra-PFC infusions were made at a rate of 0.25 µL/min for 2-min (0.5 µL total). Bilateral intra-MS infusions were made using needles projecting 5.5 mm at a rate of 0.125 µL/min for 2-min (0.25 µL total). Needles were kept in place for 2-min following all infusions, after which the stylets were replaced.

Systemic injections of NBI 35965 or CNO. NBI 35965 (Million et al., 2003) and CNO were injected subcutaneously (1 mL/kg) 60-min and 45-min before testing, respectively. Animals were injected in their homecage in the colony room, and transported to the testing room shortly before testing began.

Dual treatments of CNO and D-Phe-CRF or NBI 35965. For experiments involving dual intra-PFC and systemic treatments, animals were transported to the testing room, first infused with D-Phe-CRF or vehicle, followed by a CNO or vehicle injection. Animals were returned to their home-cage for 30-min before testing. For experiments involving dual systemic treatments, animals received injections of NBI 35965, followed by a 10-min wait, and then injected with CNO. Animals were tested 40-min later.

Histology and Immunohistochemistry

Nissl Staining. For experiments involving intracranial infusions of CRF or CRF antagonists, animals were deeply anesthetized with isoflurane and transcardially perfused with 10% wt/vol formaldehyde and brains stored in formaldehyde for at least 24-hrs before sectioning. Injector needle placement was verified in 40 µm thick coronal sections stained with Neutral Red dye (Sigma-Aldrich, St. Louis, MO).

Fluorescence microscopy. For experiments involving chemogenetic manipulation of CRF neurons, animals were perfused transcardially with 250 mL chilled heparinized saline (1 unit heparin/mL 0.9% saline) followed by 500 mL of 4% paraformaldehyde in 0.01 M phosphate buffer (PB; pH 7.4). Brains were removed, stored in paraformaldehyde overnight, and taken through graded sucrose solutions (20-30% sucrose in 0.01 M PB, pH 7.4). For the group of animals receiving intra-PFC viral infusions and intra-PFC cannulae implants, alternating 40 µm thick coronal sections were collected through the rostrocaudal extent of the PFC to confirm injector needle placement using Nissl staining and to examine viral spread using a BX51 Olympus light and reflected fluorescence microscope. The spread of virus was mapped throughout the rostrocaudal and dorsoventral extent of the PFC to ensure that the majority of

viral expression was restricted to one PFC quadrant (rostral dmPFC versus caudal dmPFC). In most cases, the radius of the viral spread was approximately 500 μm and well confined to a particular dmPFC quadrant.

Statistical Analyses

Data from a given experiment were included only when histological analyses verified accurate placement of the transgene reporter protein (mCherry), injector tracts, and minimal tissue damage. In studies examining the effects of ICV CRF and D-Phe-CRF on sustained attention, some animals were part of pilot studies to determine working doses and thus, did not receive every drug dose. Therefore, treatment effects could not be analyzed using either a between- or within-subjects analyses of variance (ANOVA). To address this, we estimated a linear mixed-effects model using the lmer package in R. For all other studies, treatment effects were analyzed using a repeated measures ANOVA. When statistical significance was indicated, comparisons between drug dose and vehicle were determined using Bonferroni corrected t-tests.

Results

Global CRF receptor actions in sustained attention

We first confirmed that when distributed globally in the brain (ICV), CRF impairs sustained attention performance using our procedures, similar to previous reports (Cole et al., 2016). Animals were treated with vehicle (n=26), 0.025 μg (n=16), 0.2 μg (n=17), and 1 μg (n=8) ICV CRF. ICV CRF exerted a non-monotonic, dose-dependent impairment in sustained attention performance as measured by d' (Figure 1; $F_{1,11.1} = 26.5$; $P < 0.001$). Specifically, at the lowest dose tested (25 ng), ICV CRF elicited a small, but significant improvement in

performance as measured by d' relative to vehicle ($P = 0.02$). In contrast, the two higher doses of CRF (0.2 and 1 μg) elicited robust, dose-dependent decreases in d' that were accompanied by decreases in the proportion of hits ($F_{1,49.1} = 8.4$, $P = 0.005$) and increases in proportion of false alarms ($F_{1,53.8} = 61.0$, $P < 0.001$). In addition, we observed significant increases in response latency ($F_{1,22.1} = 47.2$, $P < 0.001$) and trial omissions ($F_{1,21.8} = 11.4$, $P = 0.003$; Figure 1). Thus, consistent with prior studies, CRF signaling in the brain robustly disrupts sustained attention.

We next investigated whether *endogenous* CRF signaling in the brain modulates sustained attention by testing the effects of vehicle (n=18) and ICV-administered D-Phe-CRF (2 μg, n=12; 10 μg, n=18), a non-selective $CRF_{1/2}$ antagonist, in this task. Doses of D-Phe-CRF were based on our previous studies examining the working memory actions of CRF antagonists (Hupalo and Berridge, 2016). As shown in Figure 2A, we observed a dose-dependent improvement in performance as measured by d' that was significant at the 10 μg dose ($F_{1,29.1} = 7.3$, $P = 0.01$). This largely resulted from a significant increase in the proportion of hits ($F_{1,29.0} = 4.6$, $P = 0.04$), while false alarms were not significantly affected ($F_{1,29.1} = 1.1$, $P = 0.3$).

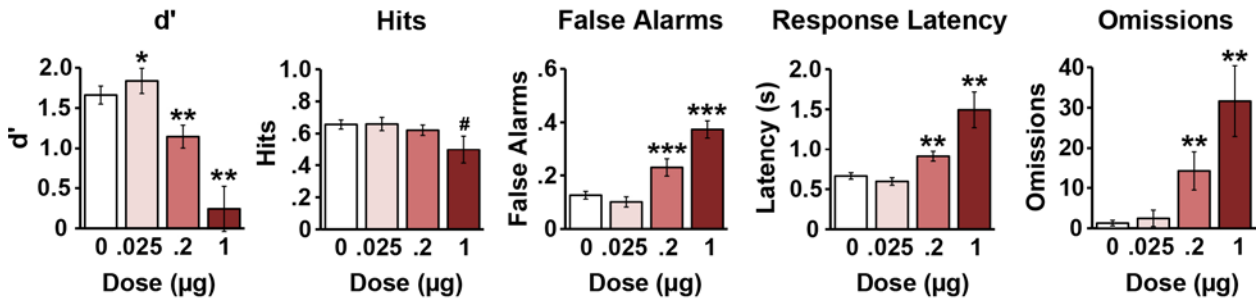

Figure 1. ICV CRF impairs sustained attention. *Left to right*: Shown are the effects of ICV administered vehicle (n=26) and varying doses of CRF (0.025 μg, n=16; 0.2 μg, n=17; 1 μg, n=8). Results represent mean ± SEM values for each response measure. At the lowest dose, CRF significantly improved performance as measured by d' ($P = 0.02$), while the 0.2 μg and 1 μg doses of CRF robustly impaired d'. These effects are driven by dose-dependent decreases in the proportion of hits and increases in proportion of false alarms. ICV CRF also elicited significant increases in response latency and trial omissions. $^{\#}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle.

Additionally, ICV CRF receptor antagonism dose-dependently decreased response latency ($F_{1,29.1}$ = 6.2, P = 0.02) and trial omissions ($F_{1,26.2}$ = 4.3, P = 0.04).

To better assess the potential clinical utility of CRF antagonists, we also examined whether a systemic CRF antagonist improves sustained attention performance (Figure 2B), as has previously been observed for working memory (Hupalo and Berridge, 2016). Systemic injections of the CRF_1-selective antagonist, NBI 35965, similarly enhanced d' in a dose-dependent manner ($F_{2,12}$ = 4.5, P = 0.05), with a magnitude comparable to that seen with clinically-relevant doses of the ADHD drug, methylphenidate (Berridge et al., 2006). This action was accompanied by non-significant increases in the proportion of hits ($F_{2,12}$ = 3.5, P = 0.09) and

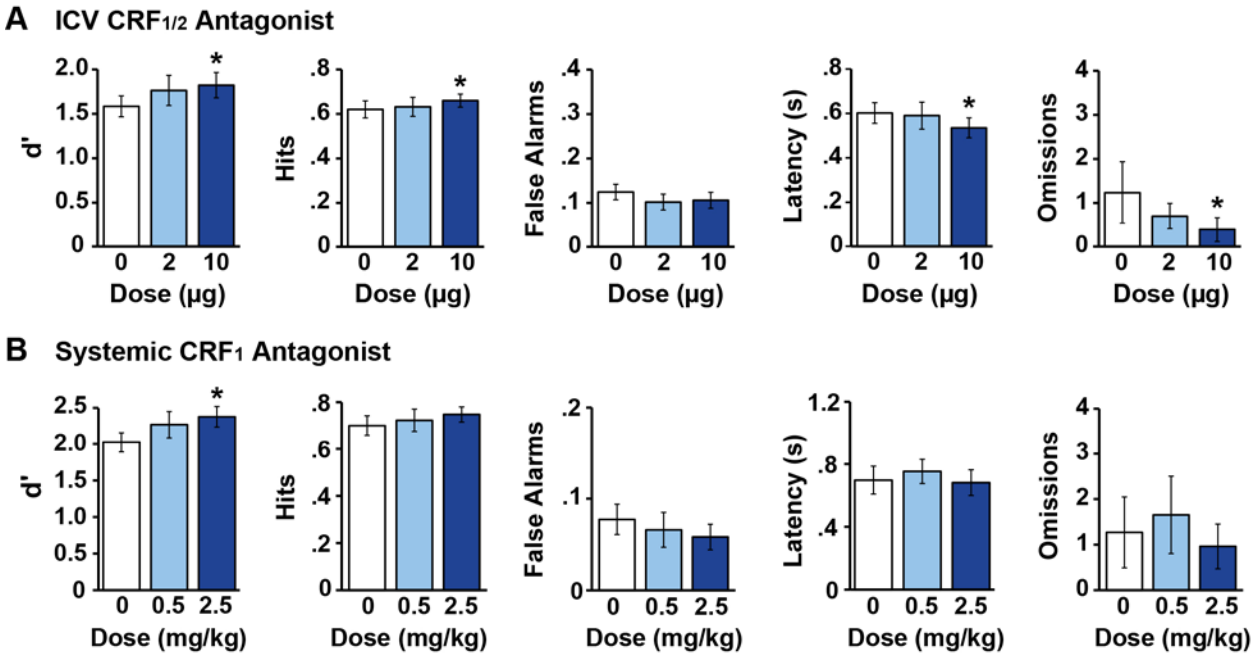

Figure 2. Global CRF receptor blockade improves sustained attention. ***A***) Shown are the sustained attention effects of vehicle (n=18) and varying doses of ICV administered D-Phe-CRF, a non-selective $CRF_{1/2}$ antagonist (2 µg, n=12; 10 µg, n=18). D-Phe-CRF dose-dependently improved performance as measured by d' and the proportion of hits. D-Phe-CRF had no significant effects on the proportion of false alarms, while significantly decreasing response latency and trial omissions (out of 100). ***B***) Sustained attention effects of systemic CRF_1-selective antagonist, NBI 35965. This antagonist elicited a significant, dose-dependent improvement in d' scores relative to vehicle (n=13). This was accompanied by a non-significant increase in the proportion of hits and a decrease in proportion of false alarms. Data shown represent mean ± SEM values. *P < 0.05 vs. vehicle.

decreases in false alarms ($F_{2,12}$ = 0.97, P = 0.34). Response latencies ($F_{2,12}$ = 3.6, P = 0.08) and trial omissions ($F_{2,12}$ = 0.35, P = 0.56) were not significantly affected (Figure 2B).

Role of PFC CRF neurons in sustained attention

Additional studies examined whether CRF neurons within the dmPFC impact sustained attention. For this, we used a dual AAV approach to chemogenetically activate PFC CRF neurons (CRF-Cre + DIO-hM3Dq-mCherry). Given we previously observed a rostrocaudal gradient of CRF actions in the dmPFC in working memory (Hupalo and Berridge, 2016), we targeted CRF neurons in either the rostral or caudal dmPFC. Thus, animals received viral infusions targeting the caudal (AP +3.2 to +2.2, Figure 3A) or rostral (AP +4.4 to 3.4, Figure 3B) dmPFC. An additional group of animals received infusions of a control virus lacking the DREADD transgene (CRF-Cre + DIO-mCherry).

Four weeks after surgery, animals began receiving treatments of vehicle and varying doses of CNO (0.3, 3 mg/kg) and underwent sustained attention testing. Chemogenetic activation of CRF neurons in the caudal dmPFC (n=8) dose-dependently impaired performance as measured by d' relative to vehicle ($F_{2,6}$ = 5.1, P = 0.02) and viral controls (n=6; $F_{1,12}$ = 7.1, P = 0.02; Figure 3A). This was accompanied by a significant decrease in the proportion of hits ($F_{2,6}$ = 3.7, P = 0.05), while false alarm rates ($F_{2,6}$ = 1.8, P = 0.2), response latency ($F_{2,6}$ = 2.4, P = 0.1), and trial omissions ($F_{2,6}$ = 3.0, P = 0.1) were not significantly affected (Table S1A). To confirm that these effects were due to activation of CRF cell bodies in the PFC, we also infused CNO locally into the PFC (0.1, 1 mM; n=9). We observed similar dose-dependent decreases in d' ($F_{2,7}$ = 7.3, P = 0.005; Figure 3A) and proportion of hits ($F_{2,7}$ = 3.6, P = 0.05; Table S1B). Similar to

that seen with systemic CNO, proportion of false alarms ($F_{2,7}$ = 2.6, P = 0.1), response latency ($F_{2,7}$ = 0.4, P = 0.7), and trial omissions ($F_{2,7}$ = 0.43, P = 0.5) were unaffected (Table S1B).

Meanwhile, activation of rostral dmPFC CRF neurons (n=5) using systemic injections of CNO elicited no significant changes in task performance as measured by d' relative to vehicle ($F_{2,4}$ = 0.04, P = 0.8) and mCherry controls (n=4; $F_{1,7}$ = 1.2, P = 0.3; Figure 3B). There were no significant effects on the proportion of hits ($F_{2,4}$ = 2.7, P = 0.2), false alarms ($F_{2,4}$ = 2.2, P = 0.2), response latency ($F_{2,4}$ = 0.5, P = 0.6), or trial omissions ($F_{2,4}$ = 0.2, P = 0.7; Table S2A). Activation of rostral dmPFC CRF neurons using intra-PFC infusions of CNO also had no significant effects on sustained attention performance (d', $F_{2,4}$ = 0.6, P = 0.5; hits, $F_{2,4}$ = 0.2, P = 0.7; false alarms, $F_{2,4}$ = 0.2, P = 0.6; response latency, $F_{2,4}$ = 1.4, P = 0.3; trial omissions, $F_{2,4}$ =

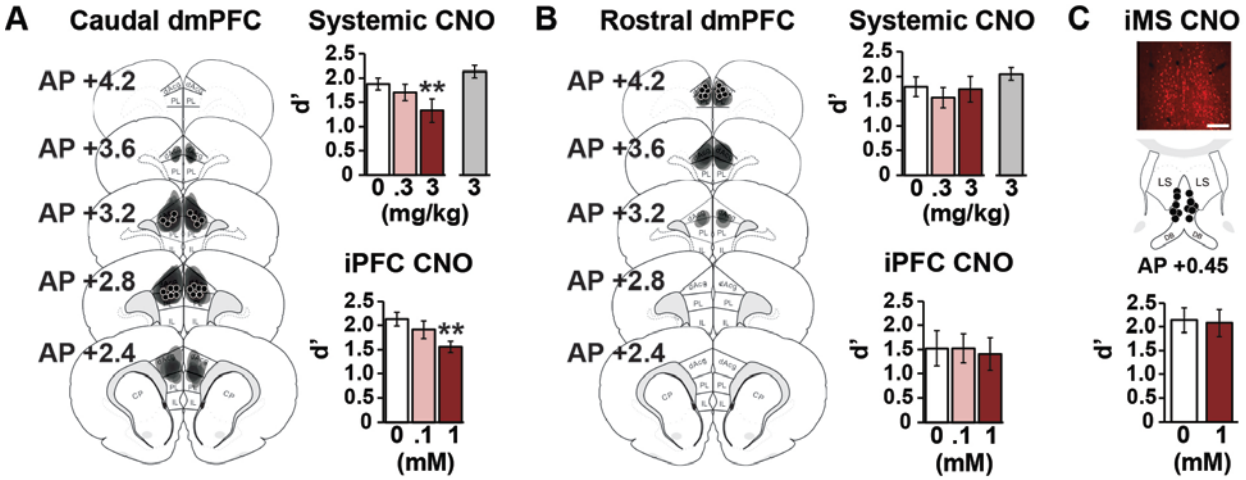

Figure 3. CRF neurons in the caudal dmPFC impair sustained attention. ***A***) *Left*, schematic demonstrating viral spread (shaded circles) and CNO infusion sites (black circles) in the caudal dmPFC (AP +3.2 to +2.2). *Top right*, chemogenetic activation of caudal dmPFC CRF neurons using systemic CNO (n=8) dose-dependently impairs d' relative to vehicle and viral controls (n=6, gray bar). *Bottom right*, activation of caudal dmPFC CRF neurons using local CNO infusions similarly impairs d' relative to vehicle (n=9). ***B***) *Left*, viral spread and CNO infusion sites in the rostral dmPFC (AP +3.4 to +4.4). *Right*, chemogenetic activation of rostral dmPFC CRF neurons (n=5) elicited no significant effects on d' relative to vehicle and viral controls (n=4, gray bar), regardless whether CNO was administered systemically (top) or locally into the PFC (bottom). ***C***) *Top*, retrograde mCherry cell body labeling was observed in the MS in ~30% of animals; scale bar = 200 µm. *Middle*, micrograph depicting the main body of CNO infusion sites into the MS. *Bottom*, CNO infusions into the MS (n=6) elicited no significant effects on d' relative to vehicle. Results represent mean ± SEM values. **P < 0.01 vs. vehicle.

3.4, $P = 0.1$; Figure 3B, Table S2B).

In about 30% of animals, we observed retrograde mCherry labeling of cell bodies in the medial septum (MS; Figure 3C). While MS projections to the PFC have been observed (Senut et al., 1989), to the best of our knowledge, these are the first observations to suggest that *CRF neurons* within the MS innervate the PFC. To determine whether MS CRF neurons contribute to the cognition-modulating effects of CNO, a subset of animals (n=6) received CNO infusions into the MS. Intra-MS CNO infusions had no effect on sustained attention performance as measured by d' ($F_{1,5} = 0.44$, $P = 0.5$; Figure 3C) or any other dependent measure (data not shown). Given we did not observe mCherry-positive cell bodies elsewhere in the brain, these results demonstrate that CRF neurons in the caudal dmPFC are responsible for the sustained attention-impairing effects of CRF neuronal activation observed in our studies.

Role of PFC CRF receptors in sustained attention

We next examined whether CRF neurons impact sustained attention via activation of local CRF receptors in the caudal dmPFC. Animals received intra-caudal dmPFC infusions of the CRF antagonist, D-Phe-CRF, prior to chemogenetic activation of CRF neurons in this region (via systemic CNO). As shown in Figure 4A, in animals treated with intra-PFC vehicle, 3 mg/kg CNO elicited robust decreases in task performance as measured by d' (n=9; $F_{1,8} = 14.4$, $P = 0.005$). Intra-PFC infusion of D-Phe-CRF (100 ng/hemisphere) did not block the CNO-induced impairment in sustained attention (CNO x Antag interaction, $F_{1,8} = 2.7$, $P = 0.1$). In contrast, systemic treatment with the CRF_1 selective antagonist, NBI 35965 (1 mg/kg, n=9), completely reversed the sustained attention-impairing effects of CNO (CNO x Antag interaction, $F_{1,8} = 7.9$, $P = 0.02$; Figure 4A). Collectively, these observations indicate that CRF neurons in the caudal

dmPFC impair sustained attention via projections outside this PFC subregion. Consistent with this, bilateral CRF infusions (25, 50, 250 ng) directly into the caudal dmPFC failed to modulate d' (n=10; $F_{3,9}$ = 0.08, *P* = 0.8; Figure 4B) or any other performance measure (data not shown).

To assess whether CRF neurons within the caudal dmPFC influence sustained attention via projections to other medial PFC subfields, we examined the sustained attention effects of CRF infusions into the rostral dmPFC as well as the rostral and caudal ventromedial PFC (vmPFC). As shown in Figure 4B, CRF infusions into the rostral dmPFC (n=13) failed to affect sustained attention performance (d', $F_{3,12}$ = 0.01, *P* = 0.9). Similarly, when infused into the rostral (n=8) or caudal (n=5) vmPFC, CRF had no noticeable impact on sustained attention performance in either subfield. Therefore, data from these regions were

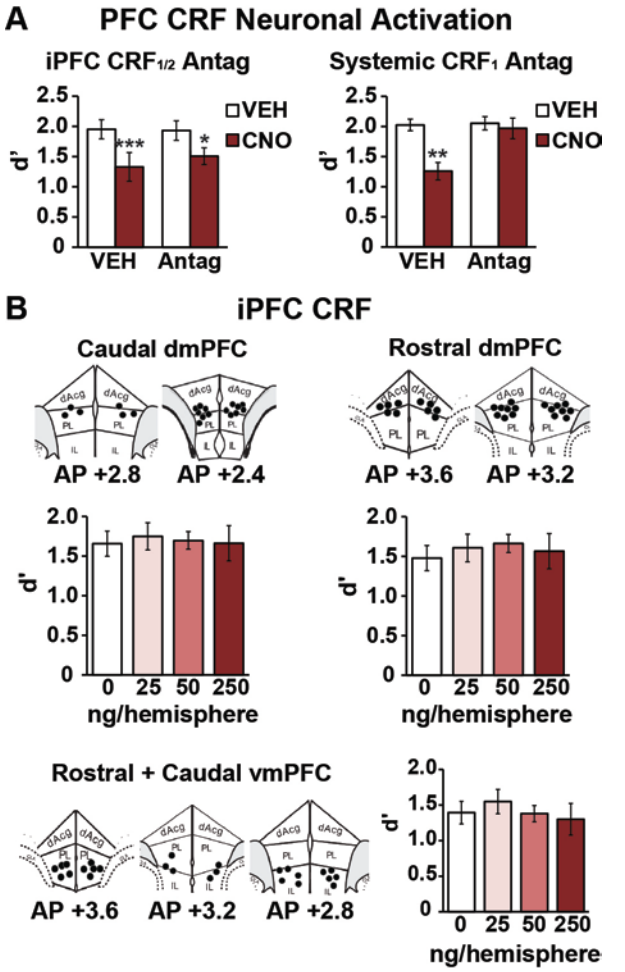

Figure 4. CRF neurons in the caudal dmPFC impair sustained attention via projections outside the PFC. ***A***) *Left*, animals received intra-caudal dmPFC infusions of vehicle or the CRF antagonist, D-Phe-CRF, prior to chemogenetic activation of CRF neurons in this region (3 mg/kg CNO). D-Phe-CRF (100 ng, n=9) did not reverse the sustained attention-impairing effects of PFC CRF neuronal activation (CNO x antagonist interaction, *P*=0.14). *Right*, systemic administration of the CRF_1 antagonist, NBI 35965 (1 mg/kg, n=9) significantly reversed the sustained attention-impairing effects of PFC CRF neuronal activation (CNO x antagonist interaction, *P* = 0.02). ***B***) CRF infusions into the caudal dmPFC (n=10), rostral dmPFC (n=13), and varying rostrocaudal fields of the vmPFC (n=13) had no significant effects on d'. Results represent mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. vehicle.

grouped (n=13; Figure 4B). Intra-vmPFC infusions of varying doses of CRF had no significant effects on d' ($F_{3,11} = 1.2$, $P = 0.3$) or any other performance measure (data not shown). Thus, these observations demonstrate that CRF receptors in the medial PFC do not modulate sustained attention.

Discussion

These studies demonstrate that CRF-synthesizing neurons within the PFC impair PFC-dependent cognition as measured in a sustained attention task. These actions are similar to CRF neuronal modulation of spatial working memory. For example, the cognition-modulatory effects of PFC CRF neurons in both tasks are topographically organized, being limited to the caudal aspect of the dmPFC. However, in contrast to that seen with working memory, the sustained attention-impairing actions of PFC CRF neurons are *not* dependent on CRF receptors in the PFC. Thus, CRF neurons within the PFC regulate distinct cognitive processes via different neural circuits. Nonetheless, the pro-cognitive effects of CRF antagonists across both sustained attention and working memory tasks mirror the actions FDA-approved drugs for ADHD (Berridge and Arnsten, 2015). Therefore, CRF antagonists may represent a novel pharmacological approach in the treatment of ADHD and other PFC-dependent cognitive disorders.

CRF Modulation of Distinct PFC-Dependent Cognitive Processes

The above-described results demonstrate that different PFC CRF neuronal circuits modulate working memory vs. sustained attention. The observation that PFC CRF receptors differentially regulate working memory vs. sustained attention is consistent with evidence

demonstrating that these tasks are regulated by different noradrenergic receptor mechanisms. For example, PFC α_1 receptor activation improves sustained attention and attention set shifting, while impairing working memory (Arnsten et al., 1999; Lapiz and Morilak, 2006; Berridge et al., 2012; Berridge and Spencer, 2016). Moreover, clinically relevant doses of methylphenidate maximally enhance working memory performance, while doses 4-fold higher than this maximally improve sustained attention in an α_1 receptor-dependent manner (Berridge et al., 2012). This likely reflects the fact that noradrenergic α_1 receptors have a lower affinity for norepinephrine (NE) than α_2 receptors, and are thus engaged at higher levels of NE (Ramos and Arnsten, 2007).

The specific cognitive processes that are supported by these different PFC circuits and receptor mechanisms are unclear. Both tests of working memory and attention require various cognitive processes for successful performance, including attention, motivation, rule learning, and switching between spatial locations. With respect to working memory, one key difference is the need maintain relevant information 'on-line' in the absence of explicit cues and in the presence of distractors. Meanwhile, the signal detection test assesses a form of focused attention with minimal external distractors and no need to maintain information. Nevertheless, these results provide strong evidence that CRF acts within distinct PFC circuits to modulate performance in tests that recruit a different set of cognitive domains.

Neurocircuitry Underlying CRF Modulation of Sustained Attention

In the present studies, global activation of CRF receptors in the brain (ICV CRF) elicited a non-monotonic impairment in sustained attention. At low doses (25 ng), ICV CRF improved sustained attention performance. In contrast, higher doses of ICV CRF (200 ng, 1 μg) elicited

robust sustained attention-impairing actions, resulting from an increase in response latencies and trial omissions in addition to impaired performance accuracy (hits, false alarms). This contrasts with that observed for chemogenetic activation of PFC CRF neurons, which primarily impaired performance accuracy on signal trials (hits), but not response latencies or trial omissions. Moreover, at the highest dose, ICV CRF elicited substantially larger impairments in overall performance (d') than seen with PFC CRF neuronal activation. These differences observed with ICV CRF vs. PFC CRF neuronal activation likely reflect the recruitment of CRF systems outside the PFC known to modulate PFC cognitive function (e.g. catecholamines and glucocorticoids) and may account for the bidirectional effects observed with ICV CRF (Valentino et al., 1983; Dunn and Berridge, 1987; Barsegyan et al., 2010).

Currently, the neurocircuitry underlying the sustained attention actions of PFC CRF neurons is unknown. In the case of working memory, the cognition-modulatory actions of PFC CRF receptors likely involve the CRF_1 subtype, given the rodent PFC contains few to no functional CRF_2 receptors (De Souza et al., 1985; Kostich et al., 1998; Van Pett et al., 2000). Meanwhile, PFC CRF neuronal regulation of sustained attention is dependent on CRF_1 receptors outside the PFC (Figure 4). Although it is unclear where in the brain CRF acts to impair sustained attention, one possible circuit involves the basal forebrain cholinergic system. The medial PFC and basal forebrain are reciprocally connected (Bloem et al., 2014) and basal forebrain cholinergic neurons modulate sustained attention by regulating acetylcholine release within the PFC (McGaughy et al., 1996; McGaughy and Sarter, 1998; Parikh et al., 2007). Moreover, basal forebrain nuclei are rich in CRF_1 receptors and PFC afferents synapse directly onto CRF_1-expressing cholinergic neurons within basal forebrain nuclei, including the substantia innominata (Sesack et al., 1989; Gaykema et al., 1991; Sauvage and Steckler, 2001; Chandler et

al., 2013). Although preliminary studies indicate that CRF receptor activation within the substantia innominata impairs aspects of sustained attention performance, this was confined to an impairment on no-signal trials (Eck et al., 2017). In contrast, PFC CRF neuronal activation primarily impaired performance on signal trials. Therefore, additional circuits may be involved in PFC CRF neuronal regulation of sustained attention. Investigation of this circuitry remains an important focus of future studies.

Sex differences in CRF Modulation of Sustained Attention

The current study used male animals to compare the actions of PFC CRF in sustained attention to a large literature documenting the modulatory actions of PFC catecholamines on PFC-dependent cognition which has utilized primarily male subjects. Yet, there exist sex differences in PFC-related psychopathology and evidence indicates females can display differential sensitivity to certain behavioral and cellular actions of CRF in the locus coeruleus and dorsal raphe nucleus (Valera et al., 2010; Valentino et al., 2012; Howerton et al., 2014). Nonetheless, there were no general sex-dependent actions of ICV CRF in sustained attention when examined across all trials, although females were slightly more sensitive to CRF (0.5 μg) during the middle part of the test session (Cole et al., 2016). Ongoing studies in our laboratory are investigating the degree to which the PFC CRF neurons modulate sustained attention in females.

Summary

Combined with our prior work, these studies demonstrate that PFC CRF neurons broadly impair distinct PFC-dependent cognitive processes as measured in tests of sustained attention

and working memory. In contrast to that seen with working memory, the sustained attention-impairing effects of PFC CRF neurons are dependent on CRF_1 receptor actions outside the PFC. Thus, PFC CRF neuronal regulation of varying PFC-dependent cognitive processes involves differing neural circuitry. Nonetheless, the ability of systemic CRF receptor antagonists to improve performance in tests of sustained attention and working memory is identical to ADHD treatments, suggesting CRF receptors may represent a novel pharmacological drug target for the treatment of ADHD and other PFC cognitive disorders.

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Supplementary Results

Supplementary Table 1. Effects of chemogenetic activation of CRF neurons in the caudal dmPFC on aspects of sustained attention performance.

A) Systemic CNO

Systemic CNO				
	VEH	**0.3 mg/kg**	**3 mg/kg**	**3 mg/kg control**
Proportion of Hits	0.69 ± 0.05	0.64 ± 0.04	0.57 ± 0.06 *,$^{+}$	0.73 ± 0.04
Proportion of False Alarms	0.11 ± 0.03	0.1 ± 0.02	0.16 ± 0.06	0.07 ± 0.01
Response Latency (s)	0.65 ± 0.25	0.66 ± 0.25	0.93 ± 0.62	0.51 ± 0.13
Trial Omissions	1.00 ± 1.00	0.25 ± 0.16	5.21 ± 2.00 $^{+}$	0 ± 0

Values represent mean ± SEM. hM3Dq group, n=8; mCherry controls, n=6. *$P < 0.05$ vs. vehicle; $^{+}P < 0.05$ vs. viral controls.

B) iPFC CNO

iPFC CNO			
	VEH	**0.1 mM**	**1 mM**
Proportion of Hits	0.65 ± 0.05	0.69 ± 0.04	0.6 ± 0.03
Proportion of False Alarms	0.05 ± 0.02	0.1 ± 0.02	0.11 ± 0.02
Response Latency (s)	0.54 ± 0.19	0.55 ± 0.15	0.58 ± 0.23
Trial Omissions	1.33 ± 0.62	0.56 ± 0.56	1.28 ± 0.75

Values represent mean ± SEM. hM3Dq group, n=9. *$P < 0.05$ vs. vehicle; $^{+}P < 0.05$ vs. viral controls.

Supplementary Table 2. Effects of chemogenetic activation of CRF neurons in the rostral dmPFC on aspects of sustained attention performance.

A) Systemic CNO

Systemic CNO				
	VEH	**0.3 mg/kg**	**3 mg/kg**	**3 mg/kg control**
Proportion of Hits	0.50 ± 0.05	0.42 ± 0.08	0.58 ± 0.02	0.75 ± 0.05
Proportion of False Alarms	0.04 ± 0.02	0.04 ± 0.02	0.08 ± 0.04	0.06 ± 0.03
Response Latency (s)	0.29 ± 0.04	0.34 ± 0.07	0.32 ± 0.04	0.6 ± 0.16
Trial Omissions	0.20 ± 0.2	0.10 ± 0.1	0.20 ± 0.2	0 ± 0

Values represent mean ± SEM. hM3Dq group, n=5; mCherry controls, n=4. There were no significant effects of any treatment on these measures of sustained attention performance.

B) iPFC CNO

iPFC CNO			
	VEH	**0.1 mM**	**1 mM**
Proportion of Hits	0.49 ± 0.11	0.5 ± 0.11	0.47 ± 0.11
Proportion of False Alarms	0.06 ± 0.03	0.08 ± 0.04	0.07 ± 0.03
Response Latency (s)	0.35 ± 0.3	0.31 ± 0.03	0.36 ± 0.04
Trial Omissions	0.1 ± 0.1	0 ± 0	1.7 ± 0.94

Values represent mean ± SEM. hM3Dq group, n=5. There were no significant effects of any treatment on these measures of sustained attention performance.

Chapter 5

Conclusions and Future Directions

Summary

The cognitive actions of CRF neurotransmission in the PFC have remained unknown, despite decades of research into the behavioral and physiological actions of this neuropeptide. These studies are the first to demonstrate that CRF neurons in the PFC regulate multiple PFC-dependent cognitive processes. Across both working memory and sustained attention, the cognitive actions of CRF neurons are topographically organized, being restricted to the caudal dmPFC. Moreover, the working memory-impairing actions of PFC CRF neurons involve local CRF release and subsequent activation of CRF_1 receptors coupled to the PKA intracellular signaling cascade. These actions are associated with robust alterations in task-related neural representations within the frontostriatal circuit. In contrast to working memory, PFC CRF neuronal modulation of sustained attention involves CRF_1 receptors *outside the PFC*. Thus, CRF modulation of different PFC-dependent cognitive processes involves distinct neuronal circuits. Nonetheless, administration of a systemic CRF_1 antagonist improves performance in tests of working memory and sustained attention, similar to the actions of FDA-approved ADHD treatments (Berridge et al., 2006; Spencer et al., 2015). Therefore, CRF receptors may represent a novel drug target for ADHD and other disorders associated with PFC-dependent cognitive dysfunction.

Circuitry Underlying Broad Cognitive Actions of PFC CRF Neurons

These studies demonstrate that different CRF neuronal circuits modulate performance in working memory vs. sustained attention tasks. Although both tasks are dependent on CRF_1 receptor actions, in the case of working memory, this involves CRF receptors in the caudal dmPFC, while sustained attention is modulated by CRF receptors outside the PFC. The precise

neurocircuitry underlying these effects is not known. One possibility is that these actions are driven by subpopulations of PFC CRF neurons with different projection targets. Indeed, our preliminary results demonstrate that PFC CRF neurons are neurochemically heterogeneous (~85% are glutamatergic, while 15% are GABAergic; Hupalo and Berridge, unpublished observations). Thus, working memory may be dependent on local GABAergic CRF neurons in the PFC, while sustained attention may be modulated by glutamatergic CRF projections outside the PFC. Alternatively, it is also possible that despite this heterogeneity, the same population of PFC CRF neurons modulate working memory and sustained attention, and rather, performance in these tasks is dependent on the areas that receive PFC CRF projections.

Currently, the region(s) where PFC CRF neurons terminate to impair sustained attention performance is not known. One possible site of CRF_1 receptor action may involve basal forebrain cholinergic systems. A body of research demonstrates that cholinergic neurons within the basal forebrain modulate sustained attention via projections to the PFC (Wenk et al., 1980; McGaughy et al., 1996; Wenk, 1997; Parikh et al., 2007). In addition, the PFC innervates CRF_1-expressing cholinergic neurons within multiple basal forebrain nuclei, including the substantia innominata (Sesack et al., 1989; Gaykema et al., 1991; Sauvage and Steckler, 2001; Chandler et al., 2013). Preliminary studies indicate that CRF receptor activation within the substantia innominata impairs aspects of sustained attention performance. However, this effect resulted from an impairment on no-signal trials (Eck et al., 2017), while PFC CRF neuronal activation impaired performance on signal trials in the present studies. Thus, additional circuits may be involved in PFC CRF neuronal regulation of sustained attention performance.

Topography of CRF actions in the PFC

The observation that PFC CRF modulation of working memory and sustained attention is restricted to the dmPFC is consistent with a well-documented dorsoventral organization of the rodent medial PFC. Specifically, the dmPFC is more closely associated with higher cognitive function while the vmPFC is more closely linked to visceral and affective processes (Voorn et al., 2004; Gabbott et al., 2005). Moreover, these results highlight a less well-known rostrocaudal topography within the medial PFC. The restriction of the cognitive actions of CRF to the caudal, but not rostral, dmPFC is similar to that seen with noradrenergic modulation of sensorimotor gating (Alsene et al., 2011). Given CRF neurons and receptors are present throughout the entire medial PFC (Swanson et al., 1983; De Souza et al., 1985), the topographical actions of CRF are likely not due to differential receptor distribution. Instead, this topography may arise from functional differences within frontostriatal and frontothalamic circuits. For example, the caudal prelimbic PFC innervates the dorsal STR more heavily than the rostral prelimbic PFC (Sesack et al., 1989). In addition, the rostral dmPFC projects heavily to the mediodorsal (MD) thalamic nucleus, while caudal dmPFC innervation of the MD is relatively sparse (Groenewegen, 1988). Combined, these observations suggest that CRF neurons and receptors within the caudal dmPFC may engage different anatomical circuits to modulate PFC-dependent cognition.

PFC CRF Modulation of Neuronal Task Representations across the Frontostriatal Circuit

These studies demonstrate that the working memory-impairing effects of PFC CRF neuronal activation are associated with a suppression of delay- and reward-related activity and a profound decrease in the population size of strongly tuned delay neurons in the dmPFC. Meanwhile, PFC CRF neuronal activation had no effects on untuned dmPFC neurons. Thus, across the broader population of dmPFC neurons, PFC CRF neuronal activation robustly

degrades the signal-to-noise representation of working memory task events. Moreover, the observation that the working memory actions of PFC CRF neurons involve local CRF receptors coupled to the PKA cascade is consistent with prior studies demonstrating robust cognitive and neurophysiological actions of PKA activity. For instance, excessive PKA signaling in the PFC impairs working memory and suppresses delay-related activity of spatially tuned dorsolateral PFC neurons (Taylor et al., 1999; Wang et al., 2007). As such, the cognition-enhancing actions of the ADHD drug, guanfacine, are associated with PKA inhibition and subsequent enhancement of delay-related activity in the PFC (Franowicz and Arnsten, 1998; Wang et al., 2007).

Furthermore, these observations suggest PFC CRF neurons do not participate in the encoding of working memory task events. For example, if PFC CRF neurons were task-sensitive, chemogenetic activation of this neuronal population would enhance PFC task-related activity. However, we observed a suppression of delay- and reward-related activity in the PFC. On the other hand, it is possible that PFC neurons classified as negatively tuned in the present studies are CRF positive, given these neurons were chemogenetically activated. The neuronal coding properties of PFC CRF neurons are unknown and remain an important topic of investigation in future studies. Currently, the most feasible approach to examine this would utilize genetically encoded calcium indicators in cognitively-tested animals.

The current studies also provide novel insight regarding the nature of working memory task representations within the dmSTR. Although the dmSTR is necessary for working memory performance (Spencer et al., 2012), the degree to which task-related activity in this region is sensitive to manipulations that impair working memory had not been explicitly examined. We observed that putative medium spiny neurons display robust task-related activation during the delay, reward, and outcome signal epochs of the task. PFC CRF neuronal activation similarly

diminished the population size of strongly tuned medium spiny neurons in the dmSTR, but elicited a weaker and non-significant suppression of delay- and reward-related activity. Combined, these observations indicate the cognition-impairing actions of caudal dmPFC CRF neurons involve a robust degradation in the ability of dmPFC neurons, and to a lesser extent dmSTR neurons, to represent key working memory task events.

Currently, the mechanisms underlying PFC CRF neuronal modulation of task-related activity downstream in the dmSTR are unknown. In the present studies, we observed no anterogradely-labeled mCherry fibers in the dmSTR, consistent with anatomical studies demonstrating few to no CRF fibers and axons in this region (Swanson et al., 1983). Thus, it is unlikely that PFC CRF neurons modulate the dmSTR via direct projections. However, it is possible that CRF directly influences layer V PFC projection neurons that terminate in the dmSTR, given CRF receptors are prominent in this layer (De Souza et al., 1985; Radulovic et al., 1998). This hypothesis remains to be tested in future studies.

Future Directions

The current studies identify interesting new questions regarding the neurocircuitry and mechanisms underlying the cognitive actions of CRF in the PFC. One important goal of future studies is to determine where in the brain CRF acts to impair sustained attention. Although the substantia innominata is one candidate structure, there exist differences in the sustained attention effects of CRF receptor activation in this region (performance impairment on no-signal trials; Eck et al., 2017) and the effects PFC CRF neuronal activation (impairment on signal trials). As such, the basal forebrain contains many clusters of cholinergic neurons, each with distinct connections and neurochemical profiles (Alheid and Heimer, 1988; Sauvage and Steckler, 2001).

Therefore, a deeper exploration of cholinergic and non-cholinergic regions that receive PFC CRF innervation is necessary. To address this, future studies will identify target areas that are activated following chemogenetic activation of PFC CRF neurons using Fos-ir. Subsequently, candidate region(s) will be targeted with CRF antagonist infusions to determine if this reverses the sustained attention-impairing effects of PFC CRF neuronal activation.

In preliminary studies, we observed that CRF neurons in the PFC are neurochemically heterogeneous. While ~15% of PFC CRF neurons are GABAergic (GAD67-ir), the majority (~85%) of PFC CRF neurons are glutamatergic (CaMKIIα-ir; Hupalo & Berridge, unpublished observations). It is unclear which of these neuronal populations, if not both, modulates PFC-dependent cognition. Ongoing studies in our laboratory are utilizing a dual virus chemogenetic approach to examine the working memory and sustained attention actions of glutamatergic (CRF-Cre + DIO-CaMKIIα-hM3Dq) vs. GABAergic (CRF-Cre + DIO-DLX-hM3Dq) CRF neurons in the PFC.

Lastly, one major goal of this thesis has been to determine whether CRF antagonists represent an alternative drug target in the treatment of ADHD. Therefore, it is promising that a systemic CRF_1 antagonist improved performance in tests of working memory and sustained attention identically to the actions of methylphenidate (Ritalin) and other ADHD drugs (Berridge and Arnsten, 2015). Nonetheless, additional studies are necessary to critically test this hypothesis. For example, an important question is whether CRF antagonists enhance working memory task-related activity in the PFC, as has been observed with the ADHD drug, guanfacine (Wang et al., 2007). Another major question is whether the cognition-enhancing actions of CRF antagonists extend to female subjects. Although the present studies included males, this allowed us to compare the cognitive actions of CRF to a large literature documenting the cognitive

actions of catecholamines, which has primarily utilized male subjects (Arnsten et al., 1999; Lapiz and Morilak, 2006; Berridge et al., 2012). However, a body of research demonstrates robust sex differences in CRF signaling across multiple brain systems (Bangasser et al., 2010; Valentino et al., 2012; Howerton et al., 2014). Although a recent study observed no general sex-dependent effects of ICV CRF in sustained attention (Cole et al., 2016), it is important to examine whether sex differences exist in the actions of CRF signaling *within the PFC*. Thus, ongoing studies in our laboratory are investigating the cognitive actions PFC CRF receptors and neurons in female rats, and whether CRF antagonists elicit similar cognition-enhancing effects in both sexes.

Although several questions remain, the present studies unambiguously demonstrate that CRF is a potent modulator of PFC-dependent cognition. Given previous wide scale efforts to develop CRF_1 antagonists for stress-related affective disorders, many CRF antagonist compounds have already passed safety tests in human subjects (Kehne and Cain, 2010; Koob and Zorrilla, 2012). Therefore, the cognitive actions of CRF antagonists could readily be examined in in ADHD and healthy individuals. This may represent a relatively low-cost path toward a potential therapeutic treatment for ADHD and other disorders associated with PFC-dependent cognitive dysfunction.

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