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The excitatory actions of corticotropin-releasing factor on ventral tegmental area dopamine neurons

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The excitatory actions of corticotropin-releasing factor on ventral tegmental area dopamine neurons

by

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The excitatory actions of corticotropin-releasing factor on ventral tegmental area dopamine neurons

By Matthew J Wanat under the direction of Dr. Antonello Bonci

Abstract:

The dopamine neurons of the ventral tegmental area (VTA) are involved with motivated behaviors and locomotion. Interestingly, stress stimulates the release of dopamine in brain regions receiving dense VTA input, which could be important in promoting escape from threatening situations. The mechanism by which stress activates the dopamine system is unknown, but many lines of evidence suggest a role for the stress-released neuropeptide, corticotropin-releasing factor (CRF). The effect of CRF on VTA dopamine neurons is not well characterized and is the major topic of this dissertation.

In order to address the effect of CRF on VTA dopamine neurons in brain slice recordings, I needed a method to confidently identify dopamine neurons. In contrast to the rat, I found that the presence of the hyperpolarization-activated, cyclic nucleotide-regulated cation current (I_h) is a reliable electrophysiological measure to identify dopamine neurons in the mouse. Furthermore, I demonstrated that CRF increased the firing of VTA dopamine neurons through a mechanism involving the CRF-receptor 1 (CRF-R1), the phospholipase C – protein kinase C (PLC – PKC pathway), and the I_h. I also found that CRF enhanced the I_h through a PKC-dependent mechanism, which did not involve changes in the voltage-dependence of activation for the I_h. Because of the role of

the VTA and dopamine release in motor behaviors, I examined the effect of CRF in the VTA on the locomotor activity of rats. Mirroring my findings from brain slice recordings, I found that intra-VTA injections of CRF-R1 agonists required PKC and I_h to increase locomotor activity. Together, these studies examined the excitatory role of CRF on VTA dopamine neurons from ion currents to behavior and further the knowledge regarding stress-related neuropeptide modulation of the dopamine system.

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Chapter 1: Introduction

The ventral tegmental area (VTA) sends projections throughout the limbic system and is one of the primary dopamine-producing cell groups in the brain (Swanson, 1982). Dopamine release is required for a number of behaviors including locomotion (Beninger, 1983; Zhou and Palmiter, 1995), and goal-directed pursuit (Denk et al., 2005; McFarland et al., 2004), as well as for optimal cognitive function (Sawaguchi and Goldman-Rakic, 1991). Diseases thought to involve dopamine dysregulation such as Parkinson's disease (Bergstrom and Garris, 2003), depression (Gershon et al., 2007), schizophrenia (Beninger, 2006), and addiction (Kalivas and McFarland, 2003; McFarland et al., 2004; McFarland and Kalivas, 2001), together highlight the importance of proper dopamine function in the central nervous system. Several, sometimes conflicting, theories developed to explain the significance of dopamine release given its role in numerous behaviors (Berridge, 2007; Salamone and Correa, 2002; Schultz, 1997; Wise, 1978; Wise, 2004); however, it is well established and uncontroversial that the dopamine system is involved with sensorimotor and motivated behaviors (Berridge, 2007; Salamone and Correa, 2002).

Intriguingly, the VTA and dopamine system is activated not only by rewarding stimuli (Schultz et al., 1997) and addictive drugs (Di Chiara and Imperato, 1988), but also by stress (Abercrombie et al., 1989; Anstrom and Woodward, 2005; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996). The stress-induced dopamine release could be involved with initiating escape from stressful and threatening situations as these behaviors are modulated by dopamine levels (Blanchard et

al., 2003; Blanchard et al., 1999). However, stress can also lead to maladaptive behavioral responses since it promotes motivated drug-seeking behaviors through activation of the VTA and subsequent dopamine release (McFarland et al., 2004). Thus, it is of great importance to determine how stress activates VTA dopamine neurons, as these findings will not only identify key neurophysiological alterations by stress, but will also highlight potential therapeutic targets to treat addiction. Although the exact mechanism by which stress activates VTA dopamine neurons is unknown, several lines of evidence suggest the stress-related neuropeptide, corticotropin-releasing factor (CRF), may be involved (Lavicky and Dunn, 1993; Wang et al., 2005; Wang et al., 2007). This chapter discusses (i) the anatomical connectivity and properties of VTA dopamine neurons, (ii) the cellular actions and hypothesized function of dopamine release in certain behaviors, (iii) dopamine, drugs of abuse, and plasticity in the VTA (iv) CRF and the stress response, (v) evidence supporting an interaction between stress, CRF, dopamine and the VTA, and finally (vi) the hypothesis and outline of this dissertation.

The VTA: composition and anatomy

The VTA and the neighboring substantia nigra (SN) are the primary dopamine producing nuclei in the brain (Swanson, 1982). However, in the rat, the VTA is not comprised solely of dopamine neurons, as 1/3 of the ~ 14,000 VTA neurons do not contain tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and as such are not dopaminergic (Swanson, 1982). The non-dopamine producing cells are likely GABA- and glutamate-producing, as markers for these neuronal subtypes, GAD65

and VGlut2, respectively, exist in the VTA (Yamaguchi et al., 2007). However, there is some debate if glutamate and dopamine are co-released from the same neurons (Kawano et al., 2006; Lapish et al., 2007) or if these neuronal subtypes exist in distinct populations (Yamaguchi et al., 2007).

The neurons of the VTA send efferent projections along three major pathways. The primary projection ascends through the medial forebrain bundle (MFB) to innervate forebrain nuclei, including the nucleus accumbens (NAcc), amygdala, bed nucleus of the stria terminalis (BNST), and prefrontal cortex (PFC) (Swanson, 1982). Another smaller projection travels dorsally to innervate the thalamus, while the final projection descends to innervate structures such as the periaqueductal gray and locus coeruleus (Swanson, 1982). Individual neurons of the VTA primarily innervate only a single nucleus and rarely do they project to the contralateral hemisphere (Swanson, 1982). The innervation from the VTA onto various nuclei is not uniform as VTA dopamine neuron input is strongest in the NAcc (~85% of VTA input), relative to either the amygdala (~50%) or the PFC (~30%) (Swanson, 1982). The afferent innervation of the VTA include many of the regions receiving VTA input such as the PFC, NAcc, BNST, lateral dorsal tegmentum (LDT) and amygdala (Omelchenko and Sesack, 2005; Phillipson, 1979). Although the connectivity of the VTA appears to be reciprocal with a number of brain regions, there are identifiable neuronal circuits. Specifically, a subset of VTA dopamine neurons receiving PFC input project back the PFC, while another subset of VTA GABA neurons receiving PFC input project to the NAcc (Carr and Sesack, 2000a). The studies discussed above

collectively highlight the VTA as a heterogeneous brain region with extensive afferent input and efferent projections within the limbic system.

Most *in vivo* studies identify dopamine neurons based on the presence of a triphasic and long-duration action potential waveform (Grace and Bunney, 1983; Grace et al., 2007). These neurons are either (i) hyperpolarized and quiescent, (ii) fire action potentials in a pacemaker-like fashion (2-10 Hz) or (iii) fire action potentials in bursts up to 15-30 Hz (Grace and Bunney, 1983). The pacemaker dopamine neuron firing is thought to give rise to the 'tonic' levels of dopamine with concentrations ranging from 5-20 nM (Parsons and Justice, 1992), while the burst firing is thought to give rise to 'phasic' elevated dopamine levels which can reach as high as 1 μM (Garris et al., 1997; Gonon, 1988). The burst firing of dopamine neurons requires glutamatergic input, activation of N-methyl-D-aspartate receptors (NMDAR), opening of high-threshold calcium currents, and finally activation of calcium-activated potassium currents to terminate the burst (Overton and Clark, 1997). Although robust immunohistochemical methods can identify dopamine, GABA, and glutamate neurons in the VTA (Yamaguchi et al., 2007), electrophysiological identification of neuronal subtypes is problematic (Margolis et al., 2006). *In vivo* juxtacellular labeling of recorded VTA neurons in the rat demonstrate that many neurons with a triphasic and long-duration waveform actually are not dopaminergic (Ungless et al., 2004), although some suggest these findings are difficult to interpret because the amplifier signal may have been over-filtered (Grace et al., 2007).

In contrast to *in vivo* recordings, dopamine neurons in brain slice preparations do not fire action potentials in bursts, but rather only exhibit spontaneous pacemaker-like action potential firing (Grace and Onn, 1989). The reported frequency of putative dopamine neuron firing in the slice varies whether one uses extracellular (3-8 Hz) (Grace and Onn, 1989), perforated-patch (2-5 Hz) (Neuhoff et al., 2002), or whole-cell recordings (1-3 Hz) (Margolis et al., 2006). Regardless of the recording technique, the firing of putative GABA neurons is significantly greater than dopamine neurons and often can be higher than 10 Hz (Grace and Onn, 1989). Although the firing rate could provide a crude segregation of neuronal subtypes, the hyperpolarization-activated, cyclic nucleotide-regulated cation current (I_h, HCN) was found in all recorded dopamine neurons and absent in GABA neurons, suggesting that the presence of I_h could be an electrophysiological test to identify dopamine neurons (Grace and Onn, 1989). The I_h arises from cation passage through a tetramer consisting of some combination of the four HCN channesl (HCN1-4) (Frere et al., 2004). Subsequent work in rodents used the presence of the I_h as a predictor of dopamine content, though it became apparent that not all cells with the I_h contained TH (Cameron et al., 1997; Margolis et al., 2006). Surprisingly, a rigorous study found that only ~50% of rat VTA neurons with the I_h are dopaminergic, suggesting that the standard electrophysiological test for dopamine content was unreliable (Margolis et al., 2006). In summary, the VTA was originally thought to consist of easily identifiable dopamine and non-dopamine subtypes (Grace and Onn, 1989; Swanson, 1982), but recent studies highlight the heterogeneity of the neuronal composition of the VTA in the rat (Margolis et al., 2006; Yamaguchi et al., 2007).

Dopamine

Dopamine has two receptor subtype families: the D1-like which includes the D1 and D5 receptors that predominately couple to the Gs signaling pathway, and the D2-like which includes the D2, D3, and D4 receptors that often couple to the opposing Gi/o signaling pathway (Nicola et al., 2000), though stimulation of both the D1- and D2-like pathways are required for certain behavioral (Ikemoto et al., 1997) and physiological responses (Hopf et al., 2003). Numerous studies demonstrate that dopamine can exert short and long-term alterations both pre- and post-synaptically in a variety of brain regions (Lapish et al., 2007; Nicola et al., 2000). The cellular effects of dopamine are hypothesized to modulate the effects of GABA and glutamate (Lapish et al., 2007) and to increase the signal to noise of coherent inputs in target brain regions (Nicola et al., 2000).

In part due to the great scientific interest in dopamine over that past 50 years, a variety of hypotheses have been developed to explain the functional significance of dopamine release. The anhedonia hypothesis (Wise, 1978; Wise, 2004), which suggests that dopamine is the reward neurotransmitter, is the theory of dopamine function often cited in the media (Berridge, 2007). Supporting this hypothesis, drugs of abuse that are thought to be rewarding such as cocaine, amphetamine, morphine, nicotine, and ethanol all elevate dopamine levels in brain regions receiving VTA input (Di Chiara and Imperato, 1988). However, since dopamine is released during stress (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996), it is unlikely that its release signifies only reward. Some have attempted to reconcile this

discrepancy by suggesting dopamine is released to motivate escape from the stressor, which could be negatively reinforcing (Berridge and Robinson, 1998), though since dopamine release is observed concurrently with a inescapable stressor (Weiss et al., 1997), this interpretation is likely not valid. Furthermore, dopamine release in the striatum is not required for 'liking' oral-facial responses in rodents (Berridge, 2007; Berridge et al., 1989), suggesting that although dopamine release is associated with rewarding events, it is not solely a 'reward neurotransmitter'.

So if dopamine does not equate to reward, what does its release signify? Using electrophysiological recordings in primates, Wolfram Schultz posited that dopamine is a reward prediction error signal (Schultz, 1997). Specifically, putative midbrain dopamine neurons fire action potentials in response to unexpected rewards, but with extended training the neurons fire instead to cues predicting the reward (Schultz et al., 1997). In contrast, neuron firing is reduced when the cue is presented and no reward is administered (Schultz et al., 1997), suggesting dopamine release may be involved in reinforcement learning. However, some argue that the phasic dopamine response functions instead as an attention switch to relevant stimuli because the dopamine neuron activation occurs too fast after the cue for it to compute error predictions (Redgrave et al., 1999). Alternatively, the dopamine signal could function to reinforce actions that preceded the unexpected stimulus (Redgrave and Gurney, 2006; Wise, 2004). Although dopamine may play a role in certain learning paradigms (Faure et al., 2005), it is not required for all learning as mice deficient in dopamine production still learn appetitive

associations (Robinson et al., 2005). Thus, the observed changes in dopamine neuron firing of primates could just be a response and not a cause of learning (Berridge, 2007).

The dopamine hypotheses discussed above attempt to ascribe a role for dopamine in reinforcing reward-driven learning; however, a general and less contentious dopamine hypothesis posits that dopamine release is instead tied to sensorimotor and motivating behaviors (Berridge, 2007; Salamone and Correa, 2002). It is well established that dopamine plays a key role in motor tasks as this is the primary deficit observed with those suffering from Parkinson's Disease, a disease that leads to the selective degeneration of dopamine neurons (Bergstrom and Garris, 2003). Also, mice deficient in dopamine production are catatonic and require supplements for survival (Zhou and Palmiter, 1995).

Dopamine is also involved in motivated behaviors, since antagonism of dopamine receptors (at doses that do not induce motor deficits) decreases the effort and the time delay a rat will endure for a high reward on a previously well-learned task (Denk et al., 2005). Conversely, dopamine transporter knock-down mice exhibit enhanced motivation to work for a food reward, but they do not have any alterations in operant learning paradigms (Cagniard et al., 2006). Further supporting a role of dopamine in motivation, dopamine release is observed during the onset of specific goal-directed behaviors, and artificial stimulation of dopamine release alone can initiate these behaviors (Phillips et al., 2003). Thus, one interpretation suggests the function of dopamine release may be to overcome the motivational costs required for making high effort tasks (Phillips et al., 2007; Salamone and Correa, 2002). Indeed, the sensorimotor/motivation theory is

consistent with reinforcement theories of dopamine function as the motivation to respond is a prerequisite for any reinforcing behavior (Salamone and Correa, 2002). Although numerous and sometimes conflicting theories of dopamine function have been espoused, the view that dopamine release is involved with sensorimotor and motivating behaviors is generally well accepted (Berridge, 2007) and is the view championed in this discourse. It also should be mentioned that many of the hypotheses of dopamine function on behavior often do not take into account that dopamine may have divergent effects depending on the brain region studied. Since dopamine release to a given stimulus can vary depending on the brain region studied (Horger and Roth, 1996; Inglis and Moghaddam, 1999; Thierry et al., 1976), caution should be taken when applying dopamine theories beyond anything more than a general framework for conceptualizing dopamine function.

Dopamine, drugs of abuse, and plasticity in the VTA

Scientific study of the VTA has often focused on its role in addiction since abused drugs can stimulate dopamine release in the NAcc (Di Chiara and Imperato, 1988; Kauer, 2004), and in many cases, drug-related behaviors require VTA activation and dopamine release (Kalivas and Alesdatter, 1993; Kalivas and McFarland, 2003; McFarland et al., 2004; McFarland and Kalivas, 2001). Behavioral sensitization and drug self-administration are the common behavioral paradigms used to study the pharmacology and neural circuits affected by addictive drugs. Behavioral sensitization is defined as the enhanced locomotor response to a drug injection that develops after exposure to the drug (Kauer, 2004). This phenomenon, much like human addiction, is observed with a number

of abused drugs and is long lasting, suggesting that behavioral sensitization is an appropriate model of drug-induced neuroadaptations (Kauer, 2004). Initiation of sensitization likely occurs in the VTA as local drug injections into the VTA, and not the NAcc, induce sensitization (Kalivas and Weber, 1988). Conversely, blockade of NMDAR in the VTA prevents cocaine-induced behavioral sensitization (Kalivas and Alesdatter, 1993).

Rodent drug self-administration models, although laborious relative to the passive drug exposure in behavioral sensitization paradigms, are more analogous to human drug-seeking behaviors (Kalivas and McFarland, 2003). The focus of many rodent drug-self administration procedures is to understand the mechanisms underlying relapse, or rather the reinstatement of drug-seeking behaviors after a protracted drug withdrawal. Importantly, stress-induced reinstatement of drug-seeking involves VTA activation and subsequent dopamine release in the PFC (Kalivas and McFarland, 2003; McFarland et al., 2004). Thus, addiction studies using behavioral sensitization and drug self-administration paradigms demonstrate the importance of the VTA in mediating these behaviors.

The critical role of the VTA in behavioral sensitization and drug self-administration highlights the possibility that addictive drugs could induce neuroadaptations in the VTA. The synapses onto VTA dopamine neurons can be modified through both long-term potentiation (LTP) of excitatory (Bonci and Malenka, 1999; Liu et al., 2005b) and inhibitory inputs (Nugent et al., 2007) as well as long-term depression (LTD) of excitatory inputs (Jones et al., 2000). Direct application of abused

drugs can modify the capacity for synaptic plasticity in the midbrain (Jones et al., 2000; Schilstrom et al., 2006). Specifically, amphetamine prevents the calcium-dependent, NMDAR-independent LTD on VTA dopamine neurons (Jones et al., 2000). Another study found that cocaine potentiates NMDAR currents through a D1-like receptor mechanism (Schilstrom et al., 2006). Since LTP on VTA dopamine neurons requires NMDAR activation (Bonci and Malenka, 1999), the authors suggest that the cocaine-induced increase of NMDAR currents could initiate synaptic plasticity changes that may be important in the development of addiction related behaviors (Schilstrom et al., 2006).

In order to address how *in vivo* exposure to drugs of abuse affect synaptic strength on VTA dopamine neurons, many studies use the ratio of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)/NMDAR current as a measure of synaptic plasticity (Borgland et al., 2004; Saal et al., 2003; Ungless et al., 2001). The AMPAR/NMDAR ratio is an electrophysiological measure that normalizes the relative contributions of ionotropic glutamate receptor currents so that comparisons can be made between different animals and treatment groups. Elevations of the AMPAR/NMDAR ratio are associated with a reduced capacity to induce LTP and an enhanced ability to express LTD, suggesting that this measure correlates with synaptic strength (Ungless et al., 2001), although this has recently been questioned (Liu et al., 2005b). Interestingly, a single exposure to addictive drugs such as cocaine, amphetamine, morphine, nicotine, and ethanol, but not non-addictive drugs such as fluoxetine or carbamazepine, increase the AMPAR/NMDAR ratio (Borgland et al., 2004; Saal et al., 2003; Ungless et al., 2001). The drug-induced synaptic changes can be long-lasting (5 days), and exists in discrete

brain nuclei as the change in the AMPAR/NMDAR ratio is found on VTA dopamine neurons, but not on CA1 pyramidal neurons of the hippocampus (Borgland et al., 2004; Ungless et al., 2001). Surprisingly, multiple cocaine injections do not induce a further increase in the AMPAR/NMDAR ratio and this change in synaptic strength is dependent only upon the last drug exposure (Borgland et al., 2004). Drug-induced changes in the AMPAR/NMDAR ratio in VTA dopamine neurons were hypothesized to be integral to the development of behavioral sensitization (Kauer, 2004), but a subsequent study demonstrated no correlation exists between the degree of sensitization with multiple drug exposures and the changes in the AMPAR/NMDAR ratio (Borgland et al., 2004). However, a recent finding demonstrates that long-lasting (3 weeks) increases in the AMPAR/NMDAR ratio correlates with voluntary cocaine self-administration (Chen et al., 2007). Together, these studies illustrate drugs of abuse induce neuroadaptations and that these changes could be important in the development of addiction.

CRF and the stress response

An organism's response to threatening and/or stressful stimuli is paramount to its survival and often elicits a host of defensive behaviors (Blanchard et al., 2003). In this regard, the physiological changes due to stress are widespread, as changes are observed in the sympathetic nervous system, as well as numerous brain nuclei and stem from the release of CRF (de Kloet et al., 2005). CRF is primarily produced in the paraventricular nucleus of the hypothalamus (PVN), but CRF mRNA is also present in the amygdala, lateral septum, dorsal raphe nucleus, locus coeruleus (LC), Edinger-Westphal nucleus,

BNST, periaqueductal gray, and cerebellum (Swanson et al., 1983). CRF has two known G-protein coupled receptors, the CRF-receptor 1 and 2 (CRF-R1 and CRF-R2), which are differentially distributed throughout the brain (Orozco-Cabal et al., 2006). Specifically, CRF-R1 is present in the cortex, cerebellum, hippocampus, amygdala, olfactory bulb, pituitary gland, and VTA (Potter et al., 1994), while CRF-R2 is found primarily in the lateral septum, hypothalamus, amygdala, and VTA (Lovenberg et al., 1995; Ungless et al., 2003). In addition to the CRF-R1 and CRF-R2, a CRF-binding protein (CRF-BP) can bind CRF and remove it from the circulatory system (Behan et al., 1995). CRF-BP is membrane-associated in the brain and found in the cerebral cortex, lateral septum, amygdala, and VTA (Potter et al., 1992). The function of the CRF-BP in the central nervous system is not completely understood, though it can modify CRF signaling through the other CRF receptors (Ungless et al., 2003). The CRF-R1 and CRF-R2 can both stimulate a variety of intracellular signaling pathways, though predominately they activate the cAMP-protein kinase A (cAMP-PKA) pathway (Blank et al., 2003; Hauger et al., 2006). Though less frequently observed, both the CRF-R1 and CRF-R2 couple to the phospholipase C - protein kinase C (PLC-PKC) signal transduction pathway (Blank et al., 2003; Hauger et al., 2006; Ungless et al., 2003). In cell culture systems, CRF receptors can activate the extracellular signal-regulated - microtubule associated protein kinase (Rossant et al., 1999) and the nitric oxide synthase - guanylyl cyclase pathways (Aggelidou et al., 2002).

Three other CRF-related peptides exist in the nervous system with their own unique expression patterns. Urocortin I (UCNI) mRNA is found in the Edinger-Westphal

nucleus and lateral hypothalamus (Kozicz et al., 1998); Urocortin II (UCNII) mRNA is located in the hypothalamus and LC (Reyes et al., 2001); and Urocortin III (UCNII) mRNA is present in the hypothalamus and amygdala (Li et al., 2002). The CRF family of peptides bind to the CRF receptors with different affinities (Hauger et al., 2006). CRF preferentially binds to the CRF-R1, though it can also activate the CRF-R2 at higher doses (Dautzenberg et al., 1999). UCNI activates both the CRF-R1 and CRF-R2 with equal efficacy (Dautzenberg et al., 1999). In contrast, UCNII and UCNIII only stimulate the CRF-R2 (Lewis et al., 2001; Reyes et al., 2001). Finally, the CRF-BP binds CRF, UCNI, but not UCNIII (Behan et al., 1996; Jahn et al., 2004).

Although CRF and its family of peptides are present throughout the brain, CRF release from the PVN is responsible for initiating the classical 'fight or flight' behavioral response to stress. Specifically, limbic and/or brainstem pathway activation of the PVN stimulates CRF release into the portal vessel system where it acts on the CRF-R1 in the anterior pituitary that in turn produces and secretes adrenocorticotropic hormone (ACTH) into the blood stream. ACTH stimulates the adrenal cortex to produce corticosteroids, which reach every organ through the circulatory system to initiate the observed stress-induced adaptations including mobilization of energy stores, suppression of the immune system, and vasoconstriction (de Kloet et al., 2005). Corticosteroids also induce genomic alterations through binding to either the high affinity mineralocorticoid receptor or the ubiquitous low affinity glucocorticoid receptors throughout the brain. Collectively, this pathway is referred to as the hypothalamic-pituitary-adrenal (HPA) axis and constitutes the basic physiological stress response (de Kloet et al., 2005).

Stress, CRF, dopamine and the VTA

Stress not only activates the HPA axis, but also induces changes in extra-hypothalamic brain regions, including the midbrain dopamine system (Horger and Roth, 1996; Sarnyai et al., 2001). For example, a host of laboratory stressors such as foot-shock, handling, tail-flick, and social defeat reliably increase dopamine levels in brain regions receiving dense input from the VTA such as the PFC, NAcc, and amygdala (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996). Supporting these microdialysis studies, restraint stress increases putative VTA dopamine neuron firing in conscious rats (Anstrom and Woodward, 2005). Interestingly, the dopamine projection from the midbrain to the PFC (mesocortical) as opposed to midbrain projection to the NAcc (mesoaccumbens) is especially sensitive to stress (Horger and Roth, 1996; Thierry et al., 1976). During stress, CRF is released not only in the pituitary to activate the HPA axis, but also in the VTA (de Kloet et al., 2005; Wang et al., 2005). Additionally, intracerebroventricular (i.c.v.) injections of CRF increases dopamine levels in the PFC (Lavicky and Dunn, 1993). Together these studies provide evidence that stress-induced activation of the dopamine system could be mediated by CRF acting on VTA dopamine neurons.

Further supporting its excitatory actions on the VTA, stress enhances dopamine- and drug-dependent behaviors. Stress itself increases cocaine-induced activity (Sorg and Kalivas, 1991), augments the rate of acquisition (Tidey and Miczek, 1997) and reinstates previously extinguished drug-seeking behaviors (McFarland et al., 2004). Additionally,

stress-induced drug-seeking requires activation of the VTA and subsequent dopamine release in the PFC (McFarland et al., 2004).

Since CRF release is integral to an organism's physiological response to a stressor, a number of studies examined the role of CRF in drug self-administration paradigms. I.C.V. injections of CRF reinstate cocaine and heroin drug-seeking behaviors (Shaham et al., 1997), while CRF-R1 antagonists prevent stress-induced cocaine, heroin, and ethanol drug-seeking behaviors (Funk et al., 2007; Le et al., 2000; Shaham et al., 1998). The effects of CRF in drug self-administration models is brain region specific as CRF promotes ethanol drinking when injected into the amygdala (Funk et al., 2006) and CRF stimulates cocaine seeking when given into the VTA (Wang et al., 2005; Wang et al., 2007) and BNST, but not in the NAcc and amygdala (Erb and Stewart, 1999). Although CRF can reinstate drug-seeking behaviors when given in a variety of limbic brain regions, the role of CRF in the VTA is likely critical since elegant inactivation studies demonstrated that VTA activation is downstream of BNST and amygdala activation in the neural circuitry required for drug-seeking behaviors (Kalivas and McFarland, 2003; McFarland et al., 2004). These studies collectively demonstrate that VTA dopamine neurons, in conjunction with the extended limbic system, are involved with both stress and CRF reinstating drug-seeking behaviors.

In addition to their role in addictive behaviors, stress, CRF, dopamine and the VTA are also involved with locomotor activity and escape behaviors. As discussed above, dopamine is important for movement (Beninger, 1983; Zhou and Palmiter, 1995), and the VTA is also involved as injections of glutamate receptor agonists into the VTA

enhance locomotion (Dunn et al., 2005). I.C.V. injections of CRF increase activity, though this effect is not observed in mice deficient for the CRF-R1 (Contarino et al., 2000). CRF injected directly into the VTA also stimulates locomotion; however, the authors from this study suggested that this effect could involve mechanisms independent of dopamine release (Kalivas et al., 1987).

CRF and dopamine are also involved in mediating behavioral responses to stressful and/or threatening stimuli. Depending on the context, mice exhibit a variety of behaviors to predators such as hiding, freezing, defensive threat, defensive attack, risk assessment and of importance to this discussion, flight (Blanchard et al., 2003). The flight/escape response is positively modulated by increases in dopamine levels (Blanchard et al., 1999) and also by i.c.v. injections of a CRF-R1 agonist (Yang et al., 2006). Conversely, the flight response is blunted when mice receive intraperitoneal (i.p.) injections of CRF-R1 antagonists (Griebel et al., 1998). In conclusion, the evidence described above highlight the interaction between stress, CRF, dopamine and the VTA, which are involved in stress-induced drug-seeking behaviors as well as flight responses to threatening/stressful situations. Thus, it is of great importance to determine how stress activates VTA dopamine neurons, as these findings will not only highlight potential therapeutic targets to treat addiction, but will also identify key neurophysiological alterations by stress.

Dissertation hypothesis and outline

The preceding sections outlined evidence supporting experimental and behaviorally relevant interactions between stress, CRF, dopamine, and the VTA. Briefly, since stress (i) stimulates the release of CRF in the VTA, (ii) increases putative VTA dopamine neuron firing and (iii) induces the release of dopamine in brain regions receiving VTA input, I hypothesized that CRF would directly excite VTA dopamine neurons that in turn would have behaviorally relevant consequences. In Chapter 2, using whole cell patch recordings from mouse brain slices, I present evidence that the presence of the I_h is a robust elecrophysiological marker of dopamine content in the VTA of mice. Also, I demonstrated that CRF increased the firing of VTA dopamine neurons involving a mechanism that requires the CRF-R1, the PLC-PKC signaling cascade, and the I_h. In Chapter 3, I observed that CRF increased the I_h through a PKC-dependent mechanism that did not involve alterations in the voltage-dependence of activation for the I_h. In Chapter 4, I demonstrate that intra-VTA injections of CRF in the rat increased locomotor activity and that this effect involves the CRF-R1, PKC activity, and the I_h. In Chapter 5, I discuss how CRF potentiated NMDAR currents of VTA dopamine neurons in mouse brain slices. This effect required actin depolymerization and in contrast to a previous report, the I_h did not correlate with the ability of CRF to increase NMDAR currents. In addition, I determined the effect of a prior *in vivo* cocaine exposure on the ability of CRF to potentiate NMDAR currents. In Chapter 6, I used fast-scan cyclic voltammetry to assay dopamine fluctuations in the NAcc. Specifically, I identified a functional connection between the LDT, VTA, and NAcc, as well as determined how intra-VTA

CRF injections affected dopamine release in the NAcc in rats. Chapter 7 summarizes the results from my studies involving the actions of CRF in the VTA. Further experimental directions are also discussed. Finally in the Appendix, I examined neuroadaptations that developed in VTA dopamine neurons in response to *in vivo* ethanol and cocaine exposure.

Chapter 2. CRF increased the firing of VTA dopamine neurons

Abstract:

CRF is released throughout the brain during stress, including onto the dopamine cells of the VTA. Although the mechanism is unknown, stress stimulates dopamine release in brain regions receiving VTA input. Stress-induced dopamine release can enhance escape from threatening situations, as well as promote drug-seeking behaviors. I hypothesized that stress could activate the dopamine system in part by CRF directly exciting VTA dopamine neurons. First, I demonstrated that VTA dopamine neurons in mouse brain slices could be reliably identified based on the presence of the I_h. Using whole-cell patch clamp recordings in current clamp mode, I recorded from spontaneously firing dopamine neurons and observed an increase in the firing rate with bath application of 1 μM CRF. Using pharmacology and transgenic mice, I found that CRF increased VTA dopamine neuron through a mechanism involving the CRF-R1, the PLC-PKC signaling pathway, and the I_h. These findings demonstrate the direct excitatory role of CRF on VTA dopamine neurons, which may be important for stress-induced dopamine release.

Introduction:

Stressful stimuli induce the release of the 41 amino acid peptide, corticotropin-releasing factor, which initiates the canonical stress response pathway (de Kloet et al., 2005). In addition to increasing systemic glucocorticoid levels through activation of the

HPA axis, CRF is released in numerous extra-hypothalamic brain regions (Hauger et al., 2006; Wang et al., 2005). CRF mediates its cellular effects through two known G-protein-coupled receptors, CRF-R1 and CRF-R2 (Hauger et al., 2006; Lovenberg et al., 1995; Potter et al., 1994). Additionally, CRF binds to the CRF-BP, which can sequester CRF as well as modify signaling through other CRF receptors (Behan et al., 1995; Ungless et al., 2003). CRF receptors couple to a variety of intracellular signaling cascades including the cAMP-PKA and PLC-PKC pathways (Blank et al., 2003; Hauger et al., 2006; Ungless et al., 2003).

The VTA is one of the primary dopamine producing nuclei in the brain that sends projections and receives input from a variety of limbic structures (Phillipson, 1979; Swanson, 1982). In vitro studies often use the presence of the I_h as an electrophysiological marker of dopamine content (Cameron et al., 1997; Grace and Onn, 1989); however, a recent study demonstrated that the I_h is actually a poor indicator of dopamine neurons in the VTA of the rat (Margolis et al., 2006). Although electrophysiological identification of rat dopamine neurons remains elusive, these neurons are behaviorally relevant as they are involved with locomotion (Beninger, 1983; Dunn et al., 2005; Zhou and Palmiter, 1995), motivated goal-related actions (Denk et al., 2005; Phillips et al., 2007; Salamone and Correa, 2002; Wise, 2004), are thought to play a critical role in the development of drug addiction (Kauer, 2004), but interestingly are also activated by stressful stimuli (Anstrom and Woodward, 2005; Horger and Roth, 1996). Foot-shock, handling, tail-flick, and social defeat stressors reliably increase dopamine levels in brain regions receiving dense input from the VTA such as the prefrontal cortex,

the nucleus accumbens, and the amygdala (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996). Stress-induced dopamine release not only could enhance escape from predators (Blanchard et al., 1999), but also is implicated in maladaptive drug-seeking behaviors (McFarland et al., 2004). Thus, identifying the mechanism through which stress activates midbrain dopamine neurons not only identifies key neurophysiological alterations by stress, but also might facilitate the development of therapeutic interventions to prevent maladaptive stress responses in drug addiction.

CRF is released into the VTA during stress (Wang et al., 2005), and in this regard CRF could be involved with the stress-induced increase in dopamine levels. Supporting an excitatory role of CRF on dopamine release, i.c.v. injections of CRF dose-dependently increases dopamine levels in the PFC (Lavicky and Dunn, 1993). CRF-R1, CRF-R2, and CRF-BP are present in the VTA and could mediate the cellular actions of CRF in this region (Chan et al., 2000; Ungless et al., 2003; Van Pett et al., 2000). We previously found that CRF potentiates NMDAR currents on putative VTA dopamine neurons (Ungless et al., 2003); however, it is unknown how CRF directly affects the firing activity of these neurons. Since stress (i) stimulates the release of CRF in the VTA, (ii) increases putative VTA dopamine neuron firing and (iii) induces the release of dopamine in brain regions receiving VTA input, I hypothesized that CRF would directly excite VTA dopamine neurons. In this set of experiments I determined that the I_h is an excellent electrophysiological marker for dopamine content in mice, as well as identified the mechanism by which CRF increases VTA dopamine neuron firing.

Materials and Methods:

Electrophysiology

All procedures conformed to National Institutes of Health, Ernest Gallo Clinic and Research Center, and University of Washington animal care policy standards. Horizontal VTA brain slices from 3-5-week-old C57BL/6 mice (Charles River) or P21-25 Sprague-Dawley rats (Charles River) were prepared for electrophysiological recordings. Briefly, rodents were anesthetized with halothane and immediately decapitated. All solutions used were saturated with 95% O_2 – 5% CO_2. 170 μM brain slices were cut in a chilled solution that contained, in mM: 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, and 75 sucrose. Slices recovered for ~1 hour at 32°C in an artificial cerebral spinal fluid (aCSF), with 295-305 mOsm and contained, in mM: 126 NaCl, 2.5 KCl, 1.1 NaH_2PO_4, 1.4 $MgCl_2$, 2.4 $CaCl_2$, 11 d-glucose, and 26 $NaHCO_3$. 100 μM picrotoxin was added to the aCSF before recordings to block $GABA_A$ input on recorded neurons. Whole cell patch-clamp recordings with 2-6 MΩ electrodes were made with an Axopatch 1D amplifier using Clampex 8.0 (Axon Instruments) and Igor Pro (Wavemetrics) as data acquisition programs. Firing experiments in current-clamp recording mode used an internal recording solution of 130 mM KOH, 105 mM methanesulfonic acid, 17 mM HCl, 20 mM HEPES, 0.2 mM EGTA, 2.8 mM NaCl, 2.5 mg/mL Mg-ATP, and 0.25 mg/mL Mg-GTP. I attempted voltage-clamp recordings using this internal solution, but were unable to get stable recordings over time; thus, I used a cesium-based internal solution containing: 117 mM cesium methanesulfonate, 20 mM

HEPES, 0.4 mM EGTA, 2.8 mM NaCl, 5 mM TEA-Cl, 2.5 mg/mL Mg-ATP, and 0.25 mg/mL Mg-GTP. Internal recording solutions used were at pH = 7.2 – 7.4 and at an osmolarity between 275 – 285 mOsm. For immunohistochemical-staining experiments, 1.0% biocytin was included in the recording solution. Neurons were visualized with an upright microscope using infrared differential interference contrast illumination. aCSF at 32 - 34°C was continuously perfused at a 2.0 – 2.5 mL/min over brain slices.

Immunocytochemistry

For immunohistochemical staining, brain slices were fixed in 4% formaldehyde for 2 hours. Slices were washed with phosphate-buffered saline (PBS) and refrigerated until pre-blocking the tissue with PBT (0.2% triton, bovine serum albumin, 0.2g/100mL in PBS) and 5% normal goat serum at 25°C for 2 hours on a shaker. Slices incubated at 4°C for 2 days with 1:100 rabbit anti-tyrosine hydroylase (Chemicon) and then washed with PBT. Finally, slices incubated overnight at 4°C with 1:50 FITC-anti-rabbit and 6.5 µl/mL Texas red conjugated streptavidin (Jackson immunoresearch) in PBT, then washed, mounted and visualized with a Zeiss LSM 510 META confocal microscope.

Reagents and statistical analysis

All drugs were obtained from Sigma, except human/rat CRF (Sigma and Bachem), antisauvagine-30 (Polypeptide Laboratories), ZD-7288 (Tocris), PDBU (Calbiochem), CP-154,56 (generous gift from Pfizer), and ovine CRF, d-Phe CRF, urocortin II, and CRF 6-33 (all from Bachem). Drugs were dissolved in DMSO at a final

concentration of less than 0.1% and then added to aCSF for experiments. The firing rate was determined in 10-second sweeps and averaged into 5-minute bins for statistical analysis. All values are expressed as mean ± SEM. Unless otherwise noted, statistical significance was assessed using a two-tailed unpaired Student's t-test.

Results:

Identification of VTA dopamine neurons

To examine how CRF might alter firing in midbrain dopamine neurons, I recorded from spontaneously firing neurons in the VTA from mouse horizontal brain slices (average baseline firing was 1.90 ± 0.05 Hz in 192 recorded cells). In the mouse, there is no clear delineation between the SN and the VTA, so for the current study, VTA neurons were classified as those medial to the most lateral edge of the medial terminalis (MT) (Figure 2-1). Previous work in various rodent species used the presence of the hyperpolarization-activated, cyclic nucleotide-regulated cation current (I_h) as an indicator of dopamine content (Cameron et al., 1997; Grace and Onn, 1989); however, a recent

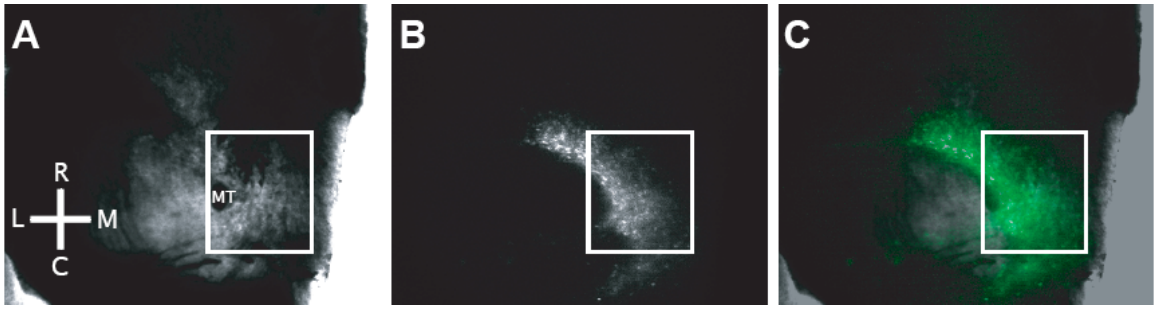

Figure 2-1: Location of recorded neurons. (A) Representative horizontal brain slice taken from a mouse expressing GFP under the TH promoter. White box indicates approximate location of recorded VTA neurons used in this study. MT is the medial terminalis. R is rostral, C is caudal, L is lateral, M is medial. (B) Fluorescence image of the same slice. Notice no clear anatomical or dopamine neuron distribution that would indicate a defined border between the SN and the VTA. (C) Panel (B) is overlaid on panel (A) in pseudocolor.

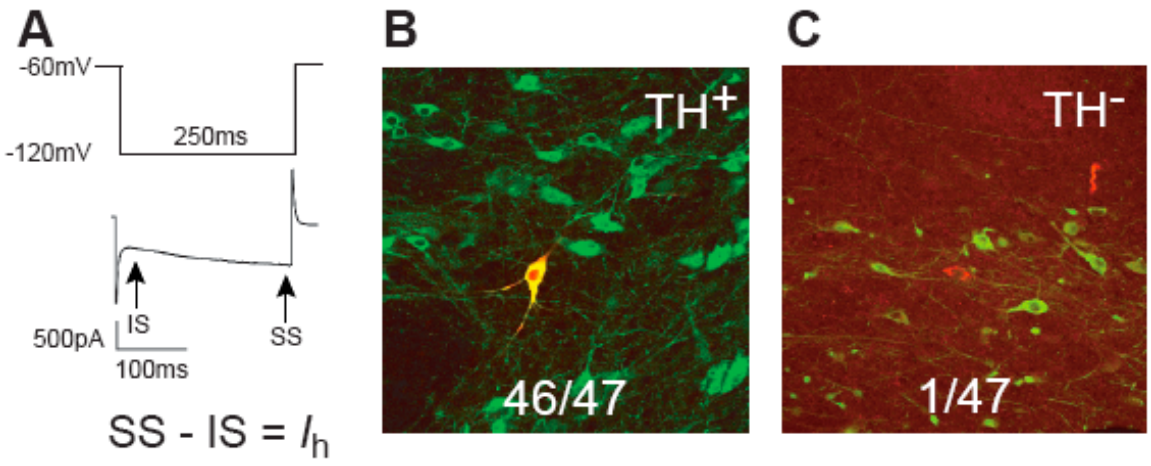

Figure 2-2: The presence of the I_h predicted dopamine content in mice. (A) Example neuron where a 250-ms hyperpolarizing voltage step from -60 mV to -120 mV elicited a slowly-developing inward current. The magnitude of I_h was calculated by subtracting the instantaneous current (IS) from the steady-state current (SS) achieved during the voltage step. (B) 46/47 recorded neurons (red) with I_h co-localized with tyrosine hydroxylase immunohistochemical staining (green). (C) 1/47 recorded neurons with I_h did not co-localize with tyrosine hydroxylase.

study has questioned the validity of this link in the rat (Margolis et al., 2006). Since mice were used in the present study, I examined whether I_h predicted dopamine content in this rodent species by using immunocytochemistry to assay for the presence of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. Unlike the rat, 98% of neurons (46/47) exhibiting an I_h in response to a hyperpolarizing voltage step also contained tyrosine hydroxylase, indicating that I_h is an excellent predictor of dopamine content in mice (Figure 2-2). Thus, for all subsequent experiments, I only recorded from neurons with I_h, since they presumably were dopaminergic.

CRF increased VTA dopamine neuron firing through the CRF-R1

Application of 1 μM CRF for 10 min, a dose previously shown to potentiate glutamate receptor currents in the VTA (Ungless et al., 2003), increased the firing rate of dopamine neurons (Figure 2-3). On average, CRF induced a 37.6 ± 5.1% increase over

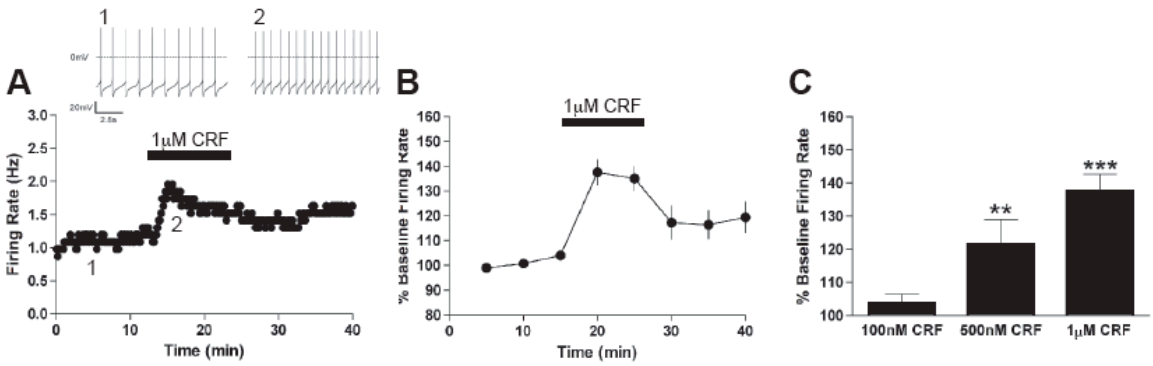

Figure 2-3: CRF increased the firing of VTA dopamine neurons. Example neuron (A) and average of 14 neurons (B) showing enhancement of VTA dopamine neuron firing by 10-min application of 1 μM CRF. (C) Significant increases over the baseline firing rate were found with 1 μM CRF (n = 14, *** $p < 0.001$) and with 500 nM CRF (n = 6, ** $p < 0.01$), but not with 100 nM CRF (n = 5).

baseline firing (Figure 2-3 B, n = 14, $p < 0.001$). A lower dose of CRF (500 nM) also significantly increased the firing by 21.5 ± 7.5% over baseline (Figure 2-3 C, n = 6, $p < 0.01$), while 100 nM CRF application was without effect on the firing rate (Figure 2-3 C, 3.8 ± 2.6% over baseline, n = 5, $p > 0.05$). All subsequent experiments used CRF at a concentration of 1 μM.

Either the CRF-R1 or the CRF-R2 receptor subtype could mediate the excitatory effect of CRF on VTA dopamine neurons, as both are present in the VTA (Ungless et al., 2003; Van Pett et al., 2000). The CRF-R1 agonist, ovine CRF (oCRF, 1 μM), significantly increased the firing rate (Figure 2-4 A,C, 29.6 ± 5.6% over baseline, n = 7, $p < 0.001$), while the CRF-R2 agonist, UCN II (1 μM), was without effect (Figure 2-4 B,C, 3.4 ± 3.0% over baseline, n = 7, $p > 0.05$), suggesting a primary role for the CRF-R1 in the CRF enhancement of VTA dopamine neuron firing. In agreement, 3 μM CP-154,526, a CRF-R1 antagonist, significantly reduced the maximal effect of CRF on the firing rate (Figure 2-5, 15.9 ± 4.6% over baseline, n = 7, $p < 0.05$ compared to CRF alone) as did 1

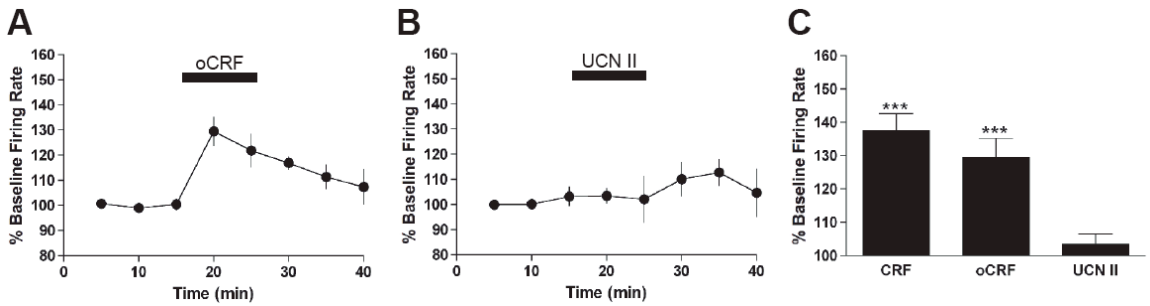

Figure 2-4: CRF-R1 but not CRF-R2 agonists increased VTA dopamine neuron firing. (A) The CRF-R1 agonist, oCRF (1 μM), increased the firing rate of VTA dopamine neurons (n = 7), while (B) the CRF-R2 agonist, urocortin II (UCN II, 1 μM) did not (n = 7). (C) Summary of the effects of CRF receptor agonists. *** $p < 0.001$ from baseline firing.

μM d-Phe-CRF, a non-specific CRF receptor antagonist (Figure 2-5, 9.5 ± 5.8% over baseline, n = 7, $p < 0.01$ to CRF alone). In contrast, 250 nM antisauvagine-30, a CRF-R2 antagonist, did not prevent the CRF-induced increase in VTA dopamine neuron firing (Figure 2-5, 30.4 ± 7.3% over baseline, n = 7, $p > 0.05$ to CRF alone). Previous work from our laboratory suggested that the CRF binding protein (CRF-BP) could participate in the cellular actions of CRF in the VTA (Ungless et al., 2003). However the CRF-BP antagonist CRF 6-33 (1 μM) did not alter the effect of CRF on the firing (33.0 ± 5.7% over baseline, n = 6, $p > 0.05$ compared to CRF alone).

Although both non-specific and CRF-R1 receptor antagonists significantly attenuated the effect of CRF on VTA dopamine neurons, they did not completely prevent the increase in firing by CRF. Thus, to avoid pharmacological concerns and to unequivocally demonstrate a CRF-R1 involvement in the CRF enhancement of firing, I examined the effect of CRF on dopamine neuron firing in mice deficient for the CRF-R1 or the CRF-R2. In the CRF-$R1^{+/+}$ mice, CRF robustly increased the firing (Figure 2-6 A,B, 38.6 ± 6.1% over baseline, n = 5, $p < 0.001$), while in the CRF-$R1^{+/-}$ mice, CRF

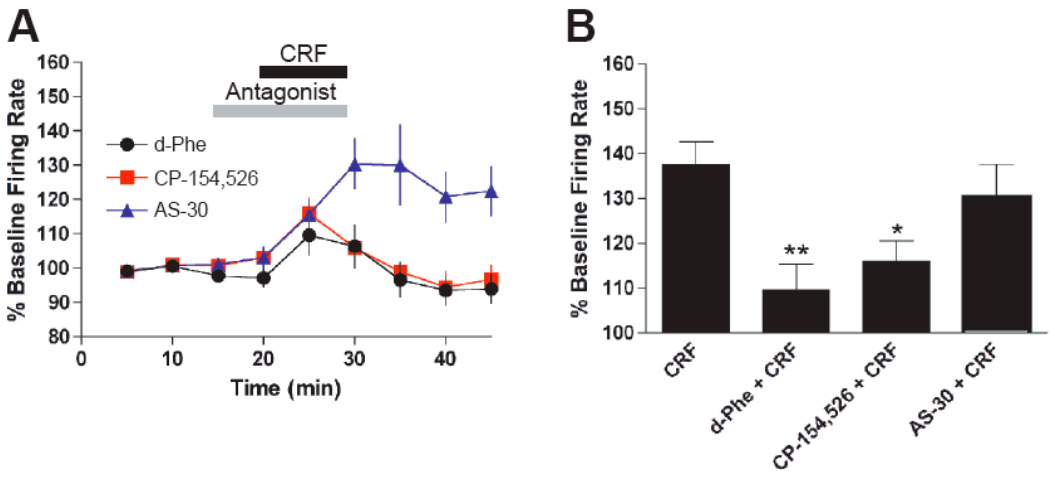

Figure 2-5: CRF-R1 antagonists attenuated the effect of CRF on VTA dopamine neuron firing. CRF receptor antagonists were applied for 5 minutes prior to and during CRF exposure. (A) The non-specific CRF receptor antagonist (d-Phe, 1 μM, black circles, n = 7) and the CRF-R1 antagonist (CP-154,526, 3 μM, red squares, n = 7), but not the CRF-R2 antagonist (antisauvagine-30, AS-30, 250 nM, blue triangles, n = 7) prevented the increase in firing by CRF. (B) Summary of the effects of CRF receptor antagonists. **, * are $p < 0.01$, $p < 0.05$ respectively.

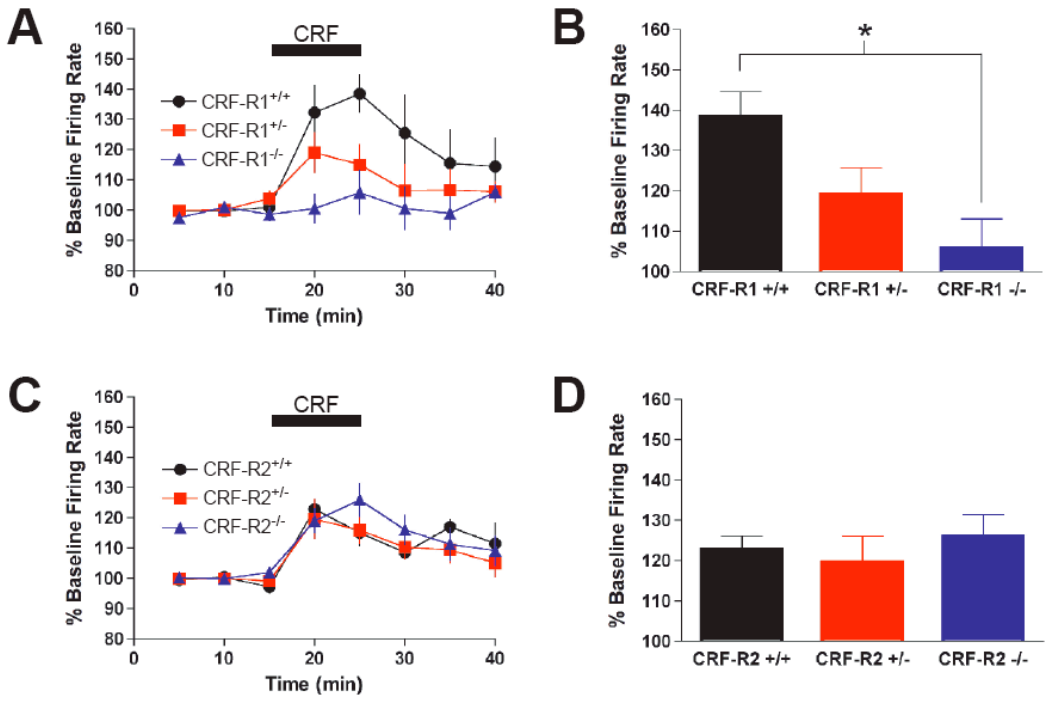

Figure 2-6: The effect of CRF on VTA dopamine neuron firing in mice deficient for the CRF-R1 or CRF-R2(A,B) CRF increased the firing of VTA dopamine neurons in CRF-R1$^{+/+}$ mice (black circles, n = 5) and in CRF-R1$^{+/-}$ mice (red squares, n = 6), though to a lesser degree than in CRF-R1$^{+/+}$ mice, and did not affect firing in CRF-R1$^{-/-}$ mice (blue triangles, n = 4). * $p < 0.05$ between CRF-R1$^{+/+}$ and CRF-R1$^{-/-}$ mice. (C,D) CRF enhanced the firing of VTA dopamine neurons to similar levels in CRF-R2$^{+/+}$ (black circles, n = 7), CRF-R2$^{+/-}$ (red squares, n = 10) animals, and CRF-R2$^{-/-}$ mice (blue triangles, n = 5).

induced a more modest though significant enhancement in the firing rate (Figure 2-6 A,B, 19.1 ± 6.6 % over baseline, n = 6, $p < 0.01$). Importantly, CRF had no effect on the firing rate in the CRF-R1$^{-/-}$ mice (Figure 2-6 A,B, 5.9 ± 7.2% over baseline, n = 4, $p < 0.05$ relative to CRF-R1$^{+/+}$). In addition, CRF augmented the baseline firing to similar levels in the CRF-R2$^{-/-}$ (Figure 2-6 C,D, 26.0 ± 5.5% over baseline, n = 7), the CRF-R2$^{+/-}$ mice (Figure 2-6 C,D, 19.6 ± 6.5% over baseline, n = 10), and the CRF-R2 $^{+/+}$ mice (Figure 2-6 C,D, 22.9 ± 3.2% over baseline, n = 5). Thus, both pharmacological and transgenic methods support a role for the CRF-R1, but not the CRF-R2 in the CRF enhancement of dopamine neuron firing.

CRF required the PLC-PKC pathway to increase VTA dopamine neuron firing

I next sought to determine the intracellular signaling pathway involved in the CRF-R1 mediated enhancement of firing in VTA dopamine neurons. Since CRF receptors predominately couple to the cAMP-PKA pathway in neuronal systems (Hauger et al., 2006), I first examined whether inhibitors of this pathway in the internal recording solution could prevent the effect of CRF. Surprisingly, CRF still increased dopamine neuron firing when cAMP signaling was blocked with 100 μM Rp-cAMPs, an inactive cAMP analog (Figure 2-7 A,C, 34.4 ± 11.1% over baseline, n = 8, $p > 0.05$ relative to control), or with 20 μM PKI, a PKA antagonist (Figure 2-7 A,C, 31.6 ± 3.8% over baseline, n = 7, $p > 0.05$ relative to control). Since the cAMP-PKA pathway was not required for CRF to increase VTA neuron firing, I next examined the involvement of the PLC-PKC pathway since CRF receptors also couple to this signaling cascade

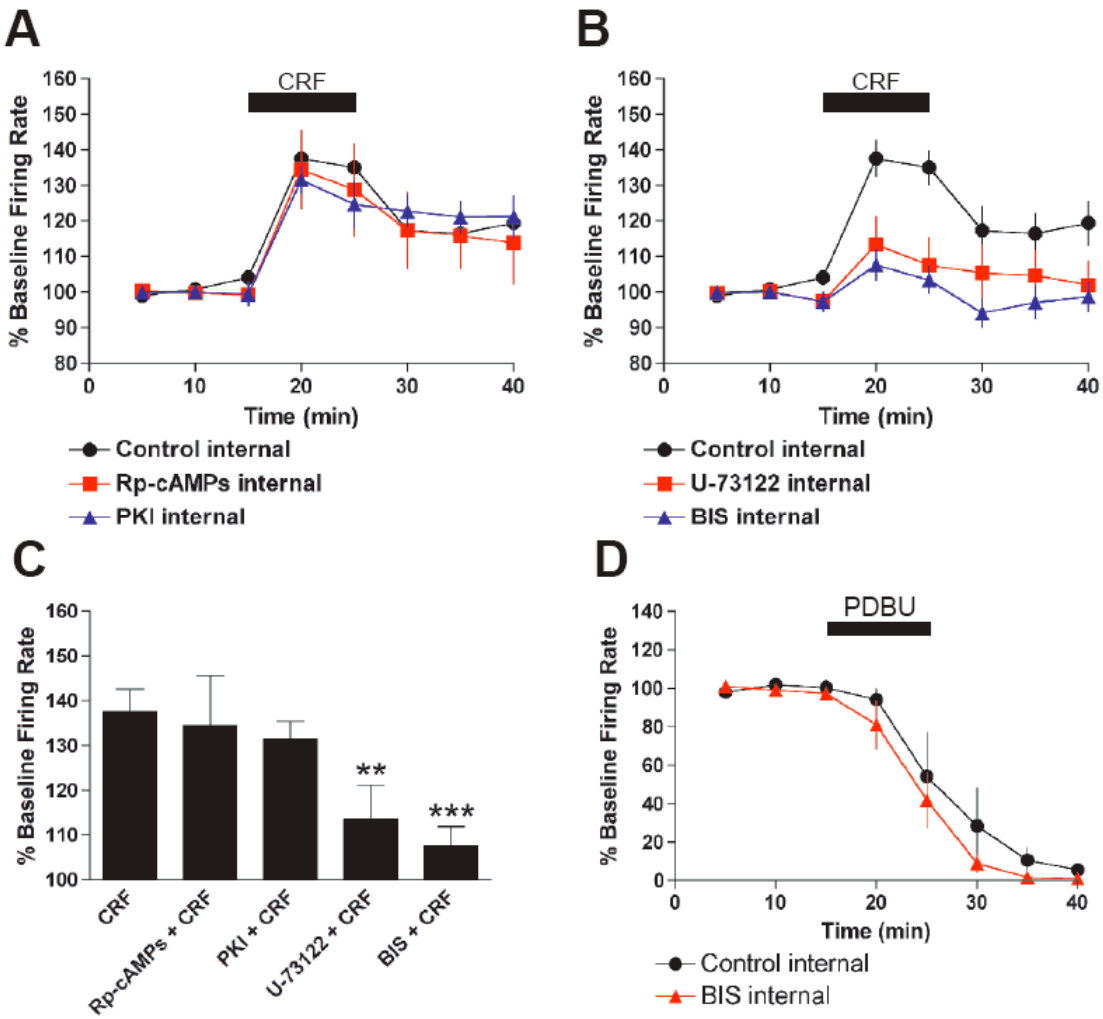

Figure 2-7: CRF required PLC and PKC activation to increase VTA dopamine neuron firing. Inhibitors to intracellular signaling pathways were included in the internal recording solution. (A) 100 μM Rp-cAMPs (red squares, n = 8), or 20 μM PKI (blue triangles, n = 7), did not prevent the effect of CRF on the firing of VTA dopamine neurons. (B) U-73122 (1 μM, red squares, n = 7) and BIS (1 μM, blue triangles, n = 8), both blocked the increase in VTA dopamine neuron firing by CRF. (C) Summary of the effects of various intracellular signaling pathway inhibitors on the CRF-mediated increase in firing. *** $p < 0.001$ relative to CRF alone. ** $p < 0.01$ relative to CRF alone. (D) 500 nM PDBU applied for 10 min eliminated the firing of VTA dopamine neurons (black circles, n = 4), even if PKC was blocked by 1 μM BIS in the internal solution, 500 nM PDBU still eliminated the firing in VTA dopamine neurons (red triangles, n = 4).

(Blank et al., 2003; Hauger et al., 2006; Ungless et al., 2003). Including the PLC antagonist, U-73122 (1 μM), in the internal recording solution significantly attenuated the effect of CRF on dopamine cell firing (Figure 2-7 B,C, 13.4 ± 7.8% over baseline, n = 7, $p < 0.05$ compared to CRF alone). Similarly, the general PKC antagonist,

bisindomaleimide (BIS, 1 μM) also significantly prevented the CRF-dependent increase in firing (Figure 2-7 B,C, 7.7 ± 4.2% over baseline, n = 8, $p < 0.01$ compared to CRF alone). Since PKC activity is required for CRF to increase VTA dopamine neuron firing, I tested if a direct PKC activator, phorbol 12,13-dibutyrate (PDBU), could similarly increase the firing rate. A 10 minute bath application of 500 nM completely abolished the firing (Figure 2-7 D, n = 4); however, this effect is likely due to non-specific effects of PDBU as the elimination of firing occurred when blocking PKC activity with 1 μM BIS in the recording solution. Although PDBU application did not recapitulate the effect of CRF on dopamine neuron firing rate, the experiments using inhibitors to PLC and PKC demonstrated a key role of this pathway in mediating the effects of CRF.

Both PKCδ (R Messing, personal communication) and PKCε (Jiang and Ye, 2003) isoforms are present in the VTA and could mediate the effects of CRF on firing rate. However, CRF still enhanced the firing of dopamine neurons in mice lacking PKCδ (Figure 2-8 A,C, 137.1 ± 12.0 % increase over baseline, n = 4) or PKCε (Figure 2-8 B,C, 124.8 ± 10.8% increase over baseline, n = 4). Although the specific PKC isoform(s)

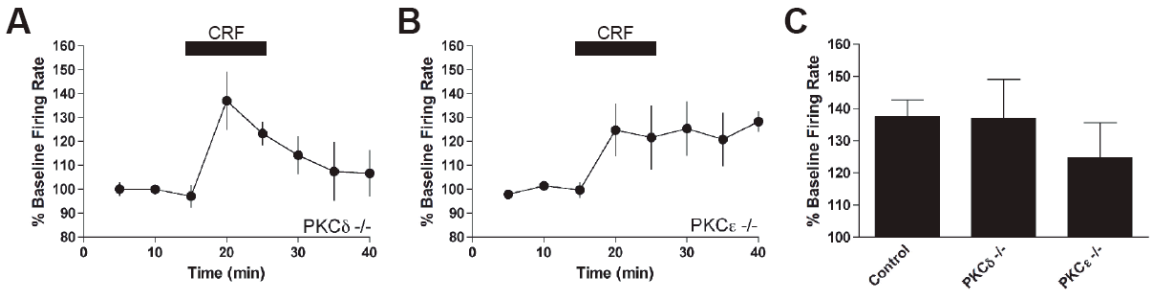

Figure 2-8: PKCδ and PKCε are not required for CRF to increase VTA dopamine neuron firing. CRF was able to increase VTA dopamine neuron firing in both (A) PKCδ−/− (n = 4) and (B)PKCε−/− (n = 4). (C) Summary of maximal changes by CRF in PKCδ-/- and PKCε-/- mice.

remains unknown, CRF activation of CRF-R1 stimulates the PLC-PKC signaling pathway to increase the firing rate of VTA dopamine neurons.

The role of various ionic conductances in the CRF-induced increase in VTA dopamine neuron firing

I next determined the ionic target through which CRF increased the firing rate of VTA dopamine neurons. The most pronounced alteration by CRF on the action potential dynamics was a significant reduction in the peak of the after-hyperpolarization potential (AHP) from -63.6 ± 0.2 mV during baseline firing to -58.6 ± 0.1 mV during CRF application (Figure 2-9 A, $n = 14$, $p < 0.001$). A number of ion currents can contribute to the generation of the AHP and regulate firing frequency including calcium-activated potassium currents ($I_{K(Ca)}$) (Sah, 1996), A-type potassium currents (I_A) (Hahn et al., 2003), inwardly-rectifying potassium currents (I_{Kir}) (Uchida et al., 2000), M-type potassium currents (I_M) (Koyama and Appel, 2006b), and I_h (Zolles et al., 2006).

Inhibiting calcium currents and $I_{K(Ca)}$ by including 10 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N,N-tetraacetic acid (BAPTA) (Tozzi et al., 2003) in the recording solution did not prevent CRF from increasing neuronal firing (Figure 2-9 B, 40.9 ± 15.4% over baseline, $n = 8$, $p > 0.05$ compared to CRF alone). Thus, although changes in $I_{K(Ca)}$ can alter the AHP and firing frequency (Sah, 1996), the CRF-R1

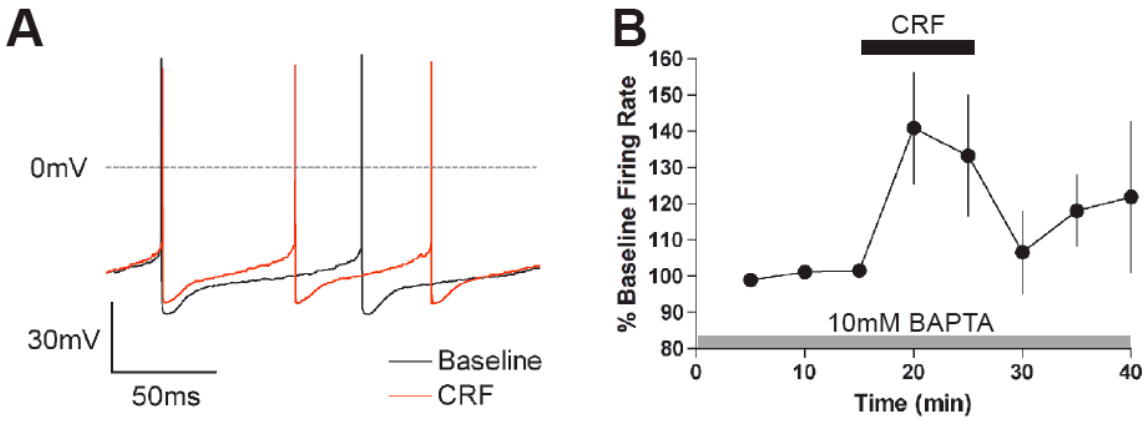

Figure 2-9: CRF reduced the AHP of VTA dopamine neurons but calcium currents are not required for CRF to increase the firing rate. (A) Overlay of action potentials from an example VTA dopamine neuron during baseline (black) and CRF application (red) that demonstrates CRF inhibition of the AHP. (B) 10 mM BAPTA in the recording solution did not alter the effect of CRF on VTA neuron firing (n = 8)

mediated increase in the firing rate was independent of changes in $I_{K(Ca)}$.

Since calcium currents and $I_{K(Ca)}$ are not required for CRF to increase VTA dopamine neuron firing, the likely candidates for the CRF-dependent modulation of the AHP are potassium currents or the I_h. In an initial set of experiments I examined whether tetraethylammonium chloride (TEA), a general blocker of numerous voltage-activated potassium currents (Coetzee et al., 1999), could occlude the effect of CRF on firing rate. Inhibition of potassium currents of the AHP should not only reduce the magnitude of the AHP, but also increase the firing rate provided the neuron does not enter depolarization block. Surprisingly, application of 1 mM TEA (10 minutes prior to and during CRF application) did not affect the firing rate (Figure 2-10 A), but did significantly attenuate the effect of CRF on VTA dopamine neurons (Figure 2-10 B,F, n = 7, 15.7 ± 2.3% increase over baseline, $p < 0.01$ relative to CRF alone). Unlike with 1 mM TEA, a higher dose of TEA (10 mM) increased the firing rate of VTA dopamine neurons (Figure 2-10 C,D). 10 mM TEA also prevented the increase in firing by CRF when examining the

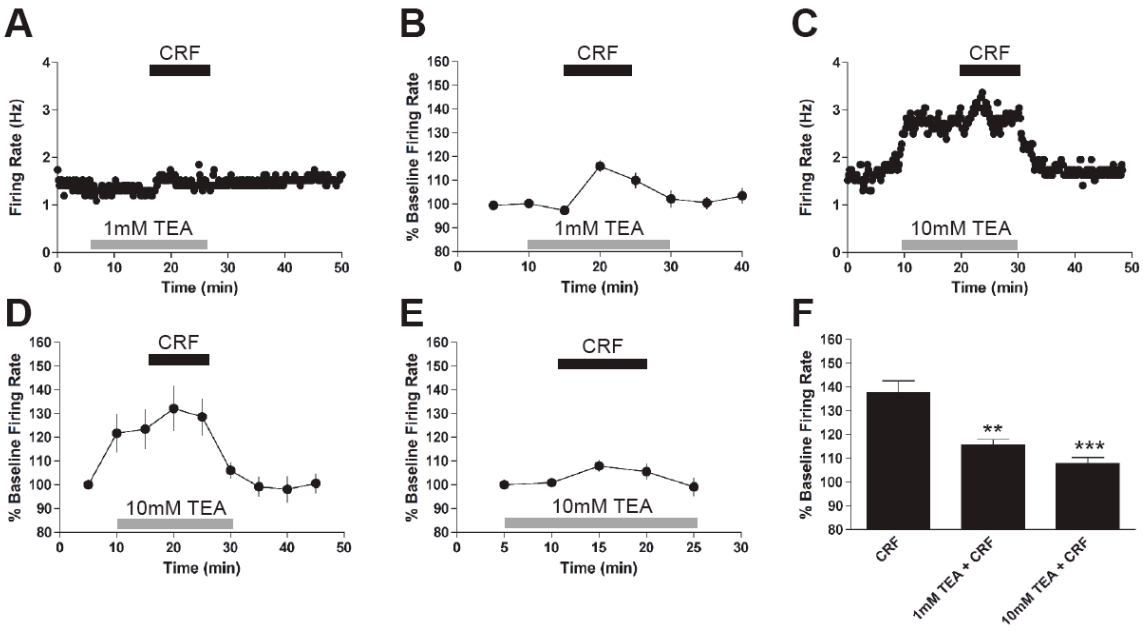

Figure 2-10: TEA attenuated the effect of CRF on VTA dopamine neuron firing. Example (A) and average of 7 neurons (B), demonstrating that 1 mM TEA did not affect firing rate, but reduced the effect of CRF. Example (C) and average of 7 neurons (D), illustrating that 10 mM TEA increases dopamine neuron firing, but also attenuated the effect of CRF. (E) Shows the same data in (D), but used the the elevated firing during 10 mM TEA application as the baseline. (F) Summary of the effects of TEA on the CRF-mediated increase in VTA dopamine neuron firing. ** $p < 0.01$, *** $p < 0.001$ relative to CRF alone.

changes in firing due to CRF relative to the elevated firing during TEA application (Figure 2-10 D,F, n = 7, 8.0 ± 2.5 % increase in firing, $p < 0.01$ relative to CRF alone). These results suggest a voltage-activated potassium current is involved with the CRF-mediated increase in VTA dopamine neuron firing; however, caution should be taken when interpreting these findings since TEA is a non-specific potassium blocker that may induce ionic conductance changes that indirectly preclude the effects of CRF on the firing rate. For this reason, I assayed if specific potassium current antagonists attenuated the enhancement of firing by CRF.

I_A currents are present in VTA dopamine neurons and can regulate the frequency of cell firing, making it another potential target for CRF (Hahn et al., 2003; Koyama and Appel, 2006a). Blocking the slow I_A with 10 μM 4-aminopyridine (4-AP) modestly

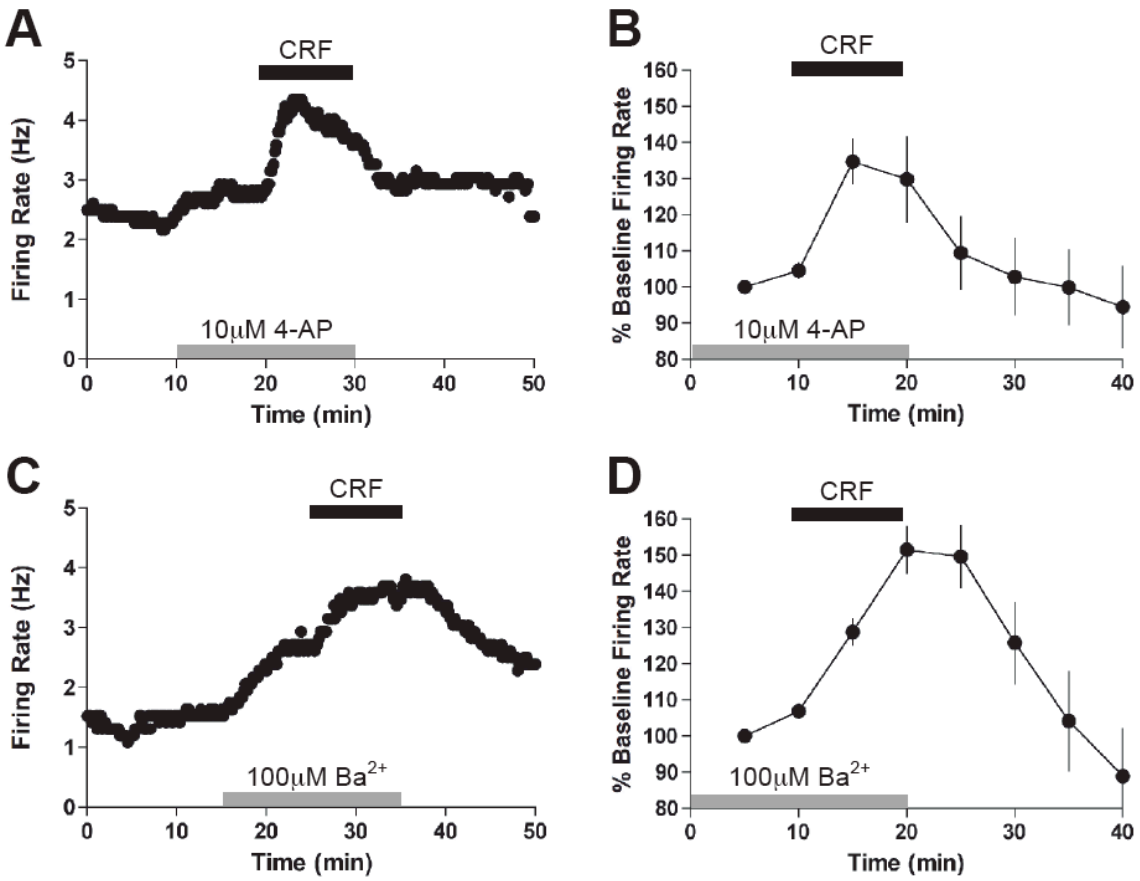

Figure 2-11: The slow I_A and I_{Kir} are not required for CRF to increase VTA dopamine neuron firing. (A) Example VTA neuron recording showing that blocking slow I_A with 10 μM 4-AP increased the firing, but did not occlude a further enhancement in firing by CRF. (B) Using the elevated firing rate during 4-AP application as a baseline, CRF increased the firing to a similar extent as under control conditions (n = 6). (C) Example VTA neuron recording demonstrating that inhibiting I_{Kir} with 300 μM Ba^{2+} increased the firing, but did not prevent a further enhancement in firing by CRF. (D) Using the elevated firing rate during the Ba^{2+} application as a baseline, CRF increased the firing to a similar extent as under control conditions (n = 8).

increased neuron firing, but did not prevent a further enhancement in firing by CRF during 4-AP application (Figure 2-11 A,B, 34.8 ± 6.3% over firing rate during 4-AP application, n = 6, $p > 0.05$ compared to CRF alone). 4-AP at higher concentrations (30 μM, 60 μM, and 100 μM) similarly increased the firing and did not attenuate the effect of CRF (increase over firing rate during 4-AP application for 30 μM: 137.7 ± 9.9%., n = 9; 60 μM: 161.6 ± 12.9%, n = 9; 100 μM: 127.8 ± 7.1, n = 5). With 4 mM 4-AP, a dose that

eliminates both fast and slow I_A (Lien et al., 2002), the spontaneous firing of VTA dopamine neurons was drastically reduced and often eliminated, preventing analysis of the CRF-mediated effects on firing. These experiments demonstrated that the slow I_A was not required for CRF to increase VTA dopamine neuron firing.

Next, I investigated the possibility that CRF increased the firing of dopamine neurons through inhibition of $I_{Kir.}$ Activation of the I_{Kir} reduces dopamine cell firing (Lacey et al., 1987; Uchida et al., 2000; Werner et al., 1996), and as expected, inhibition of I_{Kir} with 100 μM barium (Ba^{2+}) increased the firing rate in VTA dopamine neurons (Figure 2-11 C). However, similar to control conditions, CRF significantly increased the firing during Ba^{2+} application (Figure 2-11 C,D, 51.5 ± 6.5% over firing rate during Ba^{2+} application, n = 8, $p > 0.05$ compared to CRF alone). Thus, CRF did not require I_{Kir} to increase dopamine neuron firing.

The above experiments suggest CRF affected a potassium conductance that is sensitive to TEA, insensitive to 4-AP, and modulates both the AHP and firing frequency of neurons. The I_M is another potential target as this conductance fits this pharmacological profile (Coetzee et al., 1999), and regulates both the AHP and the inter-spike interval between action potentials in VTA dopamine neurons (Koyama and Appel, 2006b). Interestingly, application of the specific I_M channel blocker (10 μM XE-991) did not affect the firing of dopamine neurons (Figure 2-12 A,B), though this treatment significantly reduced the CRF-mediated increase in firing (Figure 2-12 B,C, n = 6, 11.3 ± 8.4% over baseline firing, $p < 0.01$ relative to CRF alone). If CRF primarily increased VTA dopamine firing through inhibition of I_M, then XE-991 should mimic the effect of

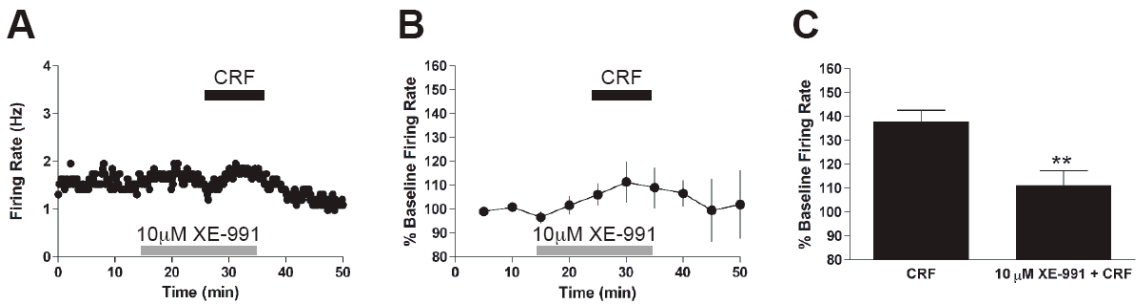

Figure 2-12: The I_M may be involved with CRF increasing VTA dopamine neuron firing. Example neuron (A) and average of 6 neurons (B) demonstrating that inhibition of I_M with 10 μM XE-991 did not affect VTA dopamine neuron firing by itself, but attenuated the CRF-mediated increase in firing. (C) Summary of the effect of I_M inhibition. ** $p < 0.01$ relative to CRF alone.

CRF, but this result was not observed. Thus, I_M could be involved with mediating the increase in firing rate through its interaction with another conductance, or perhaps XE-991 prevented the increase in firing by CRF through a non-specific pharmacological mechanism.

CRF required the I_h to increase VTA dopamine neuron firing

CRF increased VTA dopamine neuron firing independent of changes in $I_{K(Ca)}$, slow I_A, or $I_{Kir.}$ The I_h also can affect the AHP and dopamine cell firing (Neuhoff et al., 2002; Zolles et al., 2006), and could also be modulated by CRF (Qiu et al., 2005). Application of the I_h inhibitor ZD-7288 (30 μM) enhanced the AHP in VTA dopamine neurons (Figure 2-13 A). Although 30 μM ZD-7288 decreased the firing in 6/6 neurons tested, the neurons did not always attain a stable firing rate with this high dose. In order to record from stable firing neurons I pre-incubated the brain slices (> 20 minutes) and continuously applied a lower dose of ZD-7288 (1 μM). All recorded neurons lacked I_h due to the pharmacological block. Since I could not use the presence of I_h as an

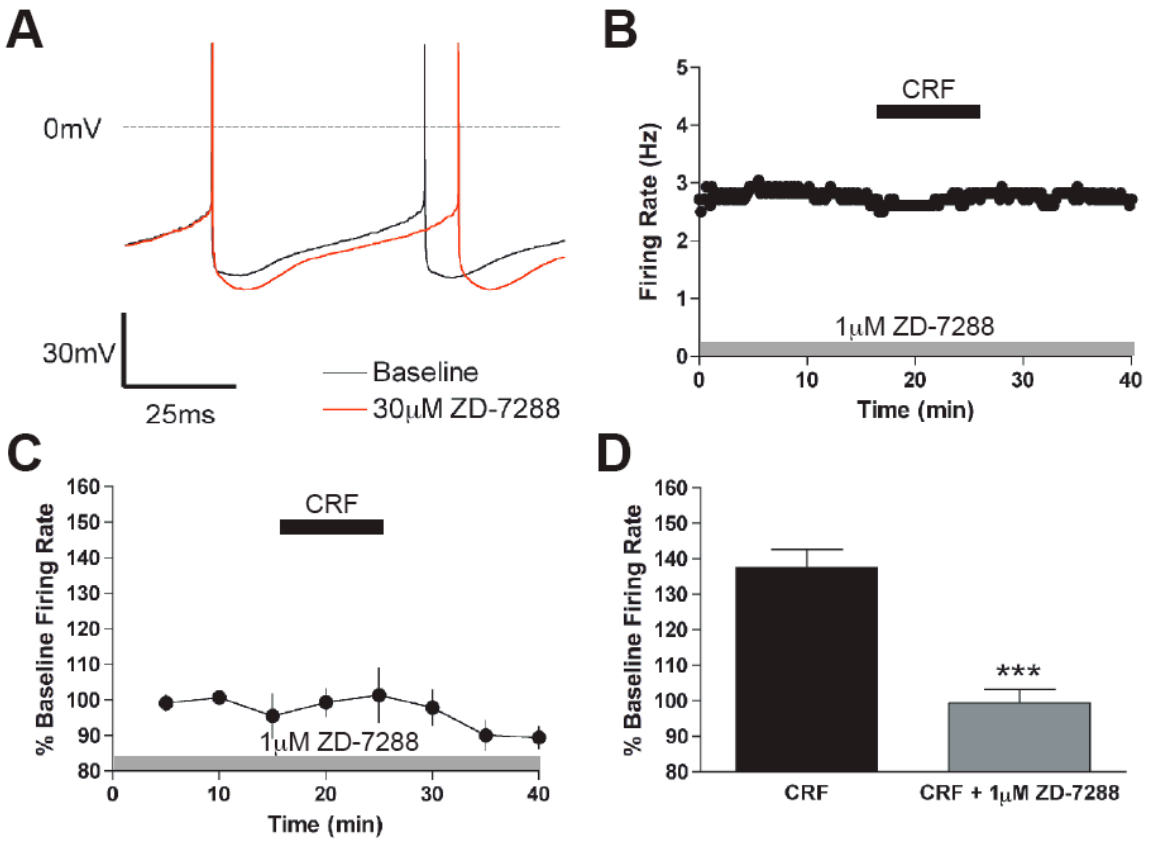

Figure 2-13: The I_h is required for CRF to increase VTA dopamine neuron firing. (A) Overlay of action potentials from an example VTA dopamine neuron during baseline (black) and 30 μM ZD-7288 application (red) that highlights the increase of the AHP after I_h inhibition. Example (B) and summary of 5 neurons (C,D) demonstrating that I_h inhibition with continuous application of 1 μM ZD-7288 prevented the increase in firing rate by CRF in VTA dopamine neurons. *** $p < 0.001$ relative to CRF alone.

electrophysiological dopamine marker, I verified that the neurons hyperpolarized in response to application of 50 μM dopamine, which is indicative of an auto-inhibitory response found in dopamine neurons (Lacey et al., 1987). When I_h was blocked, CRF did not enhance the firing rate in putative VTA dopamine neurons (Figure 2-13 B-D, -0.7 ± 3.8% over baseline, $n = 5$, $p < 0.001$ relative to CRF alone), suggesting that CRF modulated I_h to increase the firing rate. Thus, CRF required activation of the CRF-R1, stimulation of the PLC-PKC pathway, and the I_h to increase VTA dopamine neuron firing.

Discussion:

The major findings from these experiments are that (i) the I_h reliably predicted dopamine content in the mouse and that (ii) CRF enhanced VTA dopamine neuron firing involving the CRF-R1, stimulation of the PLC-PKC pathway, and the I_h. The VTA is a heterogeneous brain region in the rat containing dopamine and GABA, as well as glutamate neurons (Carr and Sesack, 2000a; Carr and Sesack, 2000b; Yamaguchi et al., 2007). The presence of the I_h is often used to identify dopamine neurons in rodents (Cameron et al., 1997; Ford et al., 2006; Grace and Onn, 1989); however, a recent study in rats found that only ~50% of VTA neurons with I_h contained TH (Margolis et al., 2006). I examined whether the I_h predicted dopamine content in mice since this was the rodent system I used because of the availability of CRF receptor transgenic mice and the fact that CRF potentiates glutamate receptor currents in mice (Ungless et al., 2003). Unlike the rat, 98% of neurons with I_h also contained TH, indicating that the presence of the I_h is an excellent indicator of dopamine content in mice.

Since I could reliably identify VTA dopamine neurons, I used whole-cell patch clamp recordings to assay the firing of the neurons. One issue with these experiments was the potential for the dialysis of important intracellular components using whole-cell recordings, as opposed to perforated-patch recordings. However, using whole-cell recordings allowed for the administration of intracellular signaling pathway inhibitors directly into the recorded the cell, which would provide further evidence that the effect of CRF is cell-autonomous.

Other groups studied the effects of CRF using lower doses in other brain regions (Aldenhoff et al., 1983; Jedema and Grace, 2004; Liu et al., 2005a), however, I chose to use 1 μM CRF for most experiments since this dose potentiates NMDAR currents (Ungless et al., 2003) and produced a maximal increase firing in VTA dopamine neurons. CRF, as well as agonists of the CRF-R1, increased dopamine cell firing in the VTA, while CRF-R2 agonists were without effect. Similarly, only non-specific CRF receptor and CRF-R1 antagonists significantly attenuated the enhanced firing by CRF, though their effect was incomplete. Perhaps a longer pre-exposure to the antagonists before CRF application would have completely eliminated the excitatory role of CRF. To unequivocally demonstrate involvement of the CRF-R1 and to avoid pharmacological concerns with the antagonists, I addressed the effect of CRF on VTA dopamine firing using CRF receptor-deficient mice. The increase in firing by CRF was completely prevented in the CRF-R1$^{-/-}$ mice, modestly inhibited in the CRF-R1$^{+/-}$ mice, and unaffected in the CRF-R1$^{+/+}$ mice. Conversely, CRF was able to augment firing in CRF-R2$^{+/+}$, CRF-R2$^{+/-}$, and CRF-R2$^{-/-}$ mice to similar levels. It should be noted that the increase in firing in the CRF-R2 transgenic mice was lower relative to the CRF-R1 transgenic and control C57BL/6 mice, though this could possibly be due to background genetic differences. Together, the findings from these experiments using CRF receptor agonists, CRF receptor antagonists, and CRF receptor-deficient mice are parsimonious with the conclusion that CRF increases the firing rate of VTA dopamine neurons through activation of the CRF-R1.

CRF receptors couple primarily to the cAMP-PKA pathway, but can also activate the PLC-PKC signaling cascade (Blank et al., 2003; Hauger et al., 2006; Nie et al., 2004; Ungless et al., 2003). Although CRF stimulates the cAMP-PKA pathway to increase neuron firing in the locus coeruleus (Jedema and Grace, 2004) and the hippocampus (Aldenhoff et al., 1983; Haug and Storm, 2000), this pathway is not involved in the VTA. Instead, I found that inhibition of PLC or PKC prevented CRF from increasing VTA dopamine neuron firing. I posited that application of PDBU, a PKC activator, would mimic the effect of CRF on firing. Unfortunately this hypothesis could not be tested directly since PDBU eliminated the firing through non-specific effects of the drug. To further identify the mechanism, I assayed for the roles of various PKC isoforms in mediating the effect of CRF on VTA dopamine neuron firing. Both PKCδ (R Messing, personal communication) and PKCε (Jiang and Ye, 2003) are present in the VTA, but these PKC isoforms are not critical for CRF to enhance dopamine neuron firing, as the lack of these proteins did not prevent the excitatory role of CRF. Thus, CRF-R1 activation stimulated the PLC-PKC pathway to increase dopamine firing.

Since CRF reduced the AHP of the action potential, I hypothesized that an ionic conductance involved with the generation of the AHP was affected by CRF. TEA reduced the effect of CRF on VTA dopamine neuron firing, suggesting a voltage-activated potassium current is modulated by CRF. These results are difficult to interpret though since TEA is a non-specific potassium blocker that could induce ionic conductance changes that indirectly preclude the effects of CRF on the firing rate. In this regard, it was necessary to assay the effects of more specific potassium channel

antagonists on the ability of CRF to increase dopamine neuron firing. Although CRF excites CA3 pyramidal hippocampal neurons by reducing the AHP through inhibition of $I_{K(Ca)}$ (Aldenhoff et al., 1983; Haug and Storm, 2000), this mechanism did not account for the effect in VTA dopamine neurons. Both the I_{Kir} and I_A are potential ionic targets for CRF since they regulate firing frequency and contribute to the AHP in dopamine neurons (Hahn et al., 2003; Koyama and Appel, 2006a; Lacey et al., 1987; Uchida et al., 2000). Additionally, CRF excites corticotropes by inhibiting I_{Kir} (Kuryshev et al., 1997). Blocking the I_{Kir} or the slow I_A predictably increased the firing of VTA dopamine neurons, but did not prevent the effect of CRF on firing. These findings along with the experiments demonstrating BAPTA did not prevent the effect of CRF indicate that the $I_{K(Ca)}$, I_{Kir} and slow I_A are not required for CRF to increase VTA dopamine neuron firing even though alterations in these conductances can modulate firing frequency. Surprisingly, inhibition of I_M with XE-991 attenuated the CRF-mediated enhancement in firing, but solely blocking I_M did not change the firing frequency. These results suggest I_M could be involved with CRF increasing dopamine neuron firing through some complex interaction with another conductance or that XE-991 mediated is actions through non-specific pharmacological means.

The HCN2, HCN3, and HCN4, but not the HCN1 are present in dopamine neurons and are the channel subunits responsible for the I_h, although the subunit composition is unknown (Franz et al., 2000). The I_h can influence the firing and magnitude of the AHP in SN dopamine neurons (Neuhoff et al., 2002). I found that the I_h also regulates firing and the AHP in VTA dopamine neurons, in contrast to a previous

study using P12-15 mice (Neuhoff et al., 2002), though the discrepancy is likely due to developmental differences since I recorded from 3-5 week-old mice. Importantly, the CRF-induced firing enhancement in VTA dopamine neurons was abolished when I_h was blocked. Although other ion currents may be altered by CRF in VTA dopamine neurons, any potential alterations in other conductances are likely not critically involved with CRF increasing the firing rate. In summary, CRF stimulated the CRF-R1, activated the PLC-PKC signaling pathway, and required the I_h, to increase VTA dopamine neuron firing.

Acknowledgements:

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Chapter 3. CRF enhanced the I_h through a PKC-dependent mechanism

Abstract:

Stress not only stimulates the release of CRF onto dopamine cells of the VTA, but also increases dopamine levels in brain regions receiving dense VTA input. Although CRF and dopamine modulate numerous behaviors, their interaction is not well understood. Consistent with an excitatory role on the dopamine system, I found that CRF increased the spontaneous firing rate of VTA dopamine neurons and involved the CRF-R1, the PLC-PKC pathway, and the I_h. The I_h is modulated by cAMP, PKA, serine-threonine protein kinases, pH, and phosphoinositides; however, no studies have characterized a PKC-dependent enhancement of the I_h. Based on the mechanism by which CRF increases VTA dopamine neuron firing, I hypothesized that CRF increases the I_h function through a PKC-dependent mechanism. Using whole-cell patch clamp recordings of VTA dopamine neurons, I demonstrated that CRF depolarizes neurons and this effect required I_h. CRF and PKC activators both increased the total I_h without changing the voltage-dependence of activation for the I_h. Finally, PKC antagonism prevented the effect of CRF on the I_h. Collectively, these experiments identify that CRF activated a novel PKC-dependent increase in the I_h that likely is responsible for CRF enhancing VTA dopamine neuron firing.

Introduction:

Stress causes a variety of physiological changes including (i) the release of CRF throughout the brain, including onto the dopamine cells of the VTA (Hauger et al., 2006; Wang et al., 2005) and (ii) the release of dopamine in brain regions receiving dense VTA input (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996). Given the role of CRF and dopamine in drug-seeking behaviors (McFarland et al., 2004; Wang et al., 2005), as well as in escape from stressful/ threatening situations (Blanchard et al., 1999; Griebel et al., 1998; Yang et al., 2006), the interactions between stress, CRF, and dopamine likely have important behavioral consequences. As described in Chapter 2, CRF increased VTA dopamine neuron firing through a mechanism involving the CRF-R1, the PLC-PKC signaling pathway, and the I_h. However, it is unknown how CRF affects the I_h directly in these neurons and if it involves the same mechanism to increase the firing rate.

Similar to other voltage-gated ion currents, the I_h arises from ion flux through a tetrameric channel containing a combination of the four cloned channel subunits (HCN1-4) (Frere et al., 2004). Originally referred to as I_f (funny current) in the heart or I_q (queer current) in the brain, the I_h is found in a variety of tissues (Frere et al., 2004; Pape, 1996). The HCN channels are permeable to both Na^+ and K^+, but even though the permeability ratio is ~ 0.2-0.4 (pNa^+/pK^+), most of the current through the HCN channels are Na^+ ions within normal physiological voltages and ionic concentrations, and as such will be depolarizing upon activation (Frere et al., 2004; Pape, 1996). The I_h has four primary roles in neuronal tissues: (i) generating pacemaker action potential firing

(McCormick and Pape, 1990), (ii) modulating resting potential (Magee, 1998), (iii) controlling dendritic summation (Magee, 1998), and (iv) regulating neurotransmitter release (Mellor et al., 2002). Although the I_h is commonly thought to generate rhythmicity and pacemaker-like firing, this is not true for all cells types studied (Frere et al., 2004). Specifically with dopamine neurons, the I_h can regulate firing frequency (Zolles et al., 2006), but is not required for pacemaker firing (Puopolo et al., 2005).

The I_h is not only regulated by voltage, but also by intracellular signaling molecules and kinases (Frere et al., 2004). The most documented modulation of I_h is by cAMP, which binds to the cytosolic C-terminal cyclic nucleotide-binding domain (CNBD) of the HCN subunits (Frere et al., 2004). Binding of cAMP to the CNBD is capable of shifting the voltage-dependence of activation for the I_h (DiFrancesco and Tortora, 1991). Additionally, protein kinases such as src (Zong et al., 2005) and PKA (Vargas and Lucero, 2002) can modulate the kinetics and gating properties of I_h activation. Recently findings from a number of laboratories demonstrated that phosphoinositides positively regulate the voltage-dependence of activation for the I_h likely through allosteric mechanisms (Fogle et al., 2007; Pian et al., 2006; Zolles et al., 2006). Little is known regarding the PKC modulation of I_h, though a couple of studies suggest that PKC activation can inhibit I_h (Cathala and Paupardin-Tritsch, 1997; Liu et al., 2003).

Since CRF required PKC activity and the I_h to increase VTA dopamine neuron firing, I hypothesize that CRF enhances I_h function through a PKC-dependent mechanism. Using whole-cell patch clamp recordings of VTA dopamine neurons, I

demonstrated that CRF and the PKC activator, PDBU, both stimulated a PKC-dependent increase in I_h that did not involve changes in the voltage-dependence of activation for the I_h. Conversely, PKC antagonism prevented the effect of CRF on the I_h. The findings from these experiments highlight a novel PKC-dependent modulation of I_h that likely is the mechanism by which CRF increased VTA dopamine neuron firing.

Materials and Methods:

Please refer to the corresponding section in Chapter 2 for specific details. Briefly, firing experiments in current-clamp recording mode used an internal recording solution of 130 mM KOH, 105 mM methanesulfonic acid, 17 mM HCl, 20 mM HEPES, 0.2 mM EGTA, 2.8 mM NaCl, 2.5 mg/mL Mg-ATP, and 0.25 mg/mL Mg-GTP. Original attempts with voltage-clamp recordings using this internal solution were unsuccessful, as stable recordings over time were difficult to maintain; thus, a cesium-based internal solution was used, containing: 117 mM cesium methanesulfonate, 20 mM HEPES, 0.4 mM EGTA, 2.8 mM NaCl, 5 mM TEA-Cl, 2.5 mg/mL Mg-ATP, and 0.25 mg/mL Mg-GTP. For the standard isolation of the I_h in this study, I used the basic aCSF (refer to Chapter 2 Material and methods) and hyperpolarized the neuron for 500 ms in 10 mV increments to -120 mV from the holding potential of -60 mV to elicit the I_h, which was calculated by subtracting the instantaneous current from the steady-state current during the voltage step. Slightly different recording conditions were used for isolating the I_h tail current. Specifically, I included 500 nM TTX, 10 mM TEA, 4 mM 4-AP, and 300 μM Ba^{2+} in the standard aCSF while hyperpolarizing the neuron for 1 s in 10 mV increments to -130 mV

from the holding potential of -60 mV. The elicited tail current arising from the offset of the voltage step was normalized to the tail current from the -130 mV step and fitted to a Boltzmann curve. Application of the modified aCSF used for I_h tail current experiments reduced the viability of the brain slices and recorded neurons (personal observations), and as such prevented long-duration experiments examining the time-course of CRF-mediated changes on I_h. Thus, the standard protocol was used to assay the time-dependent changes in I_h, while the tail current protocol was used to examine changes in the voltage-dependence of activation for the I_h. As will be presented below, the effect of CRF on the total I_h was observed with both standard and tail-current protocols.

Results:

CRF required I_h to depolarize VTA dopamine neurons

CRF required the I_h to increase VTA dopamine neuron firing (Chapter 2), but the direct effect of CRF on I_h in these neurons is unknown. Because I_h contributes to the resting membrane potential (Magee, 1998), and since the HCN subunits primarily allow passage of Na^+ ions within physiological voltage ranges (Frere et al., 2004), activation of the I_h depolarizes neurons (Qiu et al., 2005). In this regard, I examined the effect of CRF on the membrane potential and if any changes were dependent upon the I_h. To ensure only healthy neurons were studied, I recorded from spontaneously firing mouse VTA dopamine neurons. Application of 500 nM tetrodotoxin (TTX) blocked voltage-dependent Na^+ channels and abolished dopamine neuron firing. Under these conditions, CRF induced a small but significant depolarization (Figure 3-1 A, 1.9 ± 0.2 mV, n = 6, p

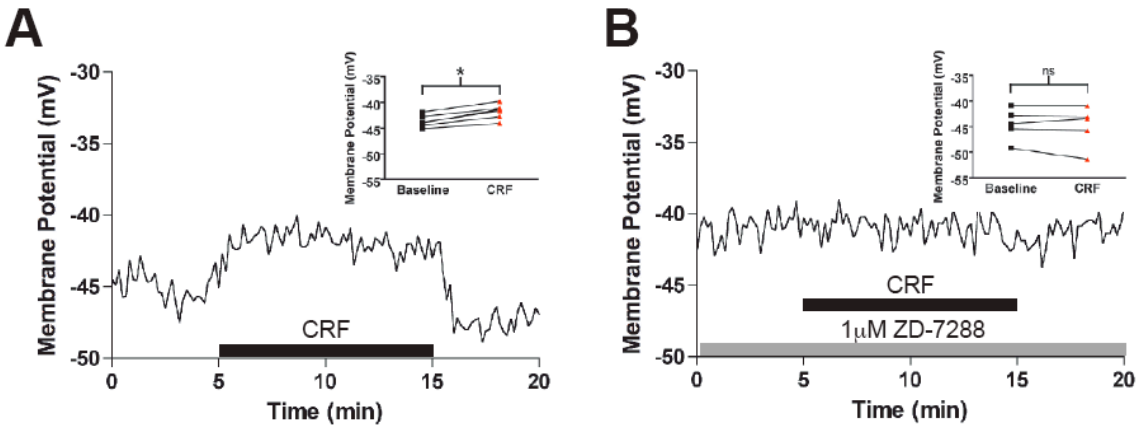

Figure 3-1: CRF required I_h to depolarize VTA dopamine neurons. (A) An example VTA dopamine neuron showing depolarization during CRF application in the presence of 500 nM TTX. Inset shows significant depolarization by CRF in 6 tested neurons. * $p < 0.05$. (B) When I_h was blocked, the membrane potential was not changed by CRF in an example VTA neuron. Inset shows the changes in membrane potential by CRF with I_h inhibition (n = 5).

< 0.05). Incubating the brain slices (> 20 minutes) and continuously applying 1 μM ZD-7288 eliminated the I_h. Since I could not use the presence of I_h as an electrophysiological dopamine marker, I verified that the neurons hyperpolarized in response to application of 50 μM dopamine, which is indicative of an auto-inhibitory response found in dopamine neurons (Lacey et al., 1987). When I_h was pharmacologically inhibited, CRF was unable to affect the membrane potential (Figure 3-1 B, -0.3 ± 0.5 mV, n = 5, $p > 0.05$), demonstrating that CRF required I_h to depolarize VTA dopamine neurons.

CRF increased the I_h independent of changes in the voltage-dependence of activation

Since VTA dopamine neurons are depolarized by CRF in a mechanism involving the I_h, I next examined the direct effect of CRF on I_h. A hyperpolarizing current injection (250 pA, 2s) elicited a current sag due to I_h activation (Neuhoff et al., 2002), which was

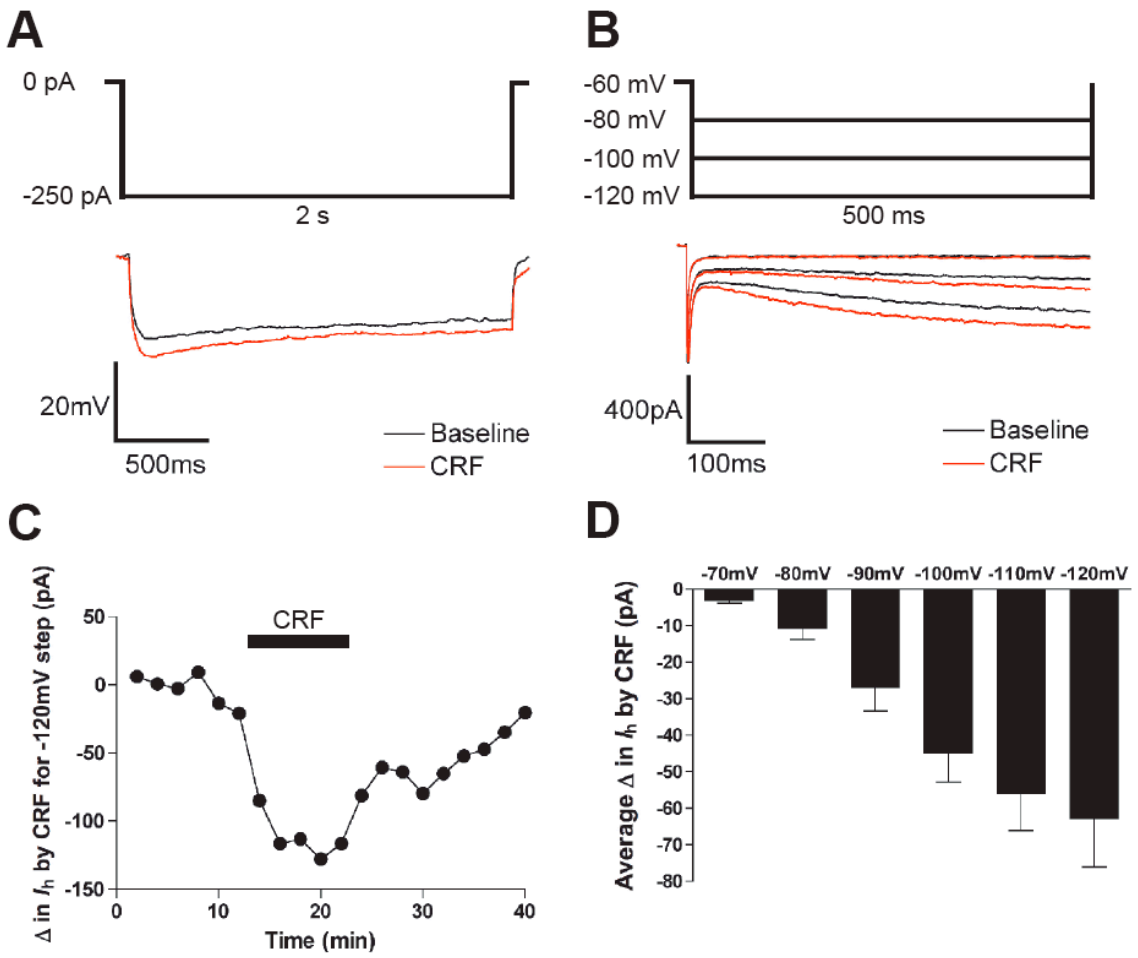

Figure 3-2: CRF enhanced the I_h. A) Hyperpolarizing current injection (2 s, 250 pA) elicited a current sag that represents activation of I_h (black), which is enhanced by CRF (red). (B) Hyperpolarizing voltage steps (500 ms) from a holding potential of -60 mV to -80, -100, and -120 mV activates the slowly-developing I_h (black), which is increased by CRF (red). (C) The increase in the I_h by CRF is reversible and follows a similar time course as the effect of CRF on the firing rate (same neuron as in B). (D) Summary of the CRF enhancement of I_h at all voltage steps tested (n = 7).

clearly enhanced by CRF (Figure 3-2 A). In addition, using voltage-clamp recordings, I measured I_h generated in response to hyperpolarizing the neuron for 500 ms in 10 mV increments to -120 mV from the holding potential of -60 mV. CRF reversibly enhanced the I_h and the duration of this effect was remarkably similar to how CRF increased the firing rate (Figure 3-2 B-C, and for comparison Figure 2-3). Additionally, CRF increased I_h over a range of voltages tested with an average enhancement of 62.8 ± 13.3 pA

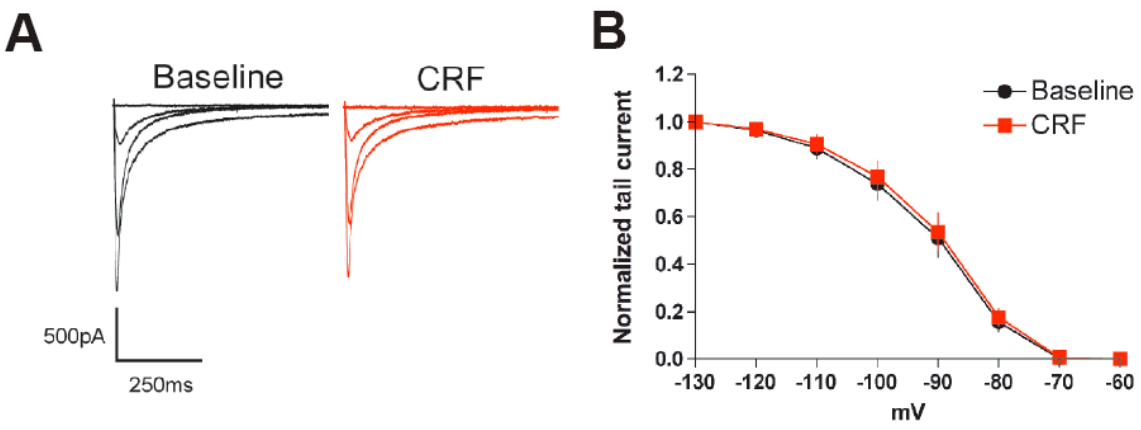

Figure 3-3: CRF did not affect the voltage-dependence of activation for the I_h. (A) Example neuron demonstrating that CRF does not alter the tail current elicited by the offset of hyperpolarizing voltage steps (1 s) from -130, -90, -80, and -60 mV to the holding potential of -60 mV. (B) Summary of the effect of CRF on I_h tail currents in 9 neurons.

measured at the -120 mV voltage step (Figure 3-2 D, n = 7). The CRF enhancement in I_h could be due to changes in the total current or a shift in the voltage-dependence of activation. For experiments analyzing the gating properties of I_h, I isolated I_h tail currents elicited by hyperpolarization steps (1 s) in the presence of 500 nM TTX, 10 mM TEA, 4 mM 4-AP, and 300 μM Ba^{2+}. In 9 neurons tested, CRF enhanced I_h (-52.9 ± 13.6 pA measured at the -120 mV voltage step), but there were no changes in the voltage-dependence of activation since CRF did not alter the Boltzmann sigmoidal fit values for the $V_{1/2}$ (Figure 3-3, baseline: -89.9 ± 1.2 mV; CRF: -89.0 ± 1.0 mV) or the slope factor (Figure 3-3, baseline: -7.9 ± 1.1; CRF: -7.6 ± 0.9).

The CRF enhancement of dopamine neuron firing required PKC (Chapter 2), and in agreement, PKC inhibition with BIS (1 μM) in the internal recording solution significantly attenuated the effect of CRF on I_h (Figure 3-4, -19.9 ± 5.6 pA measured at the -120 mV step, n = 9, $p < 0.01$ relative to control internal). In addition, the average baseline I_h measured at the -120 mV voltage step was significantly reduced by PKC

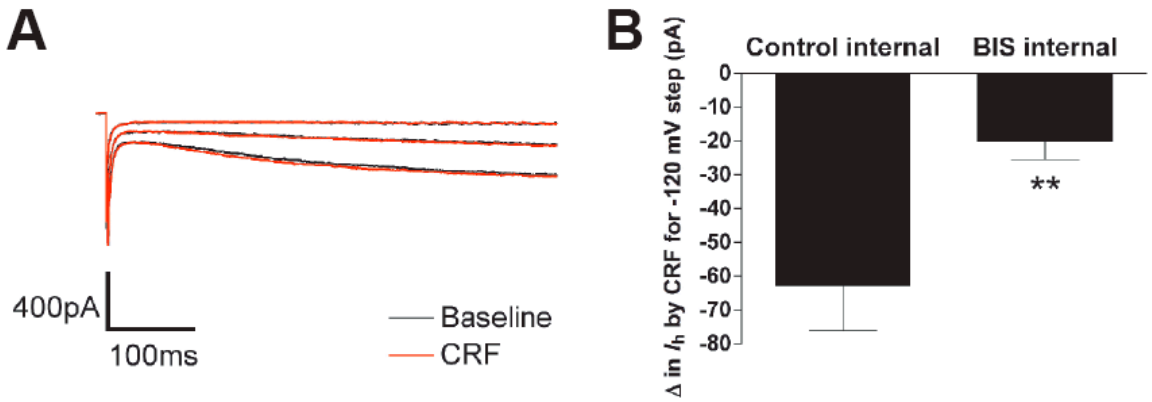

Figure 3-4: PKC antagonism reduced the effect of CRF on the I_h. (A) Example neuron demonstrating that 1 μM BIS in the internal recording solution prevented an increase in I_h by CRF. Shown are hyperpolarizing voltage steps (500 ms) to -80, -100, and -120 mV from a holding potential of -60 mV. (B) The BIS internal solution reduced the maximal effect of CRF on the I_h for the voltage step measured at -120 mV in 7 tested neurons. ** $p < 0.01$ relative to control internal.

antagonism, suggesting that PKC exerts a tonic regulation on I_h in VTA dopamine neurons (BIS: -147.1 ± 40.5 pA, n = 9; control: -264.6 ± 33.8 pA, n = 7, $p < 0.01$). Identical to control conditions, CRF did not change the Boltzmann fit values for the $V_{1/2}$ (Figure 3-5, baseline: -90.2 ± 1.4 mV; CRF: -89.5 ± 1.3 mV, n = 9) or the slope factor

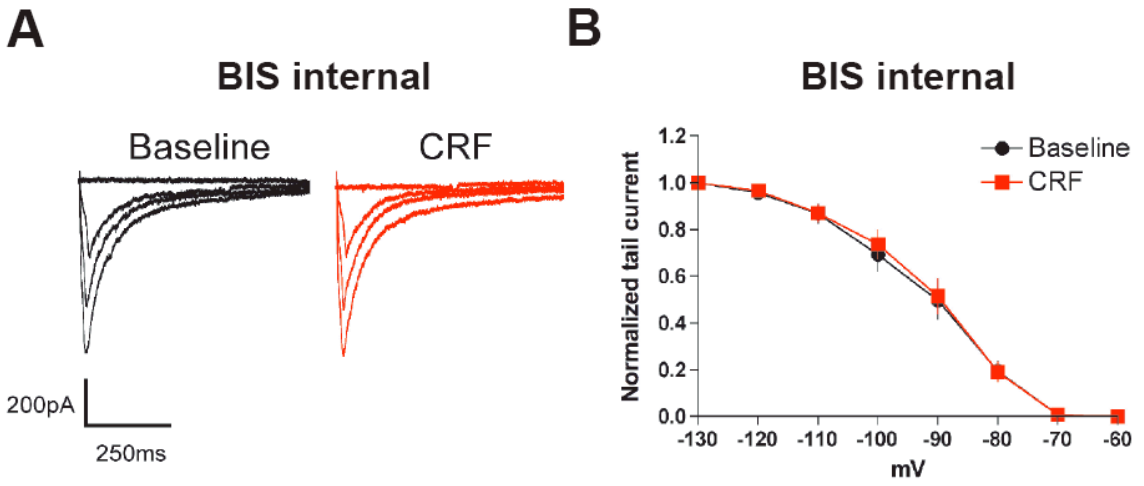

Figure 3-5: CRF did not affect the voltage-dependence of activation for the I_h when PKC activity was blocked. (A) In an example neuron, the tail current elicited by the offset of hyperpolarizing voltage steps (1 s) from -130, -90, -80, and -60 mV to the holding potential of -60 mV is not affected by CRF when including 1 μM BIS in the internal recording solution. (B) Summary of the effect of CRF on I_h tail currents in 9 neurons with the BIS internal solution

(Figure 3-5, baseline: -9.8 ± 1.5; CRF: -8.8 ± 1.3) when using the BIS internal recording solution.

A recent report demonstrated that I_h in SN dopamine neurons is under tonic regulation by phosphoinositides, which are involved in the Gq signaling pathway upstream of PKC activation (Zolles et al., 2006), thus I explored this regulatory mechanism on I_h in VTA dopamine neurons. Similar to SN neurons, I found that that PIP_2 positively modulated the voltage-dependence of activation for the I_h in VTA dopamine neurons. Specifically, 10 μM wortmannin in the internal recording solution, which inhibits PI-3 kinase and prevents PIP_2 synthesis, negatively shifted the baseline $V_{1/2}$ by ~10 mV (Figure 3-6 B, control: -89.9 ± 1.2 mV, n = 9; wortmannin: -99.7 ± 0.5 mV, n = 6). Although wortmannin shifted the voltage-dependence of activation for the I_h, CRF application did not further affect the $V_{1/2}$ (Figure 3-6, CRF: -97.7 ± 0.7 mV, n = 9)

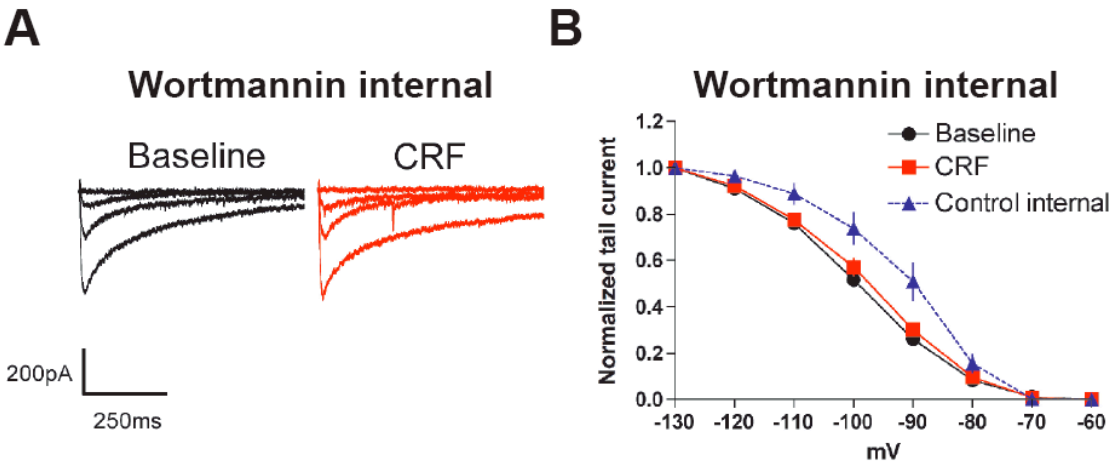

Figure 3-6: Inhibition of PIP_2 shifted the voltage-dependence of activation for the I_h, but CRF did not induce any further changes. (A) In an example neuron, the tail current elicited by the offset of hyperpolarizing voltage steps (1 s) from -130, -90, -80, and -60 mV to the holding potential of -60 mV was not affected by CRF when PIP_2 synthesis was blocked (10 μM wortmannin internal solution). (B) Wortmannin caused a hyperpolarizing shift in the voltage-dependence of activation for the I_h, but CRF did not induce any further changes (n = 6).

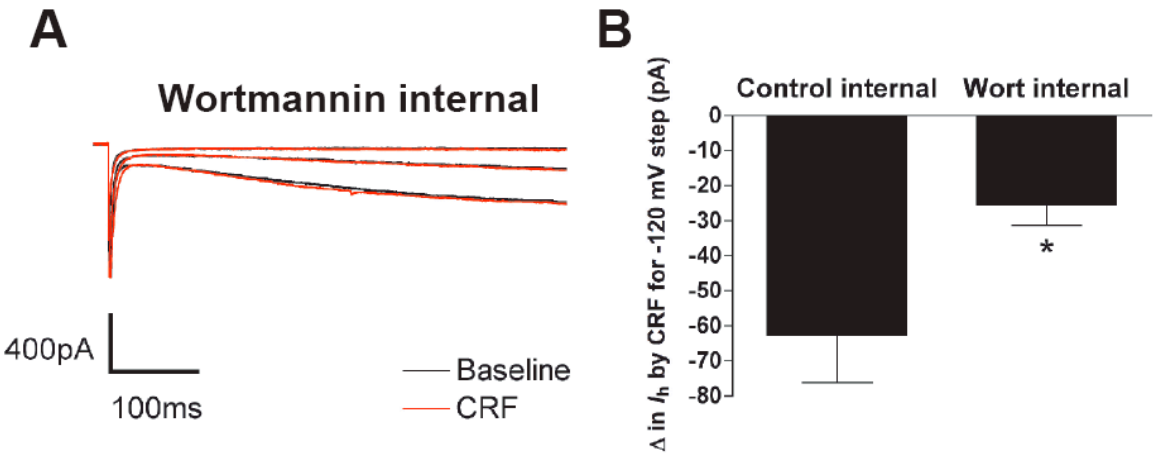

Figure 3-7: Inhibition of PIP_2 reduced the effect of CRF on the I_h. (A) Example neuron demonstrating that 10 μM wortmannin in the internal recording solution prevented an increase in I_h by CRF. Shown are hyperpolarizing voltage steps (500 ms) to -80, -100, and -120 mV from a holding potential of -60 mV. (B) The wortmannin internal solution reduced the maximal effect of CRF on the I_h for the voltage step measured at -120 mV in 9 tested neurons. * $p < 0.05$ relative to control internal.

or change the slope factor (Figure 3-6, baseline: -9.7 ± 0.5, CRF: -9.7 ± 0.8, n = 9), which was the identical result when using the control internal. Similar to PKC inhibition, wortmannin significantly attenuated the effect of CRF on I_h (Figure 3-7, -25.2 ± 5.8 pA measured at -120 mV step, n = 9, $p < 0.05$ relative to control), demonstrating that antagonism of the Gq signaling pathway through inhibition of PIP_2 synthesis or PKC prevented the CRF-induced enhancement of I_h.

Finally, because PKC is required for CRF to augment both I_h and firing in VTA dopamine neurons, I examined whether a PKC activator could recapitulate the effects of CRF. A 10 minute bath application of 500 nM PDBU enhanced the I_h at a range of voltages tested (Figure 3-8 A,B, n = 8) with a maximal average change of -41.4 ± 7.9 pA for the -120 mV step. Identical to CRF, PDBU increased the I_h without changing the voltage-dependence of activation as the Boltzmann fit values for the $V_{1/2}$ (baseline: -89.3 ± 1.5 mV; PDBU: -90.1 ± 1.5 mV) and the slope factor (baseline: -7.9 ± 1.4; PDBU: -9.5

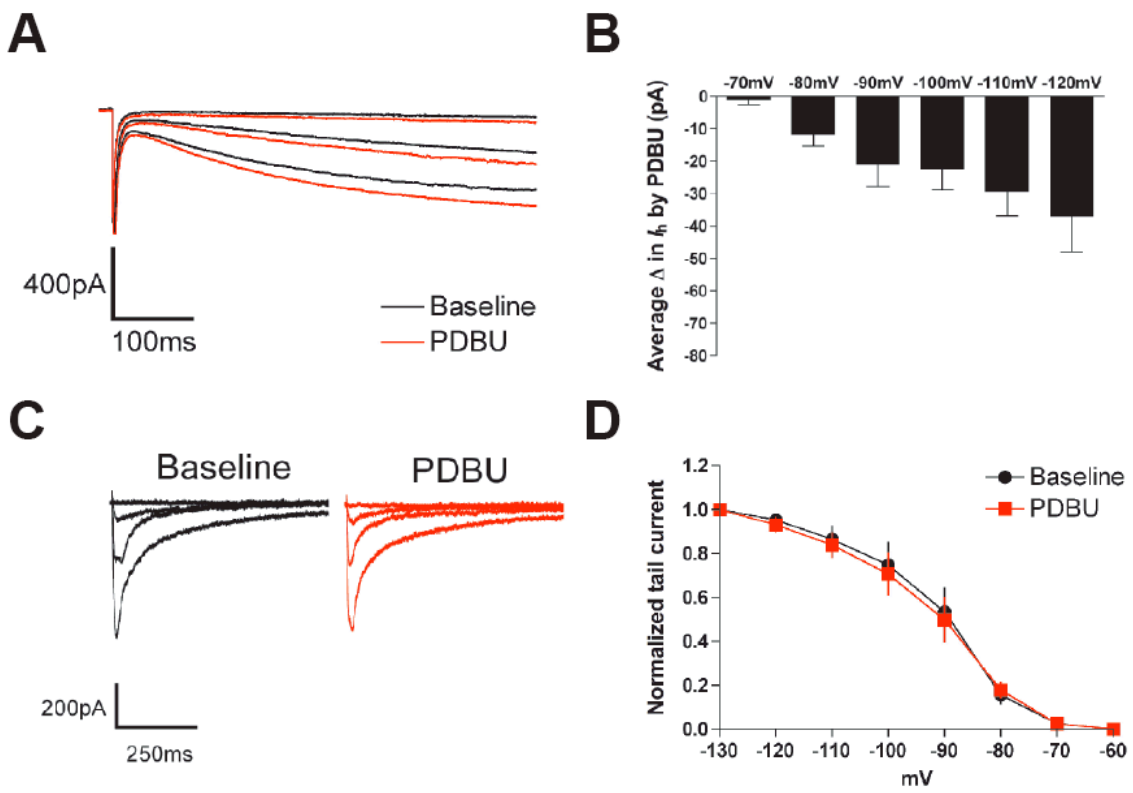

Figure 3-8: PDBU increased the I_h but did not affect the voltage-dependence of activation. (A) Direct activation of PKC with 500 nM PDBU increased the I_h elicited by hyperpolarizing voltage steps (500 ms) from a holding potential of -60 mV to -80, -100, and -120 mV in an example neuron. (B) PDBU enhanced I_h at a range of voltages tested (n = 8). (C,D) Phorbol esters did not affect the voltage-dependence of activation for thef I_h. (C) 500 nM PDBU did not change the tail current elicited by the offset of hyperpolarizing voltage steps (1 s) from -130, -90, -80, and -60 mV to the holding potential of -60 mV in an example neuron. (F) Under these conditions, PDBU did not affect voltage-dependence of activation for the I_h (n = 7).

± 1.6) were unchanged by phorbol esters (Figure 3-8 C,D, n = 7). These findings demonstrated that both CRF and PDBU enhanced the I_h through a novel PKC-dependent mechanism, without changing the voltage-dependence of activation for the I_h.

Discussion:

The key findings from this study are that CRF required I_h to depolarize VTA dopamine neurons and that CRF activated a PKC-dependent increase in the total I_h without changing the voltage-dependence of activation for the I_h. However, there are

some methodological considerations that need to be addressed. Because stable voltage clamp recordings were difficult to achieve using the potassium-based internal recording solution, I was forced to use the cesium-based solution. The cesium internal solution allows for stable voltage clamp recordings due to the blockade of numerous potassium currents, however, cesium also blocks the I_h at high extracellular concentrations (Pape, 1996). Although using a cesium internal solution was not ideal for isolating the I_h, it was necessary for this set of experiments. Another potential issue with these experiments was the usage of whole-cell patch clamp recordings as opposed to perforated-patch recordings, which prevents dialysis of potentially important intracellular components. In addition, the firing rate of dopamine neurons varies depending on whether one uses whole-cell versus perforated-patch recordings (Margolis et al., 2006; Neuhoff et al., 2002). However, I wanted to directly compare the experiments in the current chapter with those examining the changes in firing rate described in Chapter 2, and in this regard, it was best to use the same recording technique.

The I_h plays a critical role in the CRF-mediated increase in VTA dopamine neuron firing (Chapter 2). Additionally, the I_h is involved in determining the membrane potential (Magee, 1998) and I_h activation depolarizes neurons (Qiu et al., 2005), thus I posited that CRF activates the I_h, that in turn will depolarize VTA dopamine neurons. Consistent with this hypothesis, CRF induced nearly a 2 mV depolarization in the membrane potential of VTA dopamine neurons, and this effect was not observed when I_h was pharmacologically blocked.

The I_h is required for CRF to not only increase the firing rate, but also depolarize VTA dopamine neurons. However, the mechanism and the direct effect of CRF on the I_h was unknown. Based upon the requisite role of PKC activity for the CRF-mediated increase in VTA dopamine firing (Chapter 2), I expected that PKC activation positively modulated I_h function. The I_h is regulated by a number of signaling molecules (DiFrancesco and Tortora, 1991; Fogle et al., 2007; Pian et al., 2006; Zolles et al., 2006) and protein kinases (Vargas and Lucero, 2002; Zong et al., 2005). Although, some suggest PKC inhibits the I_h, (Cathala and Paupardin-Tritsch, 1997; Liu et al., 2003), no studies to date demonstrate a positive PKC-dependent modulation. Surprisingly, I found that CRF enhanced the total I_h without changing the voltage-dependence of activation. Although CRF was previously shown to augment the I_h in hypothalamic paraventricular neurons, this was through a distinct mechanism, since they observed a depolarizing shift in the voltage-dependence of activation for the I_h (Qiu et al., 2005). Blocking PKC activity in VTA dopamine neurons not only prevented the CRF-induced increase in the firing as discussed above (Chapter 2), but also prevented the increase in the I_h by CRF, whereby providing the first evidence for a PKC-dependent enhancement of the I_h. I also note that inhibition of PKC reduced the baseline I_h, suggesting that HCN channels are under tonic positive regulation by PKC. In addition, and consistent with a recent report in the SN, preventing PIP_2 synthesis in VTA dopamine neurons caused a ~ 10 mV hyperpolarizing shift in the voltage-dependence of activation for the I_h (Zolles et al., 2006). Inhibiting PIP_2 generation also blocked the increase in the I_h by CRF; however,

this is likely due to a general suppression of Gq signaling since PIP_2 is required upstream of PKC activation and not a result of a direct effect of PIP_2 on the HCN channels.

Further supporting a role for the PKC-dependent enhancement of the I_h in VTA dopamine neurons, phorbol esters increased the I_h without altering the voltage-dependence of activation. In oocytes, phorbol ester application augments phosphatidic acid and arachidonic acid production, which in turn positively shifts the voltage-dependence of activation for the I_h (Fogle et al., 2007). The modulation of the I_h by signaling lipids in oocytes is likely distinct from the mechanism of phorbol ester enhancement of the I_h in VTA dopamine neurons, because the effect in oocytes involves changes in the I_h gating properties, while I observed no such changes in VTA neurons. In a previous study, phorbol esters reduced the I_h in VTA dopamine neurons, but with a four-fold higher dose of phorbol esters than what I used in the current study, which could have non-specific effects (Liu et al., 2003). Specifically, phorbol esters can inhibit the Na^+/K^+ pump at high doses, which could alter the ionic gradient across the neuronal membrane and furthermore could indirectly affect a host of ionic conductances (Fisone et al., 1995).

Although these experiments identified a role for PKC-dependent modulation of the I_h, the specific phosphorylation target of PKC is unknown. Direct phosphorylation of the HCN channels by src kinase increase the I_h gating properties (Zong et al., 2005), but specific phosphorylation site(s) for other kinases are unknown. Some suggest that the phosphoylation event may occur on the HCN subunits and/or accessory proteins (Frere et al., 2004). Alternatively, the phosphorylation event may affect channel protein recycling, where an increase in the number of HCN channels could maximize the conductance

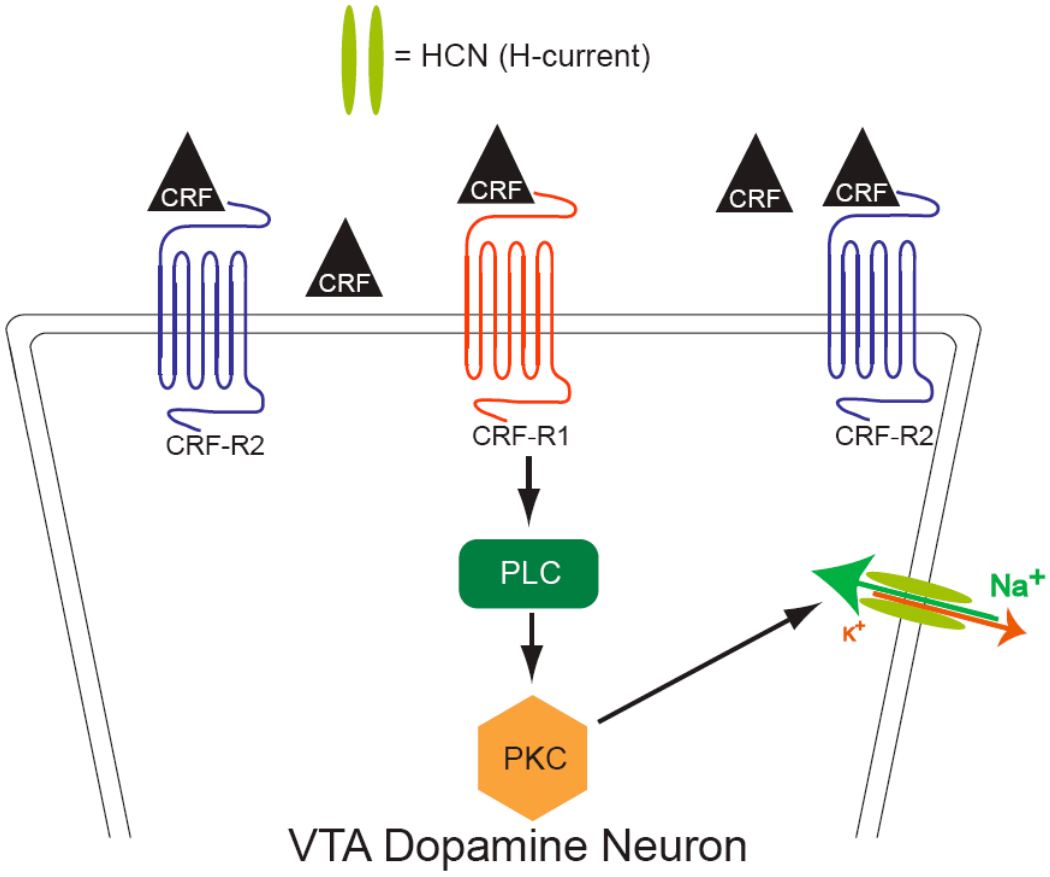

Figure 3-9: Summary figure. On VTA dopamine neurons, CRF binds to the CRF-R1, which in turn activates the PLC-PKC pathway. PKC, via an undetermined mechanism, increases the I_h that is responsible for depolarizing and increasing the firing of VTA dopamine neurons.

without changing the gating properties of the I_h (Frere et al., 2004). Regardless, further experiments will be required to determine the exact mechanism of the PKC-dependent increase in the I_h. For example, addressing the effect of PKC on I_h in a frog oocyte model system using site-specific mutations of HCN channel subunits would allow for determining if and where potential phosphorylation sites exist.

In summary, CRF stimulated a novel PKC-dependent enhancement of the I_h that was independent of changes in the voltage-dependence of activation for the I_h. These findings provide a mechanism explaining the obligatory role of both PKC and the I_h in mediating the firing rate increase by CRF in VTA dopamine neurons. Combining the

results from Chapters 2 and 3, I propose the following model that is illustrated in Figure 3-9. On VTA dopamine neurons, CRF binds to the CRF-R1, which in turn activates the PLC-PKC pathway. PKC, via an undetermined mechanism, increases the I_h that is responsible for depolarizing and increasing the firing of VTA dopamine neurons.

Acknowledgements:

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Chapter 4. CRF-R1 agonists in the VTA required PKC activity and the I_h to increase locomotor activity in rats

Abstract:

Stress stimulates the release of CRF into the VTA and increases dopamine levels in brain regions receiving dense VTA input. Additionally, my results indicate that CRF increased VTA dopamine neuron firing through a mechanism involving activation of the CRF-R1 and the PLC-PKC pathway, which in turn enhances the I_h. However, it is unknown whether the CRF-mediated increase in dopamine neuron firing has behaviorally relevant effects. Previous work demonstrated that intra-VTA injections of CRF increased locomotor activity, though the mechanism was not characterized. I hypothesized that CRF would increase locomotor activity through the same mechanism by which CRF increases VTA dopamine neuron firing. I demonstrated that similar to the mouse, CRF required PKC to increase putative VTA dopamine neuron firing in rat brain slices. Next, I observed that unilateral intra-VTA microinjections of CRF-R1, but not CRF-R2 agonists increased rat locomotor activity. Finally, intra-VTA injections of inhibitors to PKC or I_h prevented the increased activity by CRF-R1 agonists in the VTA. Together, these studies suggest that CRF acts in the VTA to increase locomotor activity through the identical mechanism by which CRF enhances VTA dopamine neuron firing in brain slices, providing a plausible link between the cellular and behavioral effects of CRF in the VTA.

Introduction:

An organism's response to threatening and/or stressful stimuli is critical to its survival and often initiates a host of defensive behaviors including, but not limited to, flight, hiding, and freezing (Blanchard et al., 2003). The physiological changes due to stress are widespread as both the sympathetic and central nervous systems are affected (de Kloet et al., 2005). Stress-induced CRF release activates the canonical stress-response pathway as well as affecting a number of brain regions including the dopamine-producing VTA (de Kloet et al., 2005; Wang et al., 2005). Additionally, a variety of laboratory stressors enhance dopamine levels in brain regions receiving VTA input (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996). Furthermore, i.c.v. injections of CRF dose-dependently increases dopamine levels in the PFC (Lavicky and Dunn, 1993). Together, these studies suggest that CRF could excite VTA dopamine neurons to increase dopamine output in response to stress. Supporting this claim, CRF increased VTA dopamine neuron firing in a mechanism requiring the CRF-R1, the PLC-PKC pathway, and the I_h (Chapters 2 and 3); however, the behavioral relevance of this effect is not yet known.

Although the VTA and dopamine release are implicated in a number of behaviors, they both are clearly involved with motor activity (Beninger, 1983; Dunn et al., 2005; Zhou and Palmiter, 1995). Specifically, the VTA is an important brain nucleus for motor behaviors as local microinjection of glutamate receptor agonists into the VTA robustly increases locomotor activity (Dunn et al., 2005). In addition, mice that do not produce dopamine are hypoactive (Zhou and Palmiter, 1995) while mice that have chronically

elevated dopamine levels exhibit hyperactivity (Zhuang et al., 2001). Importantly, increased dopamine levels promote escape and flight speed from stressful/threatening situations such as predators (Blanchard et al., 1999), suggesting that stress-induced dopamine release could be important for certain defensive behaviors. CRF is also involved with avoidance from predators since CRF-R1 antagonists inhibit (Griebel et al., 1998) and CRF-R1 agonists enhance (Yang et al., 2006) escape and flight speed from threatening and stressful situations. Finally, intra-VTA injections of CRF increased locomotor activity, although the mechanism was not fully characterized (Kalivas et al., 1987). Together, these studies identify that CRF in the VTA evokes a behaviorally relevant response of increased locomotion, which could aid in the escape from stressful situations. In this regard, I hypothesized that intra-VTA injections of CRF increased locomotor activity through the same mechanism by which CRF enhanced VTA dopamine neuron firing in brain slices. I found that CRF required PKC activity to increase putative dopamine neurons firing from the rat, demonstrating the conservation of this effect across rodent species. Additionally, unilateral intra-VTA injections of CRF-R1 agonists increased locomotor activity, which required both PKC activity and the I_h.

Materials and methods:

Electrophysiology:

Please refer to corresponding section in Chapter 2.

Intra-VTA injections and locomotor activity

For locomotor activity and microinjection experiments, 250g rats (Harlan and Charles River) underwent unilateral VTA cannulation surgeries (from bregma: -5.6 mm AP, 0.5 mm ML, -7.0 mm DV). Injector cannulas protruded 1 mm past the end of the guide cannula. All locomotor experiments consisted of a 1-hour habituation, intra-VTA injection(s), followed by 90 minutes in the locomotor chamber. In order to habituate the rats to the experimental procedure, they received a 0.5 μL vehicle injection (in mM: 154.7 Na^+, 2.9 K^+, 132.49 Cl^-, 1.1 Ca^{2+} at pH = 7.4) on the first day of testing during the week. Injections were visually monitored and were completed within the course of 4 minutes. Rats received similar intra-VTA injections of vehicle or drug (dissolved in vehicle) in a counter-balanced order on subsequent days. A minimum of 4 days passed between drug injections. The locomotor activity data is presented in 10-minute bins.

Results:

CRF required PKC to increase putative VTA dopamine neuron firing in the rat

Since the effective excitatory doses of CRF had already been determined and accurate local microinjections are more easily achieved in rats, I chose to explore the mechanism of locomotor enhancement by CRF in rats instead of mice (Kalivas et al., 1987). First, it was necessary to verify that the effect of CRF on dopamine neuron firing was also observed in rats. In agreement with the findings from mouse brain slices, CRF also increased the firing rate of putative dopamine neurons in the rat (Figure 4-1, 34.2 ± 13.1% over baseline, $n = 5$, $p < 0.01$ relative to baseline). This effect of CRF was PKC-

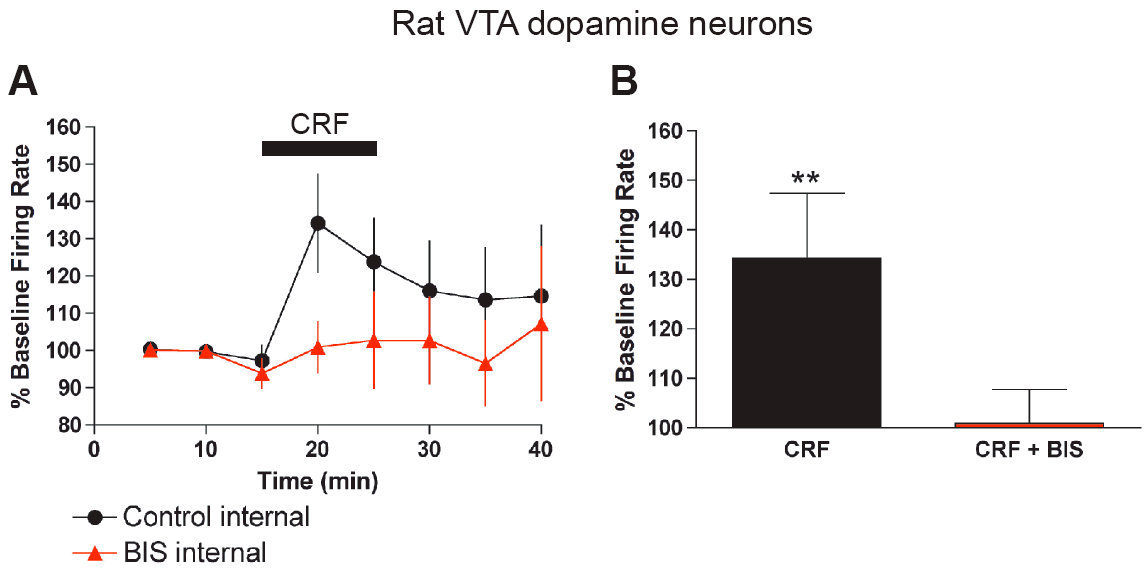

Figure 4-1: CRF increased putative VTA dopamine neuron firing through a PKC-dependent mechanism in rats. (A) Time course of firing rate changes due to CRF application (1 μM, 10 min) in neurons with control internal (black circles, n = 5) and neurons with BIS (1 μM) internal to block PKC activity (red triangles, n = 4). (B) CRF significantly increased the firing rate over baseline levels in neurons with the control internal (** $p < 0.01$), but not with those with the BIS internal.

dependent, as inclusion of a PKC antagonist (1 μM BIS) in the internal recording solution prevented the increase in firing rate (Figure 4-1, 0.9 ± 6.9 % over baseline, n = 4), suggesting a common excitatory mechanism on firing by CRF in both mice and rats.

Intra-VTA CRF-R1 agonists increase locomotor activity

Rats were implanted with cannulas aimed at the VTA for use in locomotor activity experiments (injection sites of 42 rats shown in Figure 4-2). There were no differences in activity among vehicle injection treatment groups, so vehicle injection data were combined (Figure 4-3 D, 2687 ± 256 cm traveled, n = 26). I chose to inject 0.42 nmole of CRF, a dose that elicits a significant but sub-maximal locomotor enhancement when bilaterally microinjected into the VTA (Kalivas et al., 1987). Here, unilateral microinjections of CRF produced a long-lasting, significant increase in locomotor

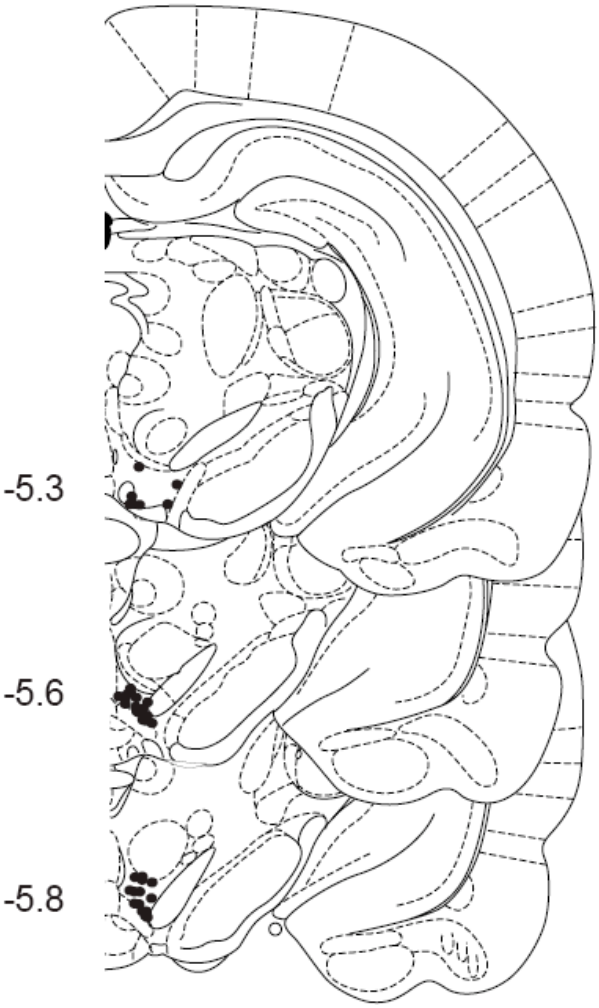

Figure 4-2: Histological representation of intra-VTA injection sites from 42 rats. Values on left column refer to distance from bregma (mm).

activity (Figure 4-3 A,D, 5018 ± 505 cm traveled, n = 9, $p < 0.001$ relative to vehicle). Similarly, 0.42 nmole injections of the CRF-R1 agonist, oCRF, significantly increased the distance traveled relative to vehicle (Figure 4-3 B,D, 6485 ± 1199 cm traveled, n = 10, $p < 0.001$). However, a 0.42 nmole injection of the CRF-R2 agonist, UCNII, was without effect on the locomotor activity (Figure 4-3 C-D, 2872 ± 270 cm traveled, n = 9, $p > 0.05$). In agreement with the *in vitro* findings demonstrating a critical role for CRF-R1 in the CRF enhancement of VTA dopamine firing (Chapter 2), CRF-R1 agonists increased locomotor activity when injected into the VTA.

Intra-VTA injections of CRF-R1 agonists involve PKC and I_h to increase locomotor activity

Finally, I wanted to determine whether CRF in the VTA activated a common intracellular pathway and ionic target to increase dopamine neuron firing and stimulate

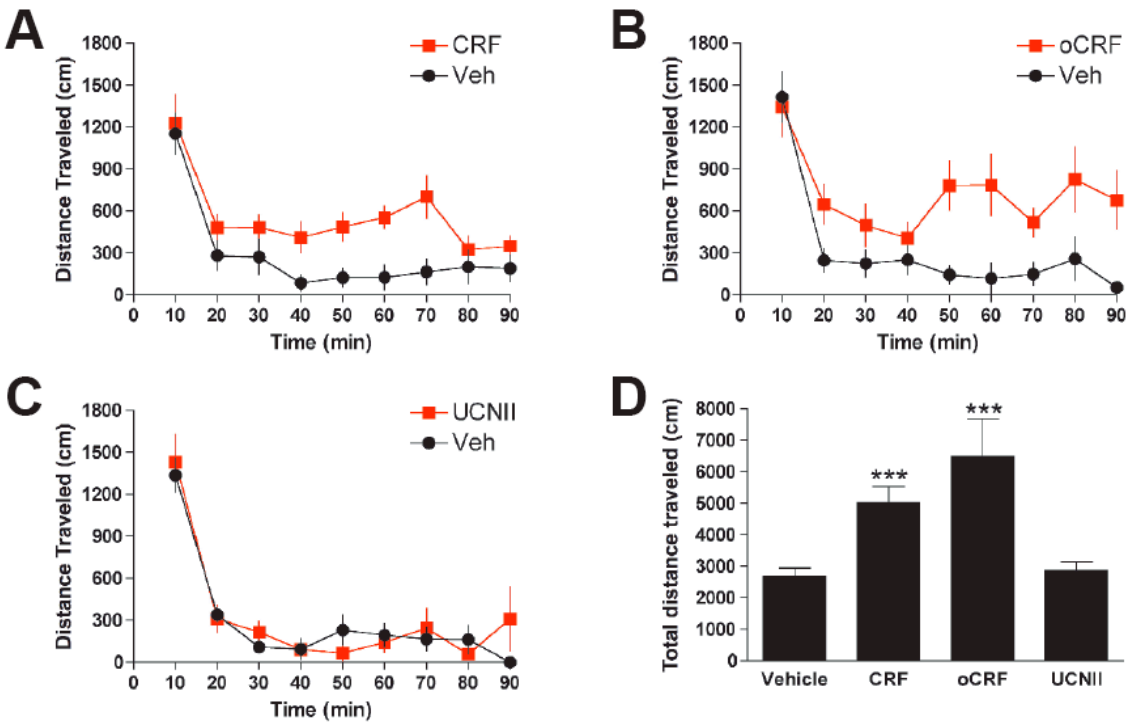

Figure 4-3: Intra-VTA injections of CRF-R1, but not CRF-R2 agonists increased locomotor activity. Locomotor activity was tracked for 90 minutes after 0.5 µL intra-VTA vehicle or drug injection(s). (A) Time course showing locomotor activity after intra-VTA injections of vehicle (Veh) and CRF (0.42 nmole, n = 9). (B) Time course showing locomotor activity after intra-VTA injections of Veh and the CRF-R1 agonist, oCRF (0.42 nmole, n = 8). (C) Time course showing locomotor activity after intra-VTA injections of Veh and the CRF-R2 agonist, Urocortin II (UCNII, 0.42 nmole, n = 9). (D) Summary of effects of intra-VTA injections of various CRF receptor agonists on total distance traveled. *** $p < 0.001$ relative to vehicle injections combined.

locomotor activity. Thus, I examined whether antagonizing PKC or I_h in the VTA could prevent the increase in locomotor activity by intra-VTA microinjections of the CRF-R1 agonist, oCRF. Vehicle microinjections 10 minutes prior to oCRF injections did not impair the ability of oCRF to increase locomotor activity (Figure 4-4, Veh - oCRF: 5212 ± 365 cm traveled, n = 9, $p > 0.05$ relative to oCRF injection). However, the soluble PKC antagonist, chelerythrin (Chel, 50ng), significantly reduced the stimulatory effect of oCRF (Figure 4-4, Chel - oCRF: 3429 ± 473 cm traveled, n = 10, $p < 0.01$ relative to Veh - oCRF injections). Chel did not cause a general suppression of activity, since there were no differences between rats receiving Chel followed by vehicle (Figure 4-4, Chel - Veh:

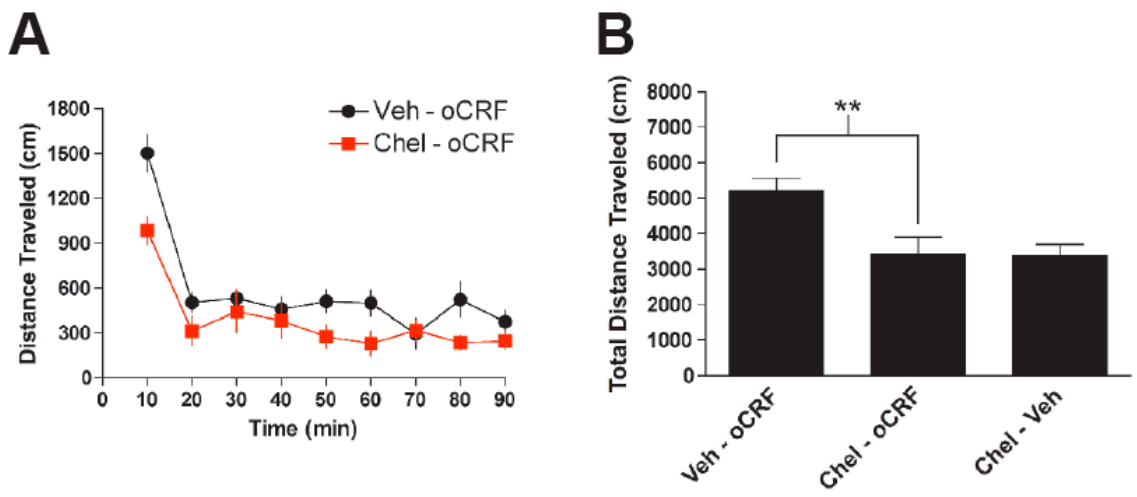

Figure 4-4: PKC antagonism reduced the effect of intra-VTA injections of oCRF on locomotor activity. (A) Time course showing enhanced locomotor activity after intra-VTA injections of Veh followed by oCRF (Veh - oCRF, n = 9). Also shown is the time course of activity following intra-VTA injections of the soluble PKC antagonist, chelerythrin (Chel, 50 ng) followed by oCRF (Chel - oCRF, n = 10). (B) Chel significantly attenuated the locomotor activating effects of oCRF (** $p < 0.01$). Similar activity was observed between Chel - oCRF treated rats and those receiving a Chel injection followed by Veh injection (Chel - Veh, n = 9).

3363 ± 341 cm traveled, n = 9) and rats receiving two vehicle injections (Veh - Veh: 2884 ± 225 cm traveled, n = 43, $p > 0.05$).

Similar to the Chel microinjection experiments, blockade of I_h by intra-VTA microinjections of ZD-7288 (50 ng) significantly attenuated the locomotor response to oCRF (Figure 4-5, ZD - oCRF: 3858 ± 495 cm traveled, n = 8, $p < 0.05$ compared to Veh - oCRF). However, injection of ZD-7288 followed by vehicle (Figure 4-5, ZD - Veh: 4002 ± 532 cm traveled, n = 8) was not different from dual-vehicle injections ($p > 0.05$), demonstrating that ZD-7288 at this dose had no effect on locomotor activity by itself. Thus, these behavioral findings demonstrated that both PKC activation and I_h are required for CRF-R1 agonists to enhance locomotor activity.

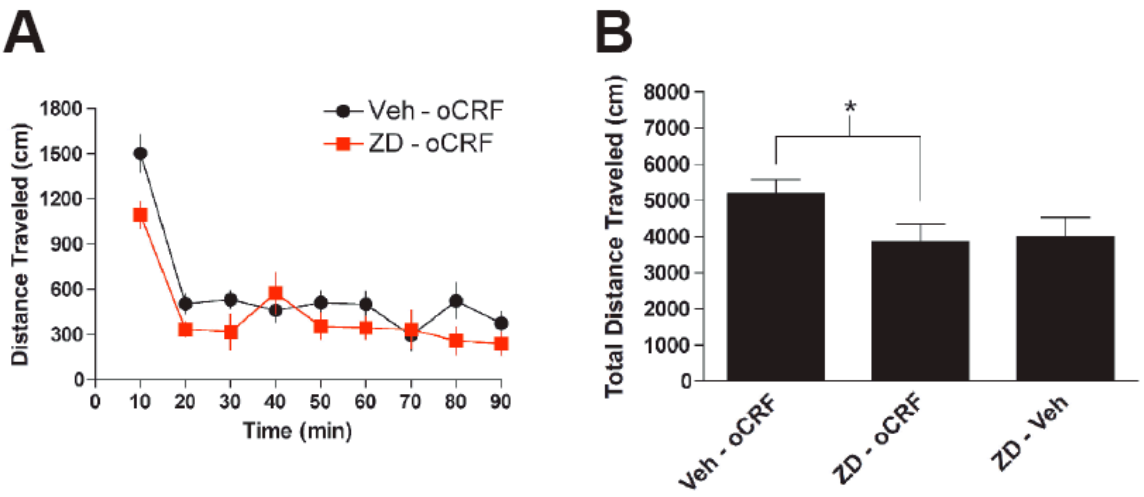

Figure 4-5: I_h antagonism reduced the effect of intra-VTA injections of oCRF on locomotor activity. (A) Time course showing locomotor activity after intra-VTA injections of Veh followed by oCRF (Veh - oCRF, n = 9). Also shown is the time course of activity following intra-VTA injections of the I_h inhibitor, ZD-7288 (ZD, 50 ng) followed by oCRF (ZD - oCRF, n = 8). (B) ZD significantly attenuated the locomotor activating effects of oCRF (* $p < 0.05$). Similar activity was observed between ZD – oCRF-treated rats and those receiving a ZD injection followed by Veh injection (ZD - Veh, n = 8).

Discussion:

These experiments first demonstrated that CRF required PKC to increase putative dopamine neuron firing in rats. In conjunction with the results outlined in Chapters 2 and 3, these findings highlight the conservation of the mechanism by which CRF increases dopamine neuron firing across rodent species. In addition, these experiments demonstrated that CRF-R1 agonists in the VTA increase locomotor activity, and that this effect involves both PKC and I_h.

Numerous theories exist about the function of dopamine release (Berridge, 2007; Redgrave and Gurney, 2006; Schultz, 2002), though abundant evidence supports a role of dopamine in motivation, movement, and general arousal (Berridge, 2007; Phillips et al., 2007; Salamone and Correa, 2002; Wise, 2004). The VTA, a major dopamine producing brain nucleus, plays a critical role in motor behaviors (Dunn et al., 2005). In addition,

dopamine-deficient mice are hypoactive (Zhou and Palmiter, 1995), while mice lacking the dopamine transporter exhibit hyperactivity (Zhuang et al., 2001).

The dopamine system is modulated by a number of behaviors (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996), and pharmacological agents (Di Chiara and Imperato, 1988), but of importance to the current study, is also activated by ventricular injections of CRF (Lavicky and Dunn, 1993). Specifically, a microdialysis study determined that i.c.v. CRF injections dose-dependently increased dopamine levels in the PFC, a brain region receiving dense VTA input (Lavicky and Dunn, 1993). Although CRF could act in the PFC to prevent reuptake of dopamine, the probable interpretation of the data suggests CRF excites VTA dopamine neurons that project to the PFC. In another study, intra-VTA injections of CRF increase locomotor activity, though the authors suggest this may be independent of dopamine release (Kalivas et al., 1987). They report that systemic dopamine antagonists prevent the CRF-induced increase in locomotion only at cataleptic doses (Kalivas et al., 1987). Using tissue punches, they observe a decrease in dopamine turnover in the PFC and increase in dopamine turnover in the NAcc, but only at 30 minutes after the intra-VTA injection of CRF (Kalivas et al., 1987). Reconciling these two studies by Kalivas and Lavicky is difficult, but microdialysis studies are more sensitive to analyte changes and have a greater temporal resolution than tissue punches studies, so caution should be used when interpreting the data from the Kalivas study. Also, given the importance of dopamine in locomotion, one could argue that systemic injections of dopamine receptor antagonists are effective only at doses that induce catalepsy. A better experiment would

be to site specifically inject dopamine receptor antagonists into the NAcc to determine if this treatment blocked the CRF-induced increase in locomotion. Although there is no simple explanation to resolve the differences between the Kalivas and Lavicky studies, the likely interpretation is that CRF directly excites VTA dopamine neurons, which increases both dopamine levels and locomotor activity.

Based upon the apparent excitatory role of CRF on the dopamine system and its ability to enhance locomotor activity, I hypothesized that CRF increased VTA dopamine neuron firing (Chapters 2 and 3) and locomotor activity through the same mechanism, involving the CRF-R1, PKC, and I_h. Consistent with my hypothesis, intra-VTA injections of CRF-R1, but not CRF-R2 agonists, increased locomotor activity in rats. This finding supports a previous study that found no locomotor-enhancing effects of ventricular CRF injections in CRF-R1 deficient mice (Contarino et al., 2000). This CRF-R1-mediated increase in activity was significantly blocked by local antagonism of PKC and the I_h in the VTA. Interestingly the antagonists to PKC and I_h primarily attenuated the initial spike of activity after the injections. I argue this activity is primarily a result of the handling stress associated with the injections, as it is unlikely this is a novelty response since the rats habituated to the same chamber for an hour prior to the injection. Thus, both the increase in locomotor activity and dopamine neuron firing by CRF in the VTA involve both PKC and I_h.

Microinjection experiments in heterogeneous brain regions like the VTA have the caveat that the specific cellular targets are unknown for the injected drugs. In this study, GABA neurons are an unlikely target since CRF did not increase the firing in putatively

GABA VTA neurons (unpublished observations). I cannot eliminate the possibility that CRF stimulated locomotor activity through a presynaptic synaptic mechanism involving PKC and the I_h. No evidence exists to either support or refute a PKC or I_h modulation of presynaptic transmitter release on VTA neurons, though both PKC and I_h can affect presynaptic terminals in other brain regions (Majewski and Iannazzo, 1998; Mellor et al., 2002). However, this is unlikely, as CRF does not affect glutamate release in the VTA of drug-naïve animals (Wang et al., 2005). Similarly, unpublished observations from the Bonci lab indicate no changes in the frequency in the miniature excitatory postsynaptic potentials in drug-naïve rodents by CRF, providing evidence against a presynaptic locus of action for CRF in the current study.

The finding that CRF in the VTA increased locomotor activity provides a mechanism explaining why both dopamine and CRF are involved in mediating escape and flight behaviors in response to stressful and threatening stimuli (Blanchard et al., 2003). Specifically, enhancing dopamine levels (Blanchard et al., 1999) and ventricular infusions of the CRF-R1 agonist, oCRF (Yang et al., 2006), increase the speed and the number of escapes from a predator, while antagonism of the CRF-R1 reduces escape behavior (Griebel et al., 1998). Thus, stimulation of the CRF and dopamine systems coupled with the canonical HPA axis function is likely a fundamental and behaviorally relevant physiological response to promote flight from threatening situations.

In summary, I propose that CRF stimulates the CRF-R1 on VTA dopamine neurons to induce a PKC- and I_h-dependent increase in locomotor activity, which is the identical mechanism by which CRF increases dopamine neuron firing in a slice.

Together these results provide a mechanistic link of the actions of CRF on VTA dopamine neurons from ion currents to behavior.

Acknowledgements:

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Chapter 5. The interaction between cocaine, CRF, and NMDAR currents on VTA dopamine neurons

Abstract:

One of the major problems with drug addiction is the high incidence of relapse, which often is precipitated by stress. Not only do both stress and drugs of abuse enhance dopamine release, but stress also sensitizes the behavioral response to drugs. A possible brain nucleus involved with the interaction between drugs of abuse and stress is the VTA. Both cocaine and CRF potentiated NMDAR currents on VTA dopamine neurons, though through distinct mechanisms. *In vivo* drug exposure induces both excitatory and inhibitory synaptic changes on VTA dopamine neurons, but how an *in vivo* drug exposure affects the ability of CRF to potentiate NMDAR currents is unknown. Here I demonstrated that a single injection of cocaine blunted the ability of CRF to increase NMDAR currents in VTA dopamine neurons. Furthermore, although stress preferentially activates the mesocortical system relative to the mesoaccumbens system in regards to dopamine release, I found no difference in the ability of CRF to affect NMDAR currents in neurons with different projection targets. In experiments designed to further identify the mechanism by which CRF potentiated NMDAR currents, I found that the I_h did not predict if a cell will respond to CRF and that the potentiation of NMDAR currents required actin depolymerization.

Introduction:

Given the emotional and financial costs arising from drug addiction in our society, a wealth of research has sought to determine the neural circuitry involved with mediating drug-related behaviors. Multiple lines of evidence demonstrate the interaction between drugs of abuse and the dopamine system. Injections of addictive drugs increase dopamine levels in the NAcc (Di Chiara and Imperato, 1988). Additionally, the VTA, a dopamine-producing brain nucleus, is required for a variety of drug-related behaviors such as behavioral sensitization (Kalivas and Alesdatter, 1993; Kalivas and Weber, 1988) and drug self-administration (McFarland et al., 2004; McFarland and Kalivas, 2001). Furthermore, *in vivo* drug exposure induces excitatory synaptic alterations onto VTA dopamine neurons that require NMDARs (Ungless et al., 2001). NMDAR currents are not only required for the expression of LTP in VTA dopamine neurons (Ungless et al., 2001), but also are potentiated by cocaine (Schilstrom et al., 2006). Together these studies indicate that NMDAR currents of VTA dopamine neurons are directly affected by addictive drugs and are required for certain drug-related behaviors.

The major concern for those suffering from addiction is the high propensity of relapse. Laboratory models of addiction in rodents demonstrate that drug exposure, previously associated drug-related cues, and stress all can reinstate drug-seeking behaviors (Kalivas and McFarland, 2003; McFarland et al., 2004; McFarland and Kalivas, 2001). Of particular interest to the current study, stress-induced drug-seeking behaviors require activation of the VTA and subsequent dopamine release in the PFC (McFarland et al., 2004). Stress also increases cocaine-induced activity (Sorg and

Kalivas, 1991) and augments the rate of acquisition of drug self-administration behaviors (Tidey and Miczek, 1997), which together demonstrate a link between stress and drug-related behaviors.

Stress could affect drug-seeking behaviors through its activation of the midbrain dopamine system, since a number of laboratory stressors increase dopamine levels in brain regions receive VTA input (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996). Interestingly, the dopamine projection from the midbrain to the PFC (mesocortical) as opposed to midbrain dopamine projection to the NAcc (mesoaccumbens) is especially sensitive to stress (Horger and Roth, 1996; Thierry et al., 1976). Stress could stimulate dopamine release through the actions of CRF, which not only is released onto the pituitary during stress to activate the HPA axis (de Kloet et al., 2005), but also is released in the VTA (Wang et al., 2005). Demonstrating the excitatory role of CRF on the dopamine system, i.c.v. injections of CRF dose-dependently increased dopamine levels in the PFC (Lavicky and Dunn, 1993). The actions of CRF in the VTA are relevant to addiction research, as CRF-related agonists with high affinity for the CRF-R2 and the CRF-BP reinstate cocaine-seeking behaviors when administered into the VTA (Wang et al., 2007).

Studying the effect of CRF on NMDAR currents in VTA dopamine neurons is of great importance to addiction research given the behaviorally relevant actions of CRF in the VTA and that NMDAR currents on VTA dopamine neurons also play a role in drug-asssociated behaviors. Previous work from the Bonci lab found that CRF requires the CRF-R2 and CRF-BP to potentiate NMDAR currents (Ungless et al., 2003), which

suggests that this could be the mechanism by which CRF reinstates cocaine-seeking behaviors (Wang et al., 2007). Thus, I wanted to determine how previous *in vivo* exposure to cocaine would affect the ability of CRF to potentiate NMDAR currents in VTA dopamine neurons.

Since cocaine and CRF increase NMDAR currents (Schilstrom et al., 2006; Ungless et al., 2003), I hypothesized that a single cocaine injection would enhance the ability of CRF to affect NMDAR currents. Surprisingly, I found that a single cocaine injection prevented the effect of CRF to increase glutamate currents on dopamine neurons. Furthermore, there was no difference in the ability of CRF to affect these currents on mesocortical versus mesoaccumbens projecting neurons. Although the I_h was previously used to classify CRF-responding and CRF non-responding VTA neurons (Ungless et al., 2003), I found that this measure does not correlate with a recorded neurons ability to be affected by CRF. Finally, I demonstrated that CRF required actin depolymerization to potentiate NMDAR currents. Together these findings add to the information regarding how stress-related neuropeptides and drugs of abuse interact on the midbrain dopamine system.

Materials and methods:

Electrophysiology:

Please refer to corresponding section in Chapter 2 for specific details. Excitatory postsynaptic currents (EPSCs) were evoked using a bipolar stimulating electrode place rostral to the recording electrode. NMDAR EPSCs were recorded at +40 mV using the

cesium-based internal recording solution and analyzed 20 ms after the stimulus to minimize the contribution of AMPA currents. *In vivo* injections of cocaine (15 mg / kg) or equal volume saline, were given 24 hrs prior to electrophysiological recordings.

DiI injections:

21-23 day old C57BL/6 mice (Charles River) were anaesthetized with isofluorane and placed in a stereotaxic apparatus. 0.5 μL of 7% Neuro-DiI (Molecular Probes) in EtOH was injected bilaterally over 20 min into the NAcc shell (relative to bregma in mm: AP +1.8; ML ± 0.9; DV -7.4) or the PFC (relative to bregma in mm: AP +2.34; ML ± 0.25; DV -2.25). The mice were singly housed until they were prepared for electrophysiological experiments about 1 week after dye injection.

Results:

Cocaine injections blunted the ability of CRF to potentiate NMDAR currents on VTA dopamine neurons

Since the VTA is a brain nucleus that could mediate the interaction between stress and drugs of abuse, I assayed how a previous injection of cocaine would affect the ability of 1 μM CRF to potentiate NMDAR currents on VTA dopamine neurons. CRF was able to increase NMDAR currents in mice that were given saline injections 24 hr prior to brain slice recordings (Figure 5-1 A,B, for example). In an average of 11 neurons, CRF

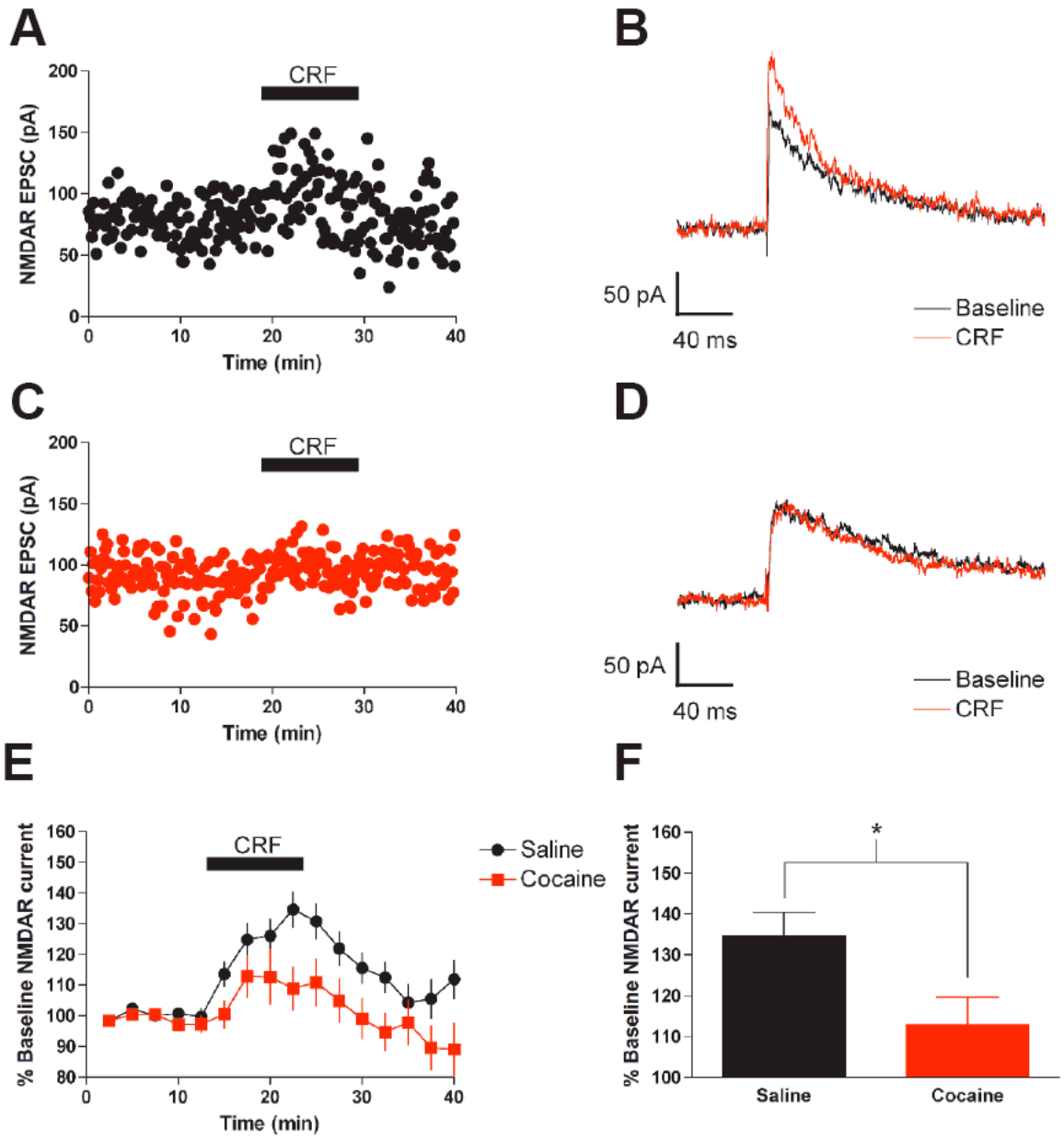

Figure 5-1: Cocaine injections reduced the effect of 1 μM CRF on NMDAR current in VTA dopamine neurons. (A,B) Example neuron from a saline injected mouse where CRF potentiated the NMDAR current evoked while stimulating EPSCs at a holding potential of +40 mV. (C,D) Example neuron from a cocaine injected mouse where CRF did not potentiate NMDAR currents. (E,F) Summary of the effect of CRF on NMDAR currents from saline injected (n = 11 cells) and cocaine injected (n = 14 cells) mice. * $p < 0.05$ between treatment groups.

increased NMDAR currents by 34.6 ± 5.6 % over baseline levels from saline injected mice (Figure 5-1 E,F). In contrast, a single cocaine injection (15 mg/kg, i.p.) reduced the effect of CRF on NMDAR currents (Figure 5-1 C,D for example). In a summary of 14

neurons tested, CRF still potentiated NMDAR currents by 12.9 ± 6.8 % over baseline levels in cocaine injected mice, but this was significantly less than that observed for saline injected rodents (Figure 5-1 E,F, $p < 0.05$ relative to saline injected). These findings demonstrate that a previous exposure to cocaine alters the capacity of VTA dopamine neurons to be affected by CRF in regards to changes in the NMDAR current.

The effect of saline and cocaine injections on the ability of CRF to potentiate NMDAR currents on mesocortical and mesoaccumbens VTA dopamine neurons

A previous study suggested that only a subpopulation of VTA dopamine neurons responded to CRF (Ungless et al., 2003). Since the mesocortical projection is more sensitive to stress relative to the mesoaccumbens projection in regards to dopamine release (Horger and Roth, 1996; Thierry et al., 1976), I hypothesized that CRF would potentiate NMDAR currents in mesocortical neurons to a greater degree than mesoaccumbens neurons. Furthermore, I hypothesized that the blunting effect of cocaine on CRF-mediated changes on the NMDAR current may arise from alterations selectively

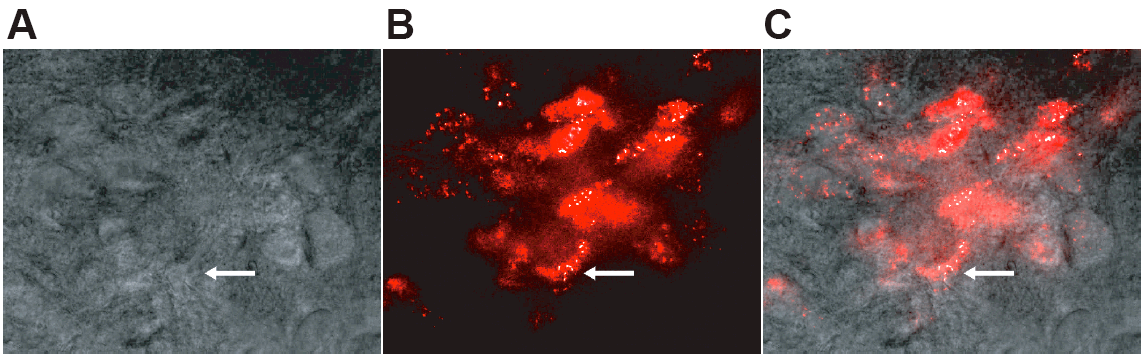

Figure 5-2: Example image of a recording from a DiI labeled cell. (A) DIC image. White arrow points to recorded neuron. (B) Same image as in (A), but showing psuedocolor DiI fluorescence. DiI staining was often punctate. (C) Overlay merge of the images in (A) and (B).

in mesoaccumbens or mesocortical neurons.

To address these concerns, mice underwent stereotaxic surgeries to inject DiI, a fluorescent retrograde label, into either the PFC or the NAcc. Mice recovered from surgery for ~ 1 week to allow for ample transport of the dye to the cell bodies in the VTA. The DiI stained the neuron in a punctate manner as evidenced in an example neuron shown in Figure 5-2. In contrast to my hypothesis, there was no difference in the ability of CRF to potentiate NMDAR currents between mesocortical (Figure 5-3 A, 21.3 ± 5.3 % over baseline, n = 11) and mesoaccumens (Figure 5-3 B, 15.6 ± 5.8 % over baseline, n = 9, $p > 0.05$ relative to mesocortical) VTA neurons from saline injected mice. Furthermore, there was no effect of cocaine injections on the ability of CRF to potentiate NMDAR currents in either mesocortical or mesoaccumbens neurons (Figure 5-3). Specifically, in cocaine injected mice, CRF increased NMDAR currents by 16.7 ± 5.4 % in mesocortical neurons (Figure 5-3 A, n = 8) and by 12.8 ± 2.9 % in mesoaccumbens

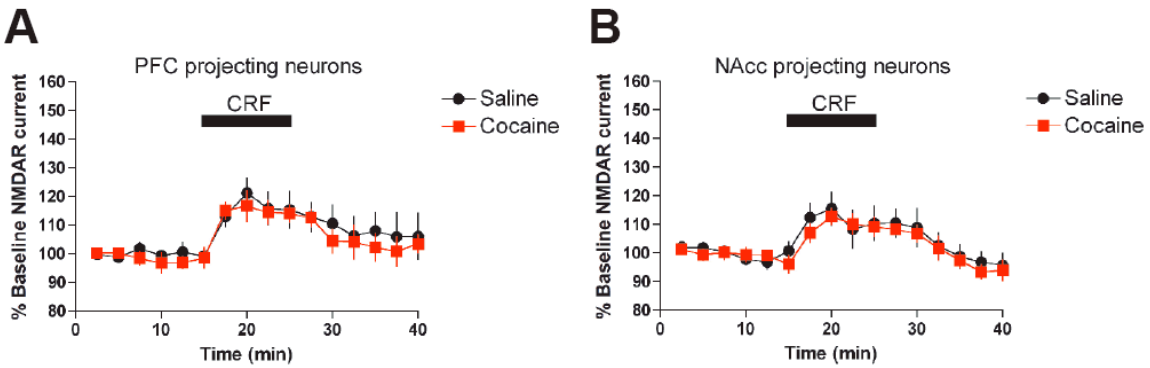

Figure 5-3: The effect of cocaine injections on the CRF-mediated increase in NMDAR currents in mesocortical and mesoaccumbens projecting VTA dopamine neurons. (A,B) Summary of the effect of 1 μM CRF on NMDAR currents in mice receiving a single saline or cocaine injection. DiI was injected into either the PFC or NAcc ~ 1wk prior to recordings. VTA neurons were recorded that were positive for DiI staining. Based on the DiI injection site, the projection target of the recorded neuron was known. (A) CRF potentiated NMDAR currents in PFC projecting neurons to the same degree in saline injected (n = 11) and cocaine injected (n = 8) mice. (B) CRF potentiated NMDAR currents in NAcc projecting neurons to the same degree in saline injected (n = 9) and cocaine injected (n = 6) mice.

neurons (Figure 5-3 B, n = 6). It should be noted the maximal effect of CRF on VTA dopamine neurons in mice undergoing the DiI injection surgery (Figure 5-3) was nearly half of that observed with mice that did not have surgery (Figure 5-1), which could potentially arise from age-related differences in the mice used or from long-lasting effects from the surgery. Thus, the lack of effect of cocaine on the CRF-mediated increase in NMDAR currents may in part be explained by the already truncated response to CRF in mice that underwent surgery. In summary, these findings indicate that CRF potentiated NMDAR currents in both mesoaccumbens and mesocortical neurons.

Further experiments on how CRF potentiated NMDAR currents in VTA dopamine neurons

The original study demonstrating that CRF potentiates NMDAR currents in VTA dopamine neurons suggested that CRF only affects a subpopulation of neurons. It was argued that two populations of VTA dopamine neurons existed: those with large I_h currents (high I_h, > 100 pA for a 250 ms hyperpolarizing step from -70 mV to -130 mV)

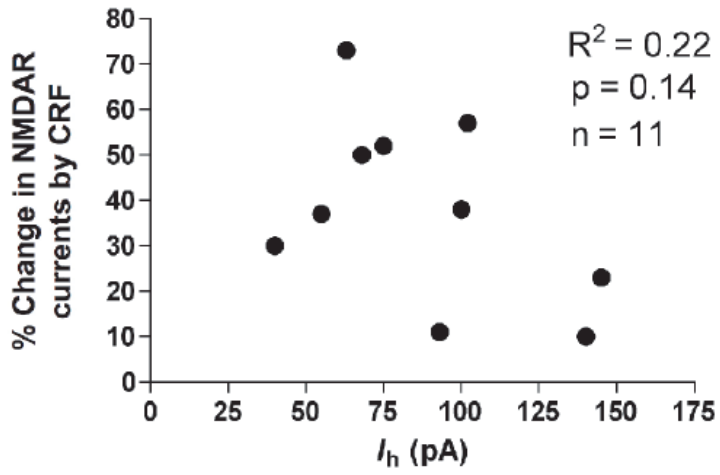

Figure 5-4: No correlation exists between the magnitude of the I_h and the observed potentiation of NMDAR currents by CRF in VTA neurons. Data is plotted from mice receiving a single saline injection prior to recordings.

and those with small I_h currents (low I_h, < 100 pA from a 250 ms hyperpolarizing step from -70 mV to -130 mV). It was further suggested that the ability of CRF to potentiate NMDAR currents roughly corresponded to the I_h, where high I_h neurons responded to CRF while low I_h neurons did not respond to CRF (Ungless et al., 2003). However, when analyzing my data from the saline injected mice, I found no significant correlation between the magnitude of the I_h and the effect of CRF on NMDAR currents (Figure 5-4, $n = 11$, $R^2 = 0.22$, $p > 0.05$).

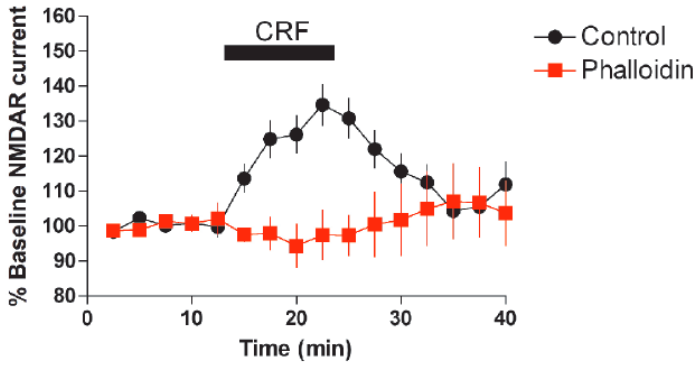

Figure 5-5: Actin depolymerization is required for CRF to increase NMDAR currents in VTA dopamine neurons. Control data is summary from 11 recorded neurons of saline injected mice. Phalloidin data is summary of 4 neurons recorded with 1 μM phalloidin in the internal recording solution

In another line of study, I further examined the intracellular mechanism by which CRF to potentiated NMDAR currents in VTA dopamine neurons. CRF induces the growth of actin filaments in cell culture systems (S Bartlett, personal communication) and actin depolymerization is required for the trafficking of NMDARs (Morishita et al., 2005), therefore I posited that CRF involves actin depolymerization to potentiate NMDAR currents. In agreement with this hypothesis, including an actin stabilizer in the internal recording solution, phalloidin (1 μM), completely abolished the ability of CRF to

potentiate NMDAR currents in VTA dopamine neurons (Figure 5-5, 0.4 ± 9.2 % over baseline, n = 4).

Discussion:

The present findings further the knowledge regarding the interaction of CRF on VTA dopamine neurons. First, I demonstrated that a single injection of cocaine given 24 hr prior to brain slice recordings significantly reduced the ability of CRF to potentiate NMDAR currents in dopamine neurons. Next, I found that both mesoaccumbens and mesocortical projecting VTA dopamine neurons both were affected by bath application of CRF. In contrast to a previous study, I determined that the I_h did not correlate with a neuron's ability to be affected by CRF. Finally, I found that stabilization of actin prevented CRF from enhancing NMDAR currents.

Since bath application of both CRF and cocaine increases NMDAR currents on VTA dopamine neurons through different mechanisms (Schilstrom et al., 2006; Ungless et al., 2003), I hypothesized that an *in vivo* exposure to cocaine would facilitate the ability of CRF to alter NMDAR currents. Instead, cocaine blunted the effect of CRF on glutamate currents of VTA dopamine neurons. Cocaine injections produce excitatory synaptic changes onto VTA dopamine neurons that can persist for at least 5 days (Borgland et al., 2004; Ungless et al., 2001) . Perhaps the cocaine injections produced synaptic changes that prevented further alterations by CRF. The exact mechanism of the interaction between cocaine, CRF, and VTA dopamine neurons is unknown and remains a question for a further study.

The mesocortical projection is especially sensitive to stressors when compared to the mesoaccumbens projection in regards to dopamine release (Horger and Roth, 1996; Thierry et al., 1976). Thus, it was surprising that CRF potentiated NMDAR currents to similar levels when comparing mesocortical and mesoaccumbens projecting VTA dopamine neurons. However, it is interesting to note that the mice with DiI injections were not as sensitive to CRF application as mice without DiI injections. This result could arise age-related differences in the mice since the DiI injected mice were ~ 1 week older than mice not receiving DiI injections. Alternatively, the surgery may be a stressor that prevented further alterations in NMDAR currents by CRF in VTA dopamine neurons. To verify the validity of these explanations, it would be necessary to test the effect of CRF on age-matched control mice not receiving DiI injections. Unlike in non-DiI injected mice, cocaine injections did not reduce the effect of CRF on NMDAR currents from DiI injected mice. This observation could similarly be explained by age-related differences or long-lasting alterations resulting from the DiI injection surgery.

In the original report outlining how CRF potentiated NMDAR currents, it was suggested that CRF only affects a subset of VTA dopamine neurons that have relatively large I_h (Ungless et al., 2003). My results indicate that no direct correlation exists between the magnitude of I_h and the degree of the CRF-mediated enhancement of NMDAR currents. One difference between my work and the original study is that I injected mice with saline and the original study used naïve mice. However, this variation is likely not important as both the Ungless study and my work found a comparable degree of potentiation of NMDAR currents by CRF. Furthermore, the I_h varies considerably

after rupturing the cell membrane in whole-cell patch clamp recordings (personal observations), and as such it is difficult to compare the magnitude of I_h without first allowing for the cell to equilibrate to whole-cell recording conditions.

In another line of experiments, I sought to determine the role of actin in mediating the changes on NMDAR currents. Including an actin stabilizer in the recording solution prevented the effect of CRF on NMDAR currents, suggesting actin plays an obligatory role in the potentiation of glutamate receptor currents of VTA dopamine neurons. This finding is consistent with a role of actin in various neuronal processes (Morishita et al., 2005).

The findings from this study described an interaction between cocaine, CRF and NMDAR currents of VTA dopamine neurons. NMDAR currents on VTA dopamine neurons are not only required for increasing excitatory synaptic strength (Bonci and Malenka, 1999; Ungless et al., 2001), but may also have behaviorally relevant roles in drug-associated behaviors (Kalivas and Alesdatter, 1993). In addition, activation of NMDAR currents is necessary for dopamine neurons to fire in action potential bursts (Overton and Clark, 1997). Burst stimulations of dopamine neurons can evoke dopamine release that is nearly six times greater than pacemaker stimulations (Gonon, 1988). Thus, the CRF-mediated increase in NMDAR currents in VTA dopamine neurons could affect plasticity, behavior, and dopamine release.

Acknowledgements:

I would also like to thank Anto and the rest of the members of the Bonci lab for their insightful discussion. I also thank Elyssa for instructing me on the DiI injection surgeries. I also would like to thank Melissa and Gilda for their help with the surgeries. This work was supported by the State of California for medical research on alcohol and substance abuse through the University of California, San Francisco (A.B) and the National Institute on Drug Abuse 5R01 DA016782-04 (A.B), 1F31 DA21464-01 (M.J.W.).

Chapter 6. Using fast-scan cyclic-voltammetry to detect changes in phasic dopamine release by intra-VTA injections of CRF

Abstract:

Dopamine neurons fire action potentials either in a pacemaker-like pattern or in bursts. The pacemaker-like firing mode is thought to contribute to basal or tonic dopamine levels. In contrast, the bursts of dopamine neuron firing are dependent upon NMDAR activation, and are likely important for phasic and more temporally precise dopamine release. Stressful stimuli increase dopamine levels throughout the brain, which may in part be due to the actions on VTA dopamine neurons by the stress-released neuropeptide, CRF. CRF increased the pacemaker-like firing of VTA dopamine neurons that could contribute to basal or tonic dopamine levels. In addition, CRF potentiated NMDAR currents in these neurons, which could increase the burst firing, as well as the phasic dopamine release events. Fast-scan cyclic-voltammetry is an electrochemical technique that allows for the *in vivo* detection of both phasic dopamine release as well as short-term tonic increases in dopamine levels. Given the excitatory role of CRF on VTA dopamine neurons, I sought to determine the effects of intra-VTA CRF injections on dopamine release in the NAcc using fast-scan cyclic-voltammetry. First, I verified the functional coordinates of the VTA while stimulating an ascending brainstem pathway. Although a number of methodological considerations need to be addressed, I surprisingly found that CRF decreased the number of phasic dopamine events relative to vehicle injections.

Introduction:

Midbrain dopamine neurons fire action potentials in two distinct patterns: single spike or pacemaker-like firing and burst firing (Grace and Bunney, 1983; Grace et al., 2007). Dopamine neurons fire action potentials around 2-10 Hz in the pacemaker-like firing mode (Grace and Bunney, 1983), which is thought to give rise to the tonic or basal levels of dopamine with concentrations ranging from 5-20 nM (Parsons and Justice, 1992). Dopamine neurons fire action potentials up to 15-30 Hz *in vivo* when in the burst-firing mode (Grace and Bunney, 1983). Bursts of dopamine neuron firing is thought to give rise to phasic elevated dopamine levels that can reach as high as 1 μM (Garris et al., 1997; Gonon, 1988). To fire in a burst, dopamine neurons require glutamatergic input, activation of NMDARs, opening of high-threshold calcium currents, and finally activation of calcium-activated potassium currents to terminate the burst (Overton and Clark, 1997). Furthermore, activation of brainstem nuclei such as the pedunculopontine tegmental nucleus (PPT) and the lateral dorsal tegmental nucleus (LDT) are involved with dopamine neuron burst generation (Lodge and Grace, 2006; Overton and Clark, 1997) and dopamine release in the NAcc and striatum (Forster and Blaha, 2000; Forster and Blaha, 2003).

A number of methods exist to detect dopamine levels in awake-behaving animals. One of the most commonly used methods is microdialysis, which has excellent selectivity and sensitivity for analyte detection (Heien and Wightman, 2006). However, microdialysis suffers from poor temporal resolution (minutes), and is better suited to detect tonic or basal dopamine levels (Heien and Wightman, 2006). In contrast,

electrochemical techniques offer excellent temporal resolution but offer poor analyte selectivity (Heien and Wightman, 2006). A variety of electrochemical detection methods have been developed including amperometry and fast-scan cyclic-voltammetry (FSCV). Amperometry involves applying a constant potential to a carbon-fiber electrode and analyzing the current as a function of time (Heien and Wightman, 2006). Amperometry has microsecond temporal resolution and is often used to study the kinetics of dopamine release and reuptake; however, this technique is non-selective since any oxidized compound will be detected with amperometry recordings (Heien and Wightman, 2006).

FSCV is similar to amperometry except that the electrode potential is ramped from -0.4 V to + 1.3 V and back down to -0.4 V at a rates greater than 100 V / s (Heien and Wightman, 2006). The rapid cycling of the voltage creates a large but stable background current on the carbon-fiber electrode (Heien and Wightman, 2006). Subtracting out the background current allows for the detection of electroactive compounds such as dopamine, on the millisecond timescale (Heien and Wightman, 2006). Another benefit of FSCV is that the analyte is both oxidized and reduced, so not only is the analyte recycled in theory, but there are potentially two electrochemical signatures for a given compound: an oxidation current and a reduction current. For example, dopamine is oxidized to dopamine-*o*-quinone that elicits an oxidizing current at about +0.6 V. Dopamine-*o*-quinone is then reduced to dopamine, which elicits a reduction current at about -0.3 V. FSCV can be used to record dopamine changes in awake-behaving rodents (Heien et al., 2005; Phillips et al., 2003), and is capable of

detecting not only phasic dopamine release events but also relatively rapid (~90 s) basal changes in dopamine levels (Heien et al., 2005).

Dopamine voltammetry recordings require a stimulating electrode in a region that evokes dopamine release in order to verify that the voltammetry electrode has been lowered to an optimal location for recordings. Most dopamine voltammetry studies elicit dopamine release by placing the stimulating electrode in the anterior VTA/SN (Heien et al., 2005; Phillips et al., 2003). However, if one wants to study pharmacological manipulations of the VTA, it would not be ideal to inject in drugs and stimulate dopamine release from the same location, since the stimulating electrode could damage the tissue near to the injection site. In this regard, it is necessary to identify a distinct pathway that when stimulated, evokes dopamine release in the NAcc.

The PPT and LDT send afferent glutamatergic, cholinergic, and GABAergic projections to midbrain dopamine neurons (Oakman et al., 1995; Omelchenko and Sesack, 2005). Although the SN primarily receives input from the PPT, the VTA receives input from both the LDT and the caudal PPT (Oakman et al., 1995). This ascending brainstem projection is physiologically relevant as both the LDT and PPT are involved with dopamine neuron burst firing (Lodge and Grace, 2006; Overton and Clark, 1997). Furthermore, amperometry recordings using extendend stimulation protocols (1 min) of the PPT (Forster and Blaha, 2003) or the LDT (Forster and Blaha, 2000) produced long-lasting changes in dopamine levels (1 hr) in the striatum and NAcc, respectively. Together, these studies indicate that the LDT and PPT input onto the midbrain dopamine

system could be a good candidate stimulation location to elicit dopamine release in the NAcc.

Stress increases dopamine levels throughout the brain (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996), which may in part be due to the actions on VTA dopamine neurons by the stress-released neuropeptide, CRF. In brain slice recordings, CRF increased VTA dopamine neuron firing (Chapter 2), and potentiated NMDAR currents (Chapter 5). I hypothesized that intra-VTA injections of CRF would increase basal dopamine levels in the NAcc since CRF increased pacemaker-like firing. Additionally, since CRF potentiated NMDAR currents, and NMDAR currents are critical for the burst firing of dopamine neurons, I hypothesized that intra-VTA injections of CRF would increase the number of phasic dopamine release events in the NAcc. I first verified that a functional connection exists between the LDT/PPT, the VTA, and the nucleus accumbens. Next, I used FSCV in awake-behaving rats to assay dopamine release in the NAcc in response to intra-VTA injections of CRF. Surprisingly, I found that CRF inhibited the number of phasic dopamine release events compared to vehicle injections, though a number of methodological issues need to be addressed.

Materials and Methods:

Surgery

All procedures conformed to National Institutes of Health and University of Washington animal care policy standards. 250-350g Sprague-Dawley rats (Harlan) were

anesthetized with urethane for PPT/LDT stimulation experiments and with isofluorane for recovery experiments, and placed in a stereotaxic apparatus. A voltammetry electrode hub was implanted over the NAcc core at AP + 1.3 mm, ML + 1.3 mm from bregma. For PPT/LDT stimulation studies, a bipolar (1 mm separation) stimulating electrode was centered at AP - 8.0 mm, ML + 1.5 from bregma, and lowered between -6.5 mm and -7.5 mm from the top of the skull. For recovery experiments, a guide cannula was cemented into the VTA (from bregma: AP -5.6 mm, ML 0.5 mm, DV -7.0 mm). The injector extended 1 mm beyond the guide cannula. Also in recovery experiments, a bipolar stimulating electrode was implanted to stimulate the medial forebrain bundle (MFB; from bregma: AP -4.6 mm, ML +1.4 mm, DV -8.0 to -8.5 mm).

Voltammetry recordings

Dopamine was detected by oxidizing it with a carbon-fiber electrode using FSCV. The electrode was held at -0.4 V against Ag/AgCl between scans and then periodically driven to +1.3 V and back in a triangular fashion at 400 V / s. Scans were repeated every 100 ms. For analyte identification, current during a voltammetric scan was plotted against the applied potential to yield a cyclic voltammagram. Once dopamine had been voltammetrically verified, the current at its peak oxidation potential was plotted against time to reveal the temporal profile of the dopamine concentration changes.

On experimental days, the rats were placed in the recording chamber and a voltammetry electrode was slowly lowered into the brain. In order to find the optimal location for the voltammetry electrode, I stimulated the MFB (24 pulses, 60 Hz, up to 400

μA) to evoke dopamine release in the NAcc while lowering the electrode. If no stimulated release was observed, I looked for phasic dopamine release events to use as a cyclic voltammegram template for the identification of further dopamine release events.

Experiments consisted of a 1 hr habituation to the recording chamber. The rats were then temporarily disconnected from the recording apparatus to give intra-VTA 0.5 μL injections of CRF (0.42 nmole), oCRF (0.42 nmole), or vehicle (in mM: 154.7 Na^+, 2.9 K^+, 132.49 Cl^-, 1.1 Ca^{2+} at pH = 7.4). Injections were visually monitored and completed within 4 minutes. The rats were then reconnected to the voltammetry recording apparatus and placed back into the recording chamber for another 2 hrs.

Results:

FSCV data analysis

An example of stimulated dopamine release detected by FSCV is presented in Figure 6-1. The pseudocolor plot represents the observed changes in the oxidation and reduction currents over 15 s. On the x-axis is time, and on the y-axis is the observed current during the triangular voltage sweep, where both the top and bottom correspond to -0.4 V and the middle corresponds to + 1.3V. Each line on the y-axis represents a single voltage sweep that was taken every 100 ms. Oxidation currents are green while reduction currents are blue/black. Below the pseudocolor plot is the time course of dopamine changes in response to the electrical stimulation (red box). This measure is calculated by following the changes to the dopamine oxidation peak over

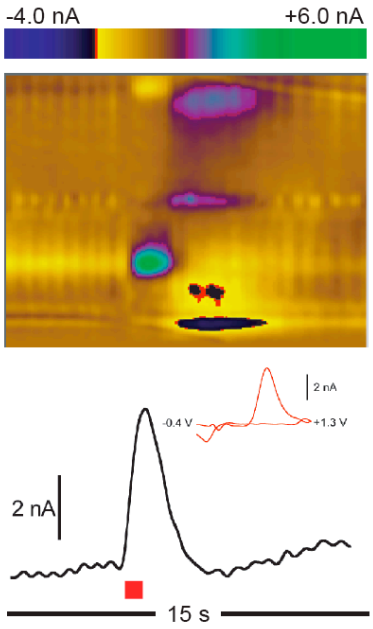

Figure 6-1: An example cyclic voltammegram of stimulated dopamine release in the NAcc. The pseudocolor plot represents the observed changes in the oxidation and reduction currents as a function of time. Below the pseudocolor plot is the time course of dopamine oxidation peak changes in response to the electrical stimulation (red box). The red traced inset is the cyclic voltammagram for dopamine that was observed during the peak of dopamine release

time. The red traced inset is the cyclic voltammagram for dopamine that was observed during the peak of dopamine release. Voltammetry data in this chapter is presented in a similar manner.

Functional connectivity of the LDT/PPT, the VTA, and NAcc

Given the anatomical (Oakman et al., 1995; Omelchenko and Sesack, 2005), physiological (Lodge and Grace, 2006; Overton and Clark, 1997), and electrochemical evidence (Forster and Blaha, 2000; Forster and Blaha, 2003), I chose to place one arm of the bipolar stimulating electrode in the caudal PPT and the other near the LDT (Figure 6-2; from bregma: AP -8.0mm, ML +1.0 mm and +2.0 mm, DV -6.5 mm to -7.5 mm) to elicit dopamine release in the NAcc. In an example urethane anesthetized rat, a 60 pulse, 60 Hz, 200 μA stimulation transiently increased dopamine levels in the NAcc (Figure 6-1), demonstrating a functional interaction between stimulation of the LDT/PPT and

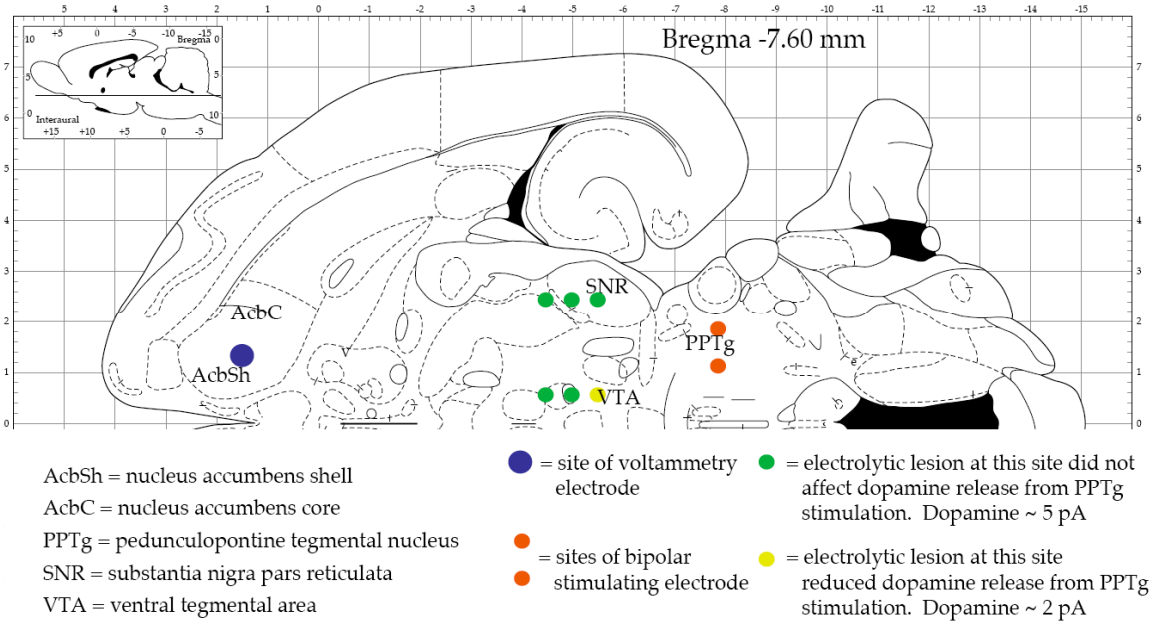

Figure 6-2: Location of the voltammetry electrode, stimulating electrode, and lesion sites with their effect on dopamine release from a representative experiment. Adapted from Paxinos and Watson, 1998.

dopamine release in the accumbens.

To further characterize the connection between the LDT/PPT and NAcc, I sought to temporarily inactivate the VTA with an injection the $GABA_B$ agonist, baclofen (50 ng, 0.5 μL, 250 μL / min), and observe a simultaneous decrease in the evoked dopamine release due to LDT/PPT stimulation. Original attempts at baclofen injections using the coordinates for the anterior VTA (from bregma: AP -5.2 mm, ML: +1.0mm, DV -7.5 mm), that are often used for evoked dopamine release in voltammetry studies (Heien et al., 2005; Phillips et al., 2003), produced no changes in dopamine release (data not shown). Thus, it was necessary to identify the anatomical location of the dopamine cells receiving the LTD/PPT input. In an example urethane anesthetized rat, electrolytic lesions did not affect dopamine release at a number of locations when using a 60 pulse, 60 Hz, 400 μA stimulation of the LDT/PPT (Figure 6-2; relative to bregma and -8.0 mm

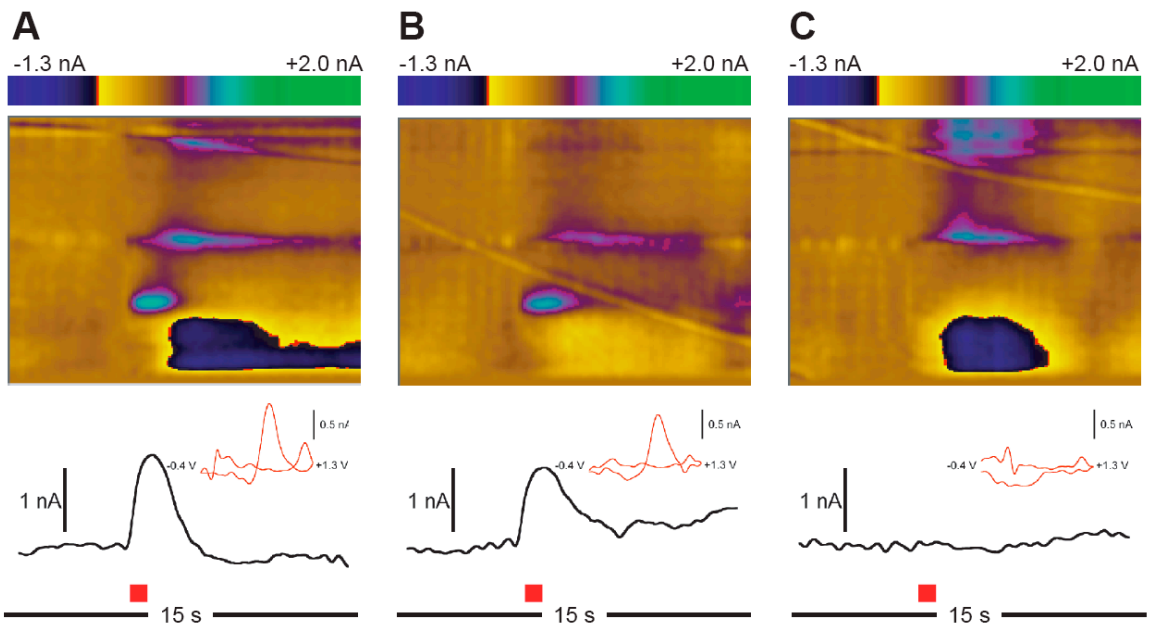

Figure 6-3: Aspiration lesion of the VTA eliminated the dopamine release in the NAcc due to LDT/PPT stimulation (60 pulses, 60 Hz, 200 μA). Lesions were focused at AP -5.6 mm, ML +/- 0.5 mm relative to bregma. (A) LDT/PPT stimulated dopamine release in the NAcc before lesions. (B) A lesion -4.0 mm from the skull did not affect the LDT/PPT stimulated dopamine release. (C) A lesion -8.0 mm from the skull eliminated LDT/PPT stimulated dopamine release.

dorsal to the skull [AP in mm, ML in mm]: [-4.5, +2.5], [-4.5, +0.5], [-5.0, +2.5], [-5.0, +0.5], and [-5.5, +2.5]). However, an electrolytic lesion at AP -5.5 mm, ML +0.5 mm, DV -8.0 mm attenuated the dopamine release due to LDT/PPT stimulation.

To further verify the functional coordinates between the LDT/PPT, the VTA, and the NAcc, in another rat I performed aspiration lesions at various depths in the brain that were focused at AP -5.6 mm and ML +0.5 mm relative to bregma. Aspiration lesions in the contralateral hemisphere (Figure 6-3A) or 4.0 mm ventral to the skull (Figure 6-3B) did not affect the dopamine release to a 60 pulse, 60 Hz, 200 μA stimulation of the LDT/PPT. In contrast, aspiration lesions 8.0 mm ventral to the skull completely eliminated the dopamine response to LDT/PPT stimulation (Figure 6-3C).

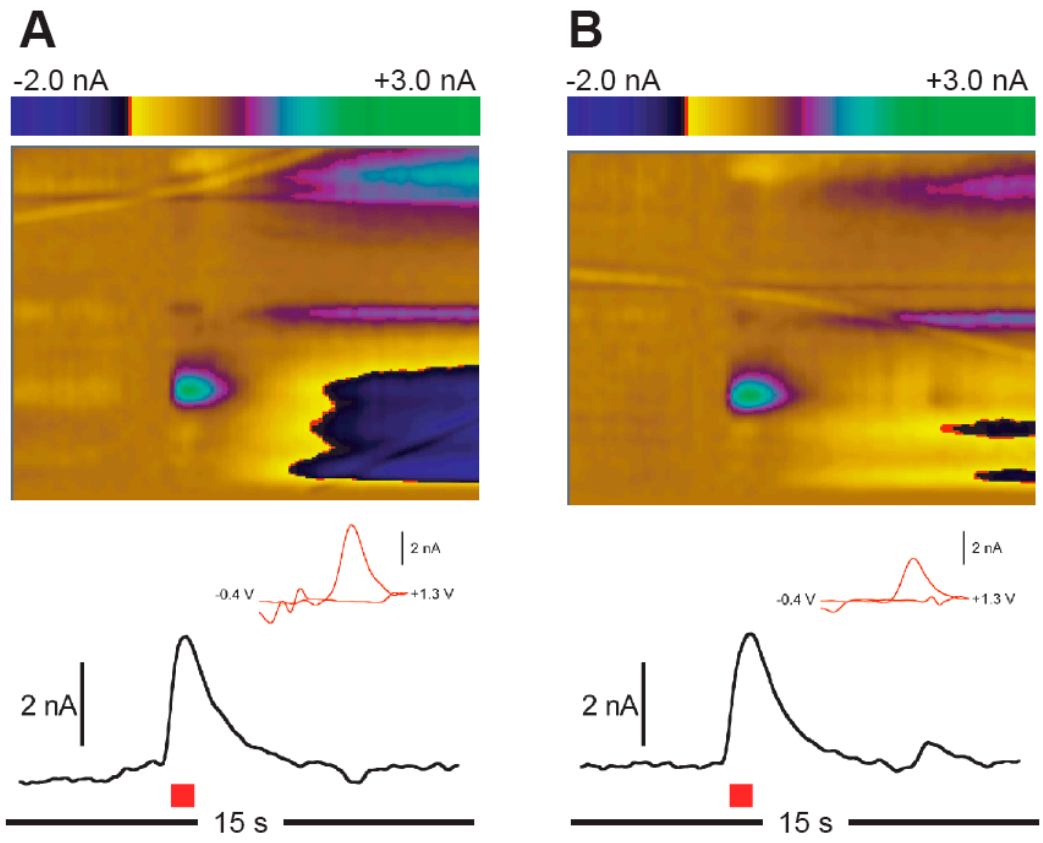

Figure 6-4: Intra-VTA aCSF injections did not affect evoked dopamine release from LDT/PPT stimulations (60 pulses, 60 Hz, 400 μA). (A) LDT/PPT stimulated dopamine release in the NAcc. (B) LDT/PPT stimulated dopamine release in the NAcc 6 minutes after 0.5 μL injections at 250 μL/min of aCSF in the VTA (AP -5.6mm, ML +0.5 mm, DV -8.0 mm from bregma).

Since I had now verified the correct functional coordinates of the VTA mediating the dopamine release in the NAcc in response to LDT/PPT stimulation, I next tested whether injections of pharmacological agents into the VTA could modulate the dopamine response. As would be expected, 0.5 μL injections at 250 μL/min of vehicle into the VTA did not affect the evoked dopamine response (Figure 6-4, LTD/PPT stimulation with 60 pulses, 60 Hz, 400 μA). However, 50 ng injections of baclofen reversibly attenuated the dopamine response to LTD/PPT stimulation (Figure 6-5, stimulation of 60 pulses, 60 Hz, 200 μA). Together, these findings indicate that stimulation of the LDT/PPT evoked dopamine release in the NAcc, which required the VTA. This finding demonstrated that

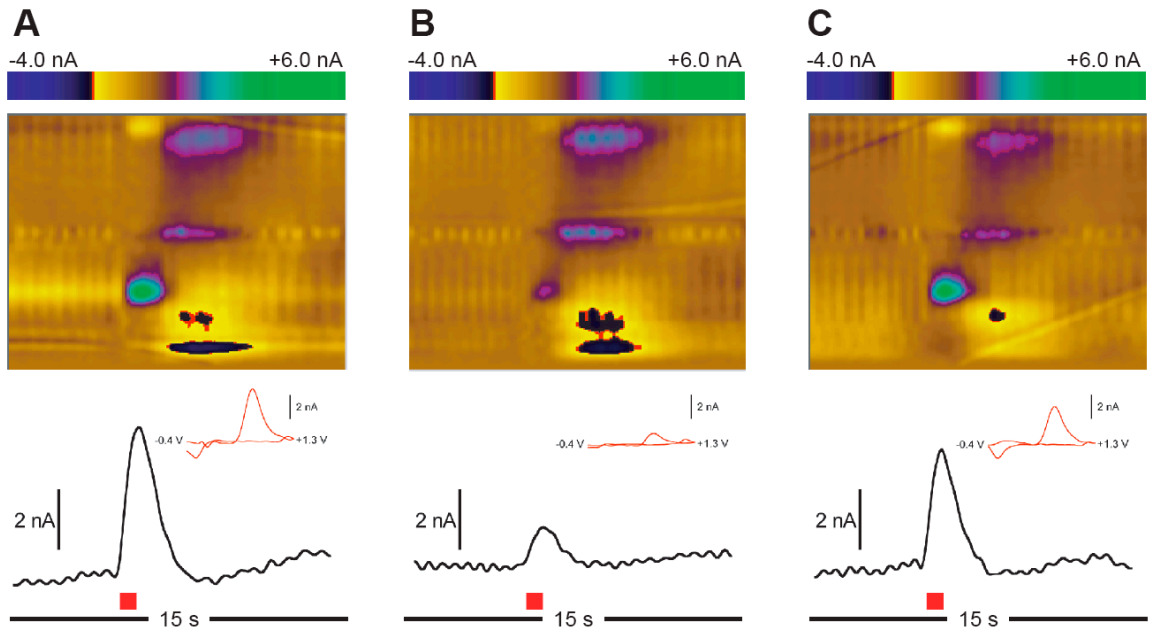

Figure 6-5: Intra-VTA baclofen injections reversibly inhibited evoked dopamine release from LDT/PPT stimulations (60 pulses, 60 Hz, 200 μA). (A) LDT/PPT stimulated dopamine release in the NAcc. (B) LDT/PPT stimulated dopamine release in the NAcc 6 minutes after 0.5 μL injections at 250 μL/min of 50 ng baclofen in the VTA (AP -5.6mm, ML +0.5 mm, DV -8.0 mm from bregma). (C) LDT/PPT stimulated dopamine release in the NAcc 48 minutes after baclofen injection.

FSCV could be used in pharmacological studies of the VTA, without the concern of possible tissue damage of the VTA due to local stimulations.

The effect of intra-VTA injections of CRF on phasic dopamine release

Based upon the studies described above, I attempted FSCV experiments in awake-behaving rats using the LDT/PPT stimulation. However, LDT/PPT stimulation elicited an aversive and drastic behavioral response that prevented the further use of this experimental setup in awake-behaving rodents. Instead, I stimulated the medial forebrain bundle (MFB), which contains the axons of midbrain dopamine neurons. This experimental approach is not ideal since the stimulation and the CRF injections would be presumably affecting the same neurons, although in different locations.

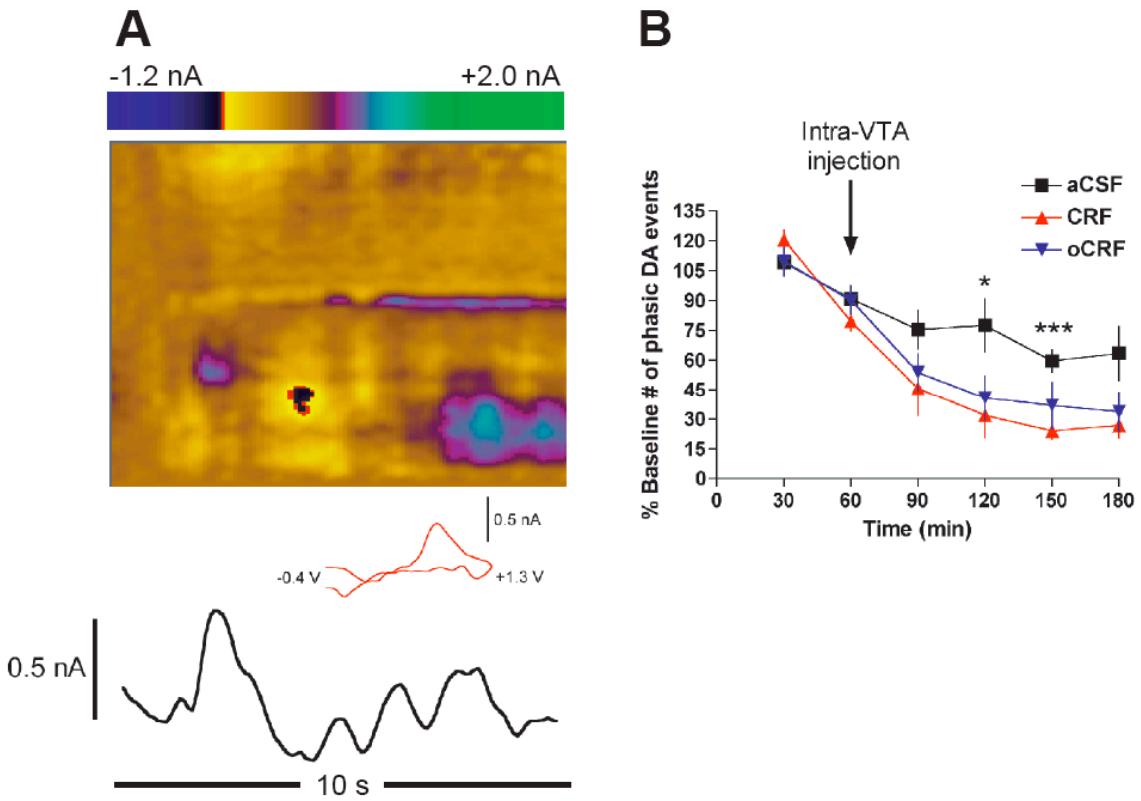

Figure 6-6: The effect of intra-VTA CRF injections of phasic dopamine release events in the NAcc. (A) An example phasic dopamine event detected in the NAcc. (B) Summary of changes in the number of phasic dopamine events in 30 min bins after intra-VTA injections of aCSF (n = 12 rats), CRF (n = 7 rats), and oCRF (n = 8 rats). Significant decreases relative to aCSF injected rats were only observed with CRF injected rats. *, ** $p < 0.05, 0.001$ relative to aCSF injected rats

After lowering the voltammetry electrode into the NAcc, rats were allowed to habituate in the chamber for 1 hr before receiving injections of the aCSF vehicle, oCRF (0.42 nmol), or CRF (0.42 nmol) into the VTA. The rats then spent another 2 hours in the recording chamber after the injection. Phasic dopamine events (Figure 6-6A) were compiled into 30 minute bins and analyzed throughout the 3 hr session in the recording chamber. The number of phasic dopamine events decreased over time in all groups (Figure 6-6B). Interestingly, injections of CRF (n = 7) and oCRF (n =8) appeared to reduce the number of observed phasic dopamine events to a greater extent than aCSF (n = 12) injections (Figure 6-6B). However, this trend was only significant for CRF injections

at 60 and 90 minutes post-injection (Figure 6-6B). In contrast to my hypothesis, intra-VTA CRF injections reduced the number of phasic dopamine events.

Discussion:

The first major finding from this study is that the VTA was required for dopamine release in the NAcc to be detected by FSCV in response to stimulation of the LDT/PPT. Previous anatomical (Oakman et al., 1995; Omelchenko and Sesack, 2005), electrophysiological (Lodge and Grace, 2006; Overton and Clark, 1997), and electrochemical studies (Forster and Blaha, 2000; Forster and Blaha, 2003) suggested a functional interaction between the LDT/PPT, the VTA, and dopamine release in the NAcc. Stimulation of the LDT/PPT allows for pharmacological studies of the VTA using FSCV without the concern of possible damage in the VTA due to local stimulations. Unfortunately, because stimulation of the LDT/PPT elicits drastic behaviors in awake-behaving rats, experiments involving direct activation of the LDT/PPT are limited to anesthetized studies.

The other finding from these experiments was that intra-VTA injections of CRF reduced the observed number of phasic dopamine events in the NAcc relative to injections of aCSF. Dopamine neurons fire action potentials in a pacemaker-like manner, which is thought to give rise to basal or tonic dopamine levels (Grace and Bunney, 1983; Grace et al., 2007). Alternatively, dopamine neurons fire action potentials in NMDAR-dependent bursts that is thought to contribute to phasic dopamine events (Grace et al., 2007; Overton and Clark, 1997). The stress-released neuropeptide, CRF, increased VTA

dopamine neuron firing (Chapter 2) and potentiated NMDAR currents (Chapter 5). Thus, I hypothesized that CRF would increase both tonic and phasic dopamine levels in the NAcc. To address this issue I used FSCV, which is an electrochemical technique that can detect both tonic (on the order of minutes) and phasic (ms) changes in dopamine release (Heien et al., 2005; Phillips et al., 2003). Because of the extended duration of these experiments (3 hrs), it was impractical to use FSCV to assess tonic changes in dopamine levels by intra-VTA CRF injections, and will be a question better addressed using microdialysis. Surprisingly, I found that intra-VTA injections of CRF decreased the number of observed phasic dopamine events in the NAcc, but a number of methodological concerns could potentially explain this unexpected result.

The primary concern with the phasic dopamine event data is that the number of detected phasic events decreased as a function of time, suggesting that FSCV may not be an ideal electrochemical technique for prolonged recordings. Although not quantified in the current study, I observed changes in the background voltammetry current in a number of experiments, which could have prevented the accurate detection of phasic dopamine events. However, recent advances in FSCV recordings have led to the development of chronically implanted voltammetry electrodes (P Phillips, personal communication). Chronic voltammetry electrodes allow for stable and long-duration voltammetric recordings, potentially minimizing the alterations in the background voltammetry current that can be observed with acute *in vivo* recordings, such as in the current study.

The intra-VTA drug injection process highlights another methodological concern with these experiments. In order to access the injection cannula, the rat had to be

unplugged from the voltammetry recording apparatus. Often upon reconnecting the rat to the voltammetry recording device after injections, the observed voltammetry recordings were not stable (~ 5 minutes), which prevented the accurate detection of phasic dopamine events during this time. Furthermore, intra-VTA drug injections required immobilizing the rat, which is an obvious confound, as this manipulation is a known stressor. Future experiments should employ chronic voltammetry electrodes and involve intra-VTA injections that do not require immobilization of the rodent.

Another possible issue with interpreting the data regarding the CRF-dependent modulation of phasic dopamine release events involves the mechanism by which phasic events are detected. Dopamine events are identified if the resulting difference between the analyzed voltammagram and the voltammagrams preceding it match a template cyclic voltammagram for dopamine. However, if basal dopamine levels are elevated, it may be difficult to observe further phasic changes in dopamine release. To summarize, although the intra-VTA injections of CRF reduced the number of phasic dopamine events in the NAcc, these results are difficult to interpret given a number of methodological issues. Further experiments using *in vivo* microdialysis, and chronically implanted voltammetry electrodes are required to definitively address the role of CRF on dopamine outflow in the NAcc.

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Chapter 7. Summary, future directions, and discussion

Stress not only stimulates dopamine release in brain regions receiving VTA input (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996), but also releases CRF throughout the brain, including into the VTA (de Kloet et al., 2005; Wang et al., 2005). This suggests that that CRF could be involved with the stress-induced increase of dopamine levels; however, the nature of the interaction between CRF and VTA dopamine neurons is not well understood. The goal of this dissertation was to characterize the effect of CRF on VTA dopamine neurons and to examine the behavioral consequences of injecting CRF into the VTA. Below is a summary and potential future directions for each of the experiments described in this thesis.

Chapter 2: CRF increased the firing of VTA dopamine neurons

In order to study the effect of CRF on VTA dopamine neurons, it is first necessary to be able to confidently identify dopamine-producing cells. Although dopamine neurons can be identified with immunhistochemical staining for TH, it is advantageous to know the neurotransmitter content of the recorded neuron with electrophysiological recordings prior to beginning experimental manipulations. Classically, the presence of the I_h has been used to identify dopamine neurons in a number of rodent species (Cameron et al., 1997; Ford et al., 2006; Grace and Onn, 1989), but a recent study questioned the validity

of this link in rats (Margolis et al., 2006). In contrast to the findings in rats, I found that the I_h is indeed an excellent predictor of dopamine content in mice.

Since I could confidently identify dopamine neurons based on the presence of the I_h, I was able to address how CRF affected the firing of VTA dopamine neurons. I found that CRF dose-dependently increased dopamine neuron firing. Using a combination of pharmacology and genetically-modified mice, I determined that CRF required the CRF-R1, and not the CRF-R2 or CRF-BP to increase the firing rate. Although CRF receptors predominately couple to the cAMP – PKA pathway (Hauger et al., 2006), I instead found that the PLC – PKC pathway is required for CRF to increase VTA dopamine neuron firing. I also demonstrated that the CRF-mediated enhancement of firing did not involve, calcium currents, the $I_{K(Ca)}$, the slow I_A, or I_{Kir}. In contrast, the I_h is required for CRF to increase dopamine neuron firing. Further supporting a role of the I_h in regulating firing frequency, high concentrations of the specific I_h antagonist (30 μM ZD-7288) reduced VTA neuron firing. Although the I_M antagonist attenuated the effect of CRF, blocking I_M by itself did not change dopamine neuron firing. This suggests that either the I_M antagonist non-specifically prevented the CRF-mediated increase in firing, or that I_M plays a permissive role the mechanism by which CRF increases VTA dopamine neuron firing.

These findings highlight future projects that would further characterize the effects of CRF on VTA dopamine neurons. Previous work demonstrated that agonists of the CRF-R2 and CRF-BP potentiate NMDAR currents though activation of the PLC-PKC pathway (Ungless et al., 2003). My findings demonstrated that CRF-R1 stimulation also

activates the PLC-PKC pathway. This suggests that in VTA dopamine neurons (i) CRF-R1 and CRF-R2/CRF-BP signaling complexes exist in distinct spatial domains or (ii) CRF-R1 and CRF-R2/CRF-BP signal through different PLC or PKC isoforms. When reliable CRF receptor antibodies become available, microscopy studies could verify if the different CRF receptors exist in different domains on the cell surface. Future experiments using PKC or PLC isoform specific inhibitors or transgenic mice could verify if different isoforms mediate the effect of CRF on firing rate and NMDAR currents. Using mice deficient for PKCδ and PKCε, I demonstrated that these PKC isoforms are likely not involved in mediating the increase in firing rate by CRF. Though, it is necessary to test the involvement of these PKC isoforms on the ability of CRF to affect NMDAR currents.

Additionally, ion currents other than I_h could be involved with increasing VTA dopamine neuron firing by CRF. Pharmacological experiments suggested that the I_M could be necessary, but this current is definitely not sufficient to modulate the firing rate of dopamine neurons. Furthermore, the ionic target(s) of CRF could be functionally coupled as this type of interaction has been described in VTA dopamine neurons (Wolfart and Roeper, 2002). In summary, CRF required the CRF-R1, the PLC-PKC pathway, and the I_h to increase VTA dopamine neuron firing, but which PKC isoform is involved and other potential affected conductances questions for future studies.

Chapter 3: CRF enhanced I_h through a PKC-dependent mechanism.

Based upon the findings in Chapter 2, I wanted to examine if CRF application on VTA dopamine neurons directly affected the I_h and if this involved PKC signaling. Indeed, CRF increased I_h, but this effect was not associated with any changes in the voltage-dependence of activation for the I_h. Although the I_h is modulated by voltage, cAMP, and protein kinases (DiFrancesco and Tortora, 1991; Frere et al., 2004; Zong et al., 2005), no studies to date have identified a PKC-dependent enhancement of the I_h. I found that PKC inhibitors prevented the effect of CRF on I_h. Furthermore, PKC activators were also able to increase the I_h without changing the voltage-dependence of activation. Together, these results demonstrated that CRF, through activation of PKC, enhanced I_h in VTA dopamine neurons.

Although these experiments demonstrated a role for PKC in enhancing I_h, the substrate for phosphorylation by PKC is unknown. Src kinases phosphorylate tyrosine residues of the HCN channels to affect the gating properties of I_h when transfected into HEK 293 cells (Zong et al., 2005). However, this has been the only demonstration of a direct phosphorylation-dependent modulation of I_h. In addition, this mechanism is likely different from the one reported here since I observed no changes in the voltage-dependence of activation for the I_h. Other studies suggesting kinase regulation of I_h rely solely on pharmacological inhibition to validate their claims (Cathala and Paupardin-Tritsch, 1997; Liu et al., 2003; Vargas and Lucero, 2002). Alternatively, it has been suggested that the kinase-dependent modulation of the I_h could arise from phosphorylation of accessory proteins to the HCN channel subunits, or on proteins involved with the trafficking of ion channels (Frere et al., 2004). Thus, further research is

needed to specifically identify the target of PKC phosphorylation, whether it is the HCN channel subunit or another protein that in turn modulates the I_h. Regardless, these experiments are better suited to cell culture or oocyte recording systems.

Chapter 4: CRF-R1 agonists in the VTA required PKC activity and the I_h to increase locomotor activity in rats

Stress elicits a number of physiological changes in the brain including (i) increasing the firing of putative VTA dopamine neurons (Anstrom and Woodward, 2005), (ii) enhancing dopamine concentrations throughout the limbic system (Abercrombie et al., 1989; Inglis and Moghaddam, 1999; McFarland et al., 2004; Tidey and Miczek, 1996), and (iii) releasing CRF into the VTA (Wang et al., 2005). Taking these findings, along with the role of the VTA and dopamine release in motor behaviors (Beninger, 1983; Dunn et al., 2005; Zhou and Palmiter, 1995), CRF could activate the dopamine system to promote activity in response to stress. Supporting this hypothesis, increasing dopamine concentrations (Blanchard et al., 1999) and i.c.v. infusion of a CRF-R1 agonist (Yang et al., 2006) both promote escape from stressful/threatening stimuli. These findings provide a behaviorally relevant basis for studying the effects of CRF in the VTA on locomotion. Although the mechanism has not been well characterized, intra-VTA injections of CRF increased locomotor activity in rats (Kalivas et al., 1987). The goal of these experiments was to determine the if the mechanism by which CRF increases VTA dopamine neuron firing also accounts for the ability of CRF to increase locomotor activity.

Since accurate cannulation placements are more easily achieved in rats, and since the effective doses of CRF had already been calculated (Kalivas et al., 1987), I chose to pursue how CRF affected activity in the rat. Identical to the mouse, CRF increased putative rat VTA dopamine neuron firing in a PKC-dependent manner, suggesting a conservation of this excitatory effect by CRF across rodent species. I found that unilateral intra-VTA injections of CRF-R1 agonists, but not CRF-R2 agonists, were able to increase locomotor activity. Furthermore, antagonism of PKC activity or the I_h was able to significantly attenuate the effect of oCRF on locomotion. In summary, the mechanism by which CRF increased locomotor activity mirrors the mechanism by which CRF increased VTA dopamine neuron firing in a brain slice.

Although these findings suggest that intra-VTA injections of CRF excite dopamine neurons to stimulate dopamine release, which in turn is responsible for the increased activity, these experiments did not prove this claim. To further support this likely interpretation of the data, it would be necessary to demonstrate that the CRF-induced increase in activity is abolished by either lesioning the NAcc-projecting dopamine neurons or by locally injecting dopamine receptor antagonists into the NAcc.

Another set of future experiments could focus on how oCRF affected the ability of a rodent to escape from a predator when given directly into the VTA, as opposed to i.c.v. infusions of the drug, which has already been shown to promote escape (Yang et al., 2006). I hypothesize that intra-VTA injections of oCRF would increase the escape speed and attempts from a predator, and that this effect would be reduced by antagonism of

PKC activity or the I_h. The findings from these experiments would demonstrate whether the VTA is required for oCRF to elicit the enhanced avoidance behavior to a stressor.

Chapter 5: The interaction between cocaine, CRF, and NMDAR currents on VTA dopamine neurons

Previous work demonstrated that CRF (Ungless et al., 2003) and cocaine (Schilstrom et al., 2006) potentiated NMDAR currents in VTA dopamine neurons, though through different mechanisms. However, it was unknown how a single injection of cocaine would affect the ability of CRF to potentiate NMDAR currents. Surprisingly, cocaine injections blunted the average effect of CRF on NMDAR currents. This experiment is difficult to examine mechanistically, since one does not know the locus (or loci) of action after i.p. drug injections. It was possible that the effect of cocaine could be mediated by changes on specific subsets of VTA dopamine neurons, as the response to CRF was not completely abolished by cocaine injections. Previous work demonstrated that stress evokes greater changes in dopamine levels in the PFC than in the NAcc (Thierry et al., 1976). Since these experiments were studying the effects of a stress-released neuropeptide, I hypothesized that cocaine could have selectively affected either the mesocortical or mesoaccumbens projecting neurons. I found that CRF potentiated NMDAR currents in both mesocortical and mesoaccumbens neurons, though cocaine injections were without effect in mice undergoing DiI injection surgeries. Interestingly, the maximal effect of CRF on NMDAR currents was reduced in mice undergoing surgery. Both the reduced efficacy of CRF and the lack of effect of cocaine injections

could arise from using older mice, or from long-lasting effects of from the DiI injection surgery. Further experiments using age-matched non-surgery mice are required to fully interpret these results. However, if the surgery was responsible for affecting the efficacy of CRF on NMDAR currents, it raises the possibility that other classical laboratory models of stress could induce similar changes.

The other set of experiments from this chapter further examined the mechanism by which CRF potentiated NMDAR currents in VTA dopamine neurons. In contrast to a previous report (Ungless et al., 2003), I found that the magnitude of the I_h does not correlate with the increase in NMDAR currents by CRF. In addition, I found that inhibiting actin depolymerization prevented the effect of CRF on NMDAR currents. This suggests that through a PKC-dependent mechanism, CRF likely involves some trafficking event to potentiate NMDAR currents, although it is possible that the observed increase in current could arise from direct phosphorylation events as well. Further experiments using synapse-specific blockers of NMDAR channels could determine if the increase in current is due to the insertion of extra-synaptic NMDAR channels (Borgland et al., 2006).

Chapter 6: Using FSCV to detect changes in phasic dopamine release by intra-VTA injections of CRF

CRF increased the firing rate and NMDAR currents in VTA dopamine neurons, and in this regard dopamine levels are likely increased in brain regions receiving VTA input. Since the VTA sends a dense dopamine projection to the NAcc (Swanson, 1982)

and because FSCV allows for the rapid detection of dopamine changes (Heien and Wightman, 2006), I chose to address how intra-VTA injections of CRF affected dopamine release in the NAcc using FSCV. Contrary to my hypothesis, I observed a reduction in the number of phasic dopamine events after CRF injection. However, as discussed in detail in Chapter 6, there are a number of methodological caveats with the experimental paradigm used. Future experiments using FSCV should employ chronically implanted voltammetry electrodes and a drug-injection system that does not involve immobilization of the rat. Although FSCV is excellent at detecting phasic dopamine changes, assaying for long-lasting tonic changes in dopamine levels is better suited for microdialysis studies.

Conclusions:

This dissertation demonstrated that CRF increased the firing rate of VTA dopamine neurons. Further experiments identified that CRF acted on the CRF-R1 to stimulate the PLC-PKC pathway. PKC activation enhanced the I_h, which in turn was responsible for increasing VTA dopamine neuron firing. Mirroring the results from brain slice recordings, CRF-R1 agonists required PKC activity and the I_h to increase locomotor activity when injected into the VTA, providing a plausible mechanistic connection from ion currents to behavior. In addition, I further examined how CRF potentiated NMDAR currents in VTA dopamine neurons.

This thesis, along with a previous study, demonstrated that CRF excited VTA dopamine neurons through two distinct mechanisms: (i) to increase the firing rate through

activation of the CRF-R1 and (ii) to potentiate the efficacy of glutamate signaling by enhancing NMDAR currents through a mechanism involving the CRF-R2 and CRF-BP (Ungless et al., 2003). Collectively, these findings identify a link between dopamine and CRF, which together have been implicated in psychiatric disorders such as depression (Banki et al., 1987; Gershon et al., 2007; Valdez, 2006), drug abuse (Funk et al., 2007; McFarland et al., 2004; Wang et al., 2005), and schizophrenia (Banki et al., 1987; Beninger, 2006). CRF-R1 antagonists have garnered significant interest as antidepressants since depression can be associated with abnormal brain levels of both dopamine and CRF (Gershon et al., 2007; Valdez, 2006). My results may also provide a possible mechanism explaining how systemic injections of CRF-R1 antagonists prevent drug seeking in ethanol-dependent rats (Funk et al., 2007), although other brain regions are also involved (Funk et al., 2006). Additionally, CRF directly exciting dopamine neurons could in part explain schizophrenic psychosis since schizophrenia is associated with a hyperactive dopamine system (Beninger, 2006) and elevated CRF levels (Banki et al., 1987). These results also provide a physiological mechanism underlying escape behaviors from threatening stimuli. In conclusion, this dissertation highlights potential therapeutic targets to prevent maladaptive stress modulation of the mesocorticolimbic system with possible benefits in the treatment of depression, addiction and schizophrenia.

Appendix. Drug-induced neural and behavioral changes

Abstract:

Numerous lines of evidence demonstrate that drugs of abuse interact with the dopamine system. Addictive drugs not only increase dopamine levels in the NAcc, but also induce synaptic changes on VTA dopamine neurons. The ability of drugs to affect dopamine neurons could be involved with certain drug-related behaviors. For example differences in how ethanol affects the properties of VTA dopamine neurons could explain why C57 mice consume greater amounts of ethanol relative to DBA mice. In the first set of experiments, I examined the effects of saline and ethanol injections on excitatory currents in both C57 and DBA mice. Surprisingly, I found that there were no changes in the paired-pulse ratio or AMPAR currents in either C57 or DBA mice after saline or ethanol injections. However, ethanol injections selectively reduced NMDAR currents in DBA mice and were without effect in C57 mice, which could be involved with the observed differences in ethanol consumption between the two strains. In another set of experiments, I explored the dose-dependent effects of a single cocaine injection on locomotor sensitization. I found that a single injection of cocaine dose-dependently increased the activity in response to a challenge injection of cocaine on the following day. Interestingly, cocaine doses that induced catalepsy still produced behavioral sensitization to the challenge injection of cocaine. Together, these studies further identify drug-induced neural and behavioral changes that may be important in the development of addiction.

Introduction:

A number of behavioral and electrophysiological studies demonstrate that drugs of abuse interact with the dopamine system. In support of this, abused drugs stimulate dopamine release in the NAcc (Di Chiara and Imperato, 1988), and drug-related behaviors such as behavioral sensitization (Kalivas and Alesdatter, 1993) and cocaine self-administration (Kalivas and McFarland, 2003; McFarland et al., 2004; McFarland and Kalivas, 2001) are dependent upon the VTA and subsequent dopamine release. Application of addictive drugs can directly affect the firing (Appel et al., 2003) and glutamate receptor currents (Schilstrom et al., 2006) of VTA dopamine neurons. Interestingly, *in vivo* drug exposure can induce long-lasting synaptic changes on these dopamine neurons (Borgland et al., 2004; Melis et al., 2002; Melis et al., 2007; Saal et al., 2003; Ungless et al., 2001). Thus, identifying the drug-induced neural and behavioral adaptations could highlight alterations involved with the development of addiction.

In particular, i.p. ethanol (EtOH) injections potentiate GABAergic synapses in VTA dopamine neurons of C57BL/6 (C57) mice, an EtOH preferring mouse strain (Melis et al., 2002). Similarly, EtOH injections also induce the same inhibitory synaptic alterations in DBA/2 (DBA) mice, an EtOH non-preferring mouse strain (Melis et al., 2007). However, C57 and DBA mice exhibit different preferences (Risinger et al., 1998) and locomotor responses (Lessov et al., 2001) towards EtOH. Although EtOH induces the same inhibitory synaptic alterations in both mouse strains (Melis et al., 2002; Melis et al., 2007), changes in excitatory synaptic currents could account for the differences in

behavioral responses between C57 and DBA mice. In the first set of experiments, I found no effect of EtOH on the paired-pulse ratio (PPR) or AMPAR currents in either strain, but NMDAR currents were selectively reduced in DBA mice exposed to EtOH.

Cocaine is another addictive drug that directly affects VTA dopamine neurons by enhancing NMDAR currents (Schilstrom et al., 2006). Furthermore, a single *in vivo* injection of cocaine not only increases the AMPAR/NMDAR ratio (Borgland et al., 2004; Ungless et al., 2001), but also induces behavioral sensitization (Jackson and Nutt, 1993). However, the time-course and cocaine dose-response leading to locomotor sensitization has not yet been explored. In the second line of experiments in this chapter, I found that a single injection of cocaine dose-dependently increased the activity in response to a challenge injection of cocaine on the following day, though this effect was pronounced only during the first 15 minutes of the hour-long session. Interestingly, cocaine doses that induced catalepsy still produced behavioral sensitization to the challenge injection of cocaine. Together, these studies further identify drug-induced neural and behavioral changes that may be important in the development of addiction.

Materials and methods:

Electrophysiology

Please refer to the corresponding section in Chapter 2 for more details. 21 day old C57BL/6 or DBA2 mice (Charles River) were given injections of ethanol (2 g/ kg, i.p.) or cocaine 24 hrs prior to slice recordings. The internal recording solution used for these experiments contained 117 mM cesium methanesulfonate, 20 mM HEPES, 0.4 mM

EGTA, 2.8 mM NaCl, 5 mM TEA-Cl, 2.5 mg/mL Mg-ATP, and 0.25 mg/mL Mg-GTP. Paired-pulse recordings used an inter-stimulus interval of 50 ms when the cell was held at -70 mV. To prevent desensitization of AMPAR by AMPA bath application, cyclothiazide (100 μM) was applied for 10 minutes prior to and during the exposure.

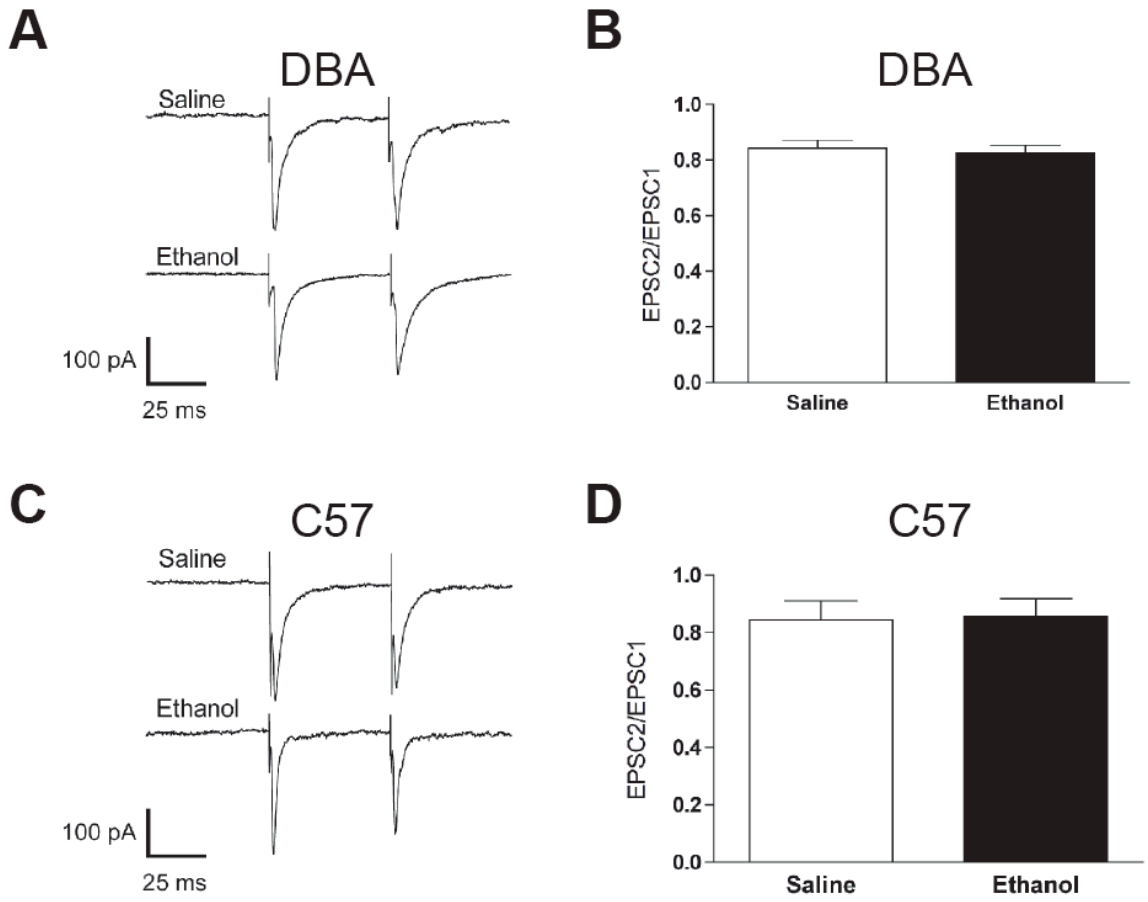
Behavioral sensitization

Mice were handled and given mock injections for at least 2 days prior to experimental manipulations. After cocaine or saline injections (i.p.), the mice were immediately placed in the locomotor chamber and activity was monitored for 1 hr. The locomotor activity data is averaged into 5 min bins.

Results:

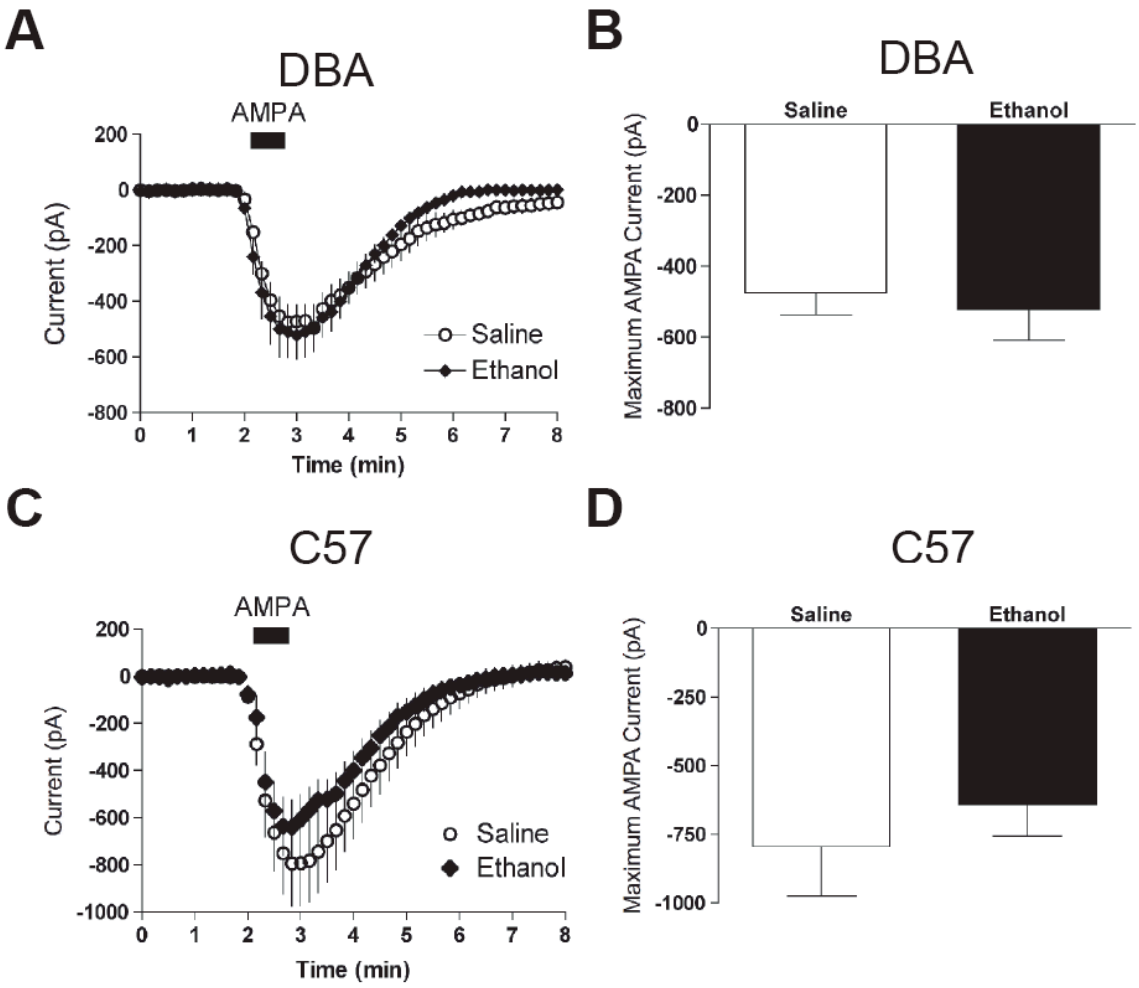
The effect of in vivo EtOH injections on PPR, AMPAR currents, and NMDAR currents in DBA and C57 mice strains

Based upon the critical role of the VTA in mediating the effects of drugs (Di Chiara and Imperato, 1988; Kalivas and Alesdatter, 1993; Kalivas and McFarland, 2003), and that DBA and C57 mice exhibit different preferences for EtOH (Risinger et al., 1998), I hypothesized that EtOH could induce differential synaptic alterations between these two mice strains. A single *in vivo* injection of EtOH (2 mg / kg, i.p.) enhanced GABAergic input onto VTA dopamine neurons in both C57 (Melis et al., 2002) and



Appendix Figure 1: The PPR was unaffected by EtOH injections in C57 and DBA mice. (A,B) In DBA mice, there was no effect of a single EtOH injection (2 g / kg, i.p.) given 24 hrs before recordings on the PPR (50 ms ISI) relative to saline injected mice. (A) Example neurons and (B) summary from saline injected (n = 7) and EtOH injected (n = 6) mice. (C,D) Same treatment as in (A,B), but with C57 mice. (C) Example neurons and (B) summary from saline injected (n = 8) and EtOH injected (n = 7) mice.

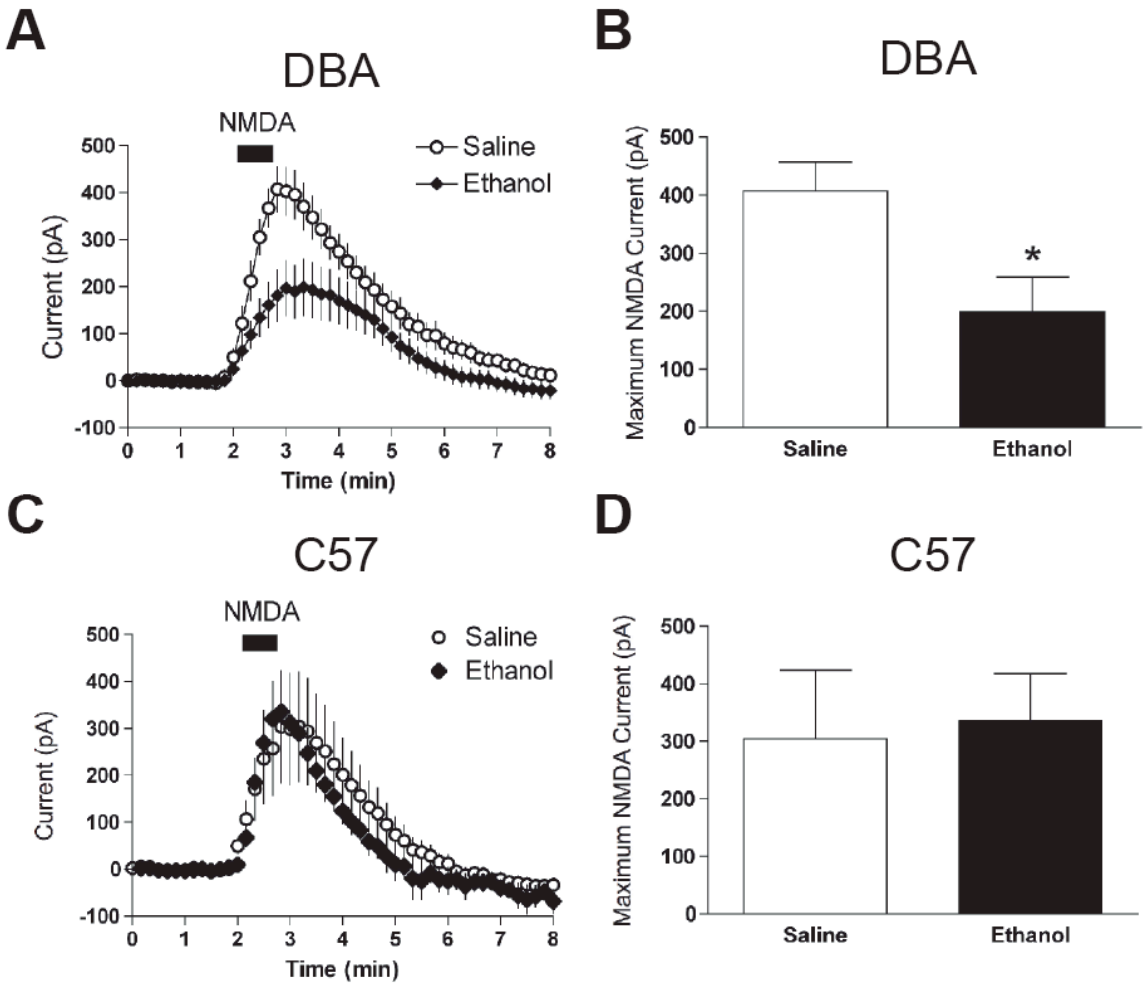
DBA mice (Melis et al., 2007), but how this treatment affects excitatory input on VTA dopamine neurons is unknown. In DBA mice, EtOH injections did not affect the PPR for EPSCs using an interstimulus interval of 50 ms relative to saline injections (Appendix Figure 1 A,B; saline EPSC2/EPSC1 was 0.84 ± 0.03, n = 7; EtOH EPSC2/EPSC1 was



Appendix Figure 2: The AMPAR current was unaffected by EtOH injections in C57 and DBA mice. Neurons held at -70 mV were exposed to 100 μM cyclothiazide for 10 minutes prior to and during a 30s bath application of 2.5 μM AMPA (A,B) In DBA mice, there was no effect of a single EtOH injection (2 g / kg, i.p.) given 24 hrs before recordings on AMPAR currents relative to saline injected mice. (A) Time course and (B) maximum current from AMPA bath application in saline injected (n = 5) and EtOH injected (n = 5) mice. (C,D) Same treatment as in (A,B), but with C57 mice. (C) Time course and (B) maximum current from AMPA bath application in saline injected (n = 4) and EtOH injected (n = 6) mice.

0.83 ± 0.03, n = 6). Similarly in C57 mice, the PPR for saline injected mice (Appendix Figure 1 C,D, 0.84 ± 0.06, n = 8) was not different from mice receiving EtOH injections (Appendix Figure 1 C,D, 0.86 ± 0.06, n = 7). These results provide evidence against a pre-synaptic modification by EtOH in both DBA and C57 mice.

To directly test if post-synaptic excitatory current alterations occurred after EtOH injections, I bath applied specific glutamate receptor agonists. In order to assay for AMPAR currents, I held VTA dopamine neurons at -70 mV and applied 100 μM cyclothiazide for 10 minutes prior to and during a 2.5 μM AMPA application (30 s) to prevent AMPAR desensitization. In DBA mice, AMPA bath application produced the same maximal change in current in both saline injected (Appendix Figure 2 A,B, -474.5 ± 62.7 pA, n = 5) and EtOH injected mice (Appendix Figure 2 A,B, -520.9 ± 86.5 pA, n = 5). This was the same result in C57 mice, as AMPA elicited similar changes in the current in both the saline injected (Appendix Figure 2 C,D, -793.4 ± 180.9 pA, n = 4) and EtOH injected mice (Appendix Figure 2 C,D, - 640.0 ± 116.1 pA, n = 6). I next analyzed the NMDAR current by holding the VTA dopamine neurons at +40 mV during a 30s application of 50 μM NMDA. Interestingly in DBA mice, EtOH injections significantly reduced the NMDA elicited current (Appendix Figure 3 A,B, 199.3 ± 60.3 pA, n = 5) relative to mice receiving saline injections (Appendix Figure 3 A,B, 407.6 ± 48.9 pA, n = 5, $p < 0.05$ relative to EtOH injection). Furthermore, this was not observed in C57 mice as NMDA evoked similar current changes in both saline injected (Appendix Figure 3 C,D, 303.8 ± 119.7 pA, n = 5) and EtOH injected mice (Appendix Figure 3 C,D, 335.7 ± 81.9 pA, n = 5).

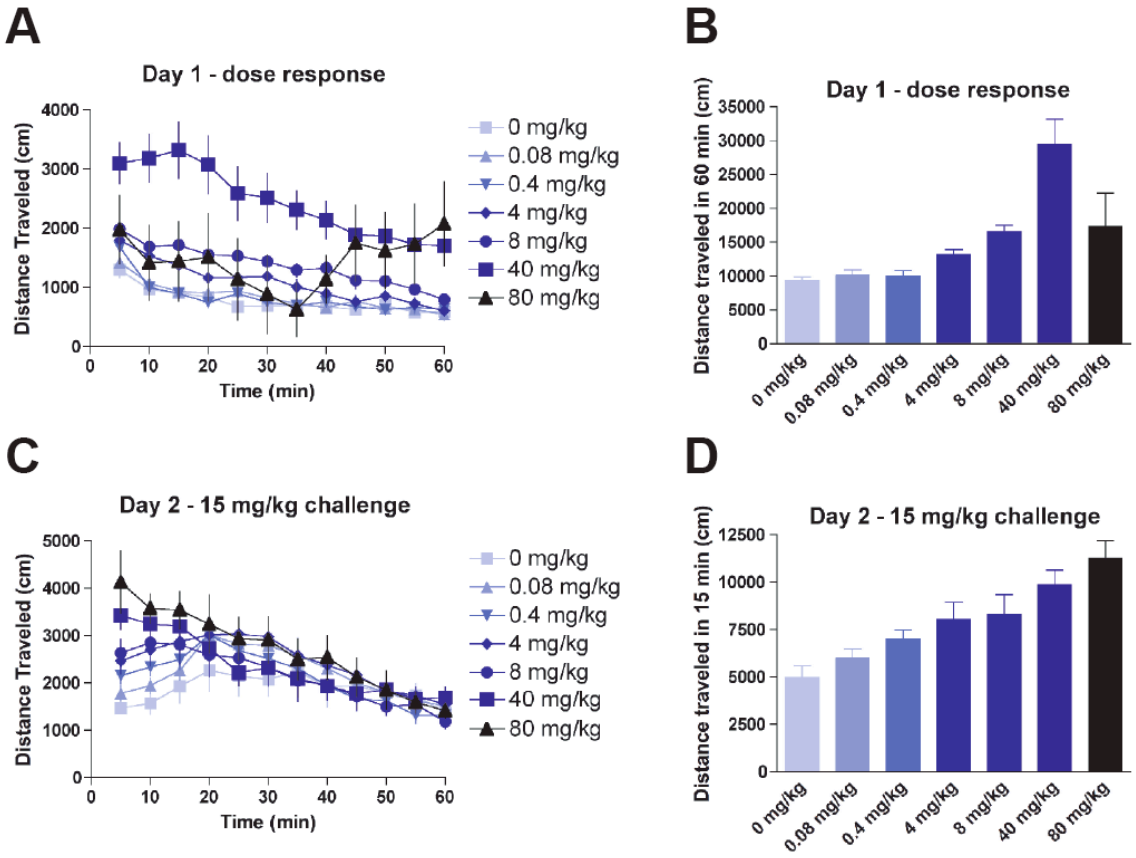


Appendix Figure 3: The NMDAR current was reduced by EtOH injections only in DBA mice. Neurons held at + 40 mV were exposed to 50 μM NMDA for 30s. (A,B) In DBA mice, a single EtOH injection (2 g / kg, i.p.) given 24 hrs before recordings significantly reduced NMDAR currents relative to saline injected mice. (A) Time course and (B) maximum current from NMDA bath application in saline injected (n = 5) and EtOH injected (n = 5) mice. * $p < 0.05$ between saline and EtOH injected mice. (C,D) Same treatment as in (A,B), but with C57 mice. (C) Time course and (B) maximum current from NMDA bath application in saline injected (n = 5) and EtOH injected (n = 5) mice.

Dose-dependent effects of cocaine on locomotor sensitization

A single injection of cocaine not only increases the AMPAR/NMDAR ratio in VTA dopamine neurons (Borgland et al., 2004; Ungless et al., 2001), but also induces behavioral sensitization (Jackson and Nutt, 1993). However, the dose-dependent relationship of cocaine injections on sensitization has not been explored. I found that cocaine injections increase locomotor activity up to doses of 40 mg / kg, but at 80 mg /

kg cocaine induces catalepsy (Appendix Figure 4 A,B, n = 6 - 9). A 15 mg / kg cocaine injection on the following day produced sensitization, but only during the first 15 minutes of the hour-long session (Appendix Figure 4 C,D, n = 6 - 9). Surprisingly, even though mice receiving the 80 mg / kg dose of cocaine were not active, they exhibited the greatest degree of sensitization due to the challenge injection of cocaine on the following day.



Appendix Figure 4: The dose-dependent effects of a single cocaine injection on locomotor sensitization. Day 1. (A) Time course of activity and (B) total distance travelled after a single injection of cocaine for a number of doses (n = 6-9 per group). Day 2. (C) The time course of activity and (D) distance travelled in first 15 minutes of locomotor session in mice receiving a challenge injection of 15 mg /kg cocaine.

Discussion:

The findings from these studies indicate that drugs of abuse produce both neural and behavioral changes. In the first set of experiments, I examined how EtOH affects excitatory glutamate currents on VTA dopamine neurons in DBA and C57 mice. Although the PPR and AMPAR currents were unchanged, EtOH injections selectively reduced the NMDAR current in DBA mice. Given the effects of EtOH on dopamine release (Di Chiara and Imperato, 1988), and the role of the VTA in drug-seeking behaviors (Kalivas and McFarland, 2003), the selective reduction of NMDAR currents in DBA mice by EtOH could be mechanistically involved with the observed difference in EtOH preference between the mouse strains (Risinger et al., 1998), though further behavioral experiments are required to verify the hypothesis. EtOH injections increase the AMPAR/NMDAR ratio in C57 mice (Saal et al., 2003). One expectation based upon the current findings is that EtOH injections would increase the AMPAR/NMDAR to a greater extent in DBA relative to C57 mice, and is an excellent experiment for further study. Additionally, EtOH does not affect the frequency or the amplitude of miniature EPSCs in DBA mice (Melis et al., 2007), but this also needs to be assessed in C57 mice.

In the second set of experiments, I examined the dose-dependence of a single cocaine injection on behavioral sensitization. Although a 40 mg / kg dose of cocaine was previously shown to produce a sensitized locomotor response to a 15 mg / kg challenge cocaine injection on the following day, the time course of this phenomena was not described (Jackson and Nutt, 1993). I found that the cocaine sensitized only the first 15 minutes of the 60 minute locomotor activity session, but that this effect depended on the

dose of cocaine received on the previous day. Interestingly, mice receiving the 80 mg / kg dose of cocaine were not as hyperactive as mice receiving the 40 mg/kg dose, but exhibited the greatest degree of sensitization on the following day. In summary, these studies demonstrated the adaptations on neural circuits and on behavior in response to *in vivo* exposure to addictive drugs.

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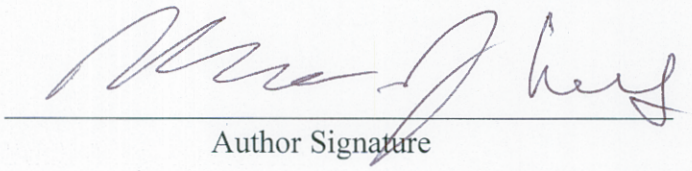

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