MODULATION OF THE MESOCORTICOLIMBIC DOPAMINE SYSTEM

BY THE CENTRAL NUCLEUS OF THE AMYGDALA:

ELECTROPHYSIOLOGICAL AND BEHAVIOURAL ANALYSES

by

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ABSTRACT

Accumulating evidence implicates the amygdala as the main brain region underlying anxiety and, in a parallel stream of research, suggests that dopamine (DA) may be a key neuromodulator of experimental anxiety. However, little is currently known about how the primary output area, the central nucleus, of the amygdala (CeA) and the mesocorticolimbic DA system interact. Chapter 2 sought to directly assess the role of the CeA in neurophysiological alterations occurring at the DA cell-body region of the ventral tegmental area (VTA). The CeA was either activated via pharmacological antagonism of $GABA_A$ receptors or inactivated via pharmacological agonism of $GABA_A/GABA_B$ receptors. Subsequently, in vivo electrophysiological measures were used to examine the modulation of VTA DA neuron (i) population activity, (ii) firing rate, and (iii) bursting. CeA activation resulted in a significant increase in the population activity and bursting of VTA DA neurons. Conversely, inactivation of the CeA resulted in a significant reduction in the population activity of these cells. These are the first electrophysiological data to demonstrate that the CeA elicits differential regulation of distinct physiological parameters of VTA DA neurons. To complement these findings, Chapter 3 describes experiments that investigated the effects of CeA inactivation, in a manner similar to that used in Chapter 2, utilizing a behavioural approach. This series of experiments employed a mesocortical DA-dependent conflict paradigm that simultaneously incorporates appetitive (Reward) and aversive (Conflict) components. CeA inactivation resulted in a significant reduction of responding during the Reward components and a significant increase in lever-pressing during the Conflict period of the conflict test (Experiment1) and its variant, the extinction of conflict test (Experiment 2). The data from Chapter 3

suggest that the CeA plays dissociable roles in mediating motivational responding under appetitive conditions and suppressing responding following exposure to aversive, conflict-inducing, stimuli. Collectively, the results from Chapter 2 and Chapter 3 offer corroborating evidence from electrophysiological and behavioural perspectives for the role of the CeA in modulating the mesocorticolimbic DA system. Ultimately, the systems approach implemented in the current study may offer novel insight into the neurophysiological mechanisms by which one area of the amygdala mediates experimental anxiety.

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CHAPTER 1

GENERAL INTRODUCTION

Anxiety, the innate fear of threatening aspects of one's environment, is an

adaptive and protective phenomenon exhibited by all species. However, when anxiety

becomes disproportional in intensity, chronic, or irreversible in response to either a

perceived or actual aversive event, it may become representative of a "debilitating

anxious state" (Millan, 2003). In this pathological form, anxiety exists as the core

defining symptom of a number of psychiatric conditions including generalized anxiety

disorder, social phobia, simple phobia, panic disorder, posttraumatic stress disorder, and

obsessive compulsive disorder. Given that anxiety disorders affect a large number of

people and can cause immense emotional, physical, and economic distress, a great deal of

scientific interest has focused on delineating neural mechanisms of these conditions. The

resulting body of literature on the neural bases of emotionality has implicated the

amygdala, a group of nuclei buried deep within the medial temporal lobe, as the key

neural substrate underlying anxiety. For example, a wealth of evidence for the amygdala

as the primary brain region underlying anxiety has come from neuroimaging and

neuropsychological studies in healthy individuals either viewing (1) face stimuli, (2)

emotionally valenced stimuli, or (3) participating in fear conditioning tests (Breier et al.,

1996; Lane et al., 1998; Morris et al., 2001; Whalen et al., 2001; Anderson et al., 2003;

Sotres-Bayon et al., 2004; Phelps et al., 2004). Complementary studies with (1) anxious

individuals, (2) those with anxiety disorders, or (3) patients with damage to the amygdala

have further supported the theory that the amygdala is the most significant mediator of

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both normal and pathological anxious states (Adolphs et al., 1994; Tillfors et al., 2001; Rauch et al., 2003; Etkin et al., 2004).

The amygdala can be subdivided into regions distinguished by their architectonic organization, histochemistry, and connectivity (for reviews, see McDonald, 1998; Alheid, 2003; Sah et al., 2003). This group of subnuclei can be loosely divided into the basolateral nuclei, the superficial or cortical-like nuclei, the centromedial nuclei, and a group of nuclei that comprise the remaining amygdalar areas (see Price, 2003; Sah et al., 2003). However, the anatomical connectivity involving the deep basolateral nuclei, comprised of the lateral nucleus (LA), the basal nucleus (B; usually denoted together as the basolateral amygdala [BLA]) and the accessory basal nucleus, and the centromedial nuclei, consisting of the central (CeA) and medial nuclei, are best understood functionally. As a result of numerous tract tracing studies examining both intra- and inter-nuclear connections, the current view of the functional organization of the amygdala is that sensory information enters the amygdaloid complex via the basolateral nuclei, where it is processed locally, and then flows in a medial to lateral direction to the centromedial nuclei, which act as the output station (Krettek & Price, 1978; McDonald, 1992; Pitkanen et al., 1997; Jolkkonen & Pitkanen, 1998; Swanson & Petrovich, 1998).

Based on the anatomical connectivity of the BLA and CeA, it is generally believed that the regulation of aversely motivated behaviour by the amygdala is mediated by one-way communication from the BLA to the CeA. The most dominant theories to date of the neural mechanisms underlying experimental anxiety are based on this serial flow of information through the amygdala (eg. Davis, 1992; LeDoux, 2000). Interestingly, most of the support for these notions comes from studies of fear

conditioning in rodents (for reviews, see Fendt & Fanselow, 1999; LeDoux, 2000; Maren, 2001). In fear conditioning paradigms, a neutral conditioned stimulus (CS; eg. a tone/light) is paired with an aversive unconditioned stimulus (US; eg. a footshock). CS-US pairings result in the association of the CS and US and, over time, the CS alone comes to elicit a range of easily measured conditioned fear responses (CRs). This simple learning task is hypothesized to be relevant to the study of certain anxiety disorders, such as simple phobias, in humans. (Davis, 1992; Davis & Whalen, 2001; Rosen & Schulkin, 1998). During fear conditioning, sensory input first reaches the LA, the site for CS-US association, and proceeds to the CeA to evoke behavioural and physiological CRs. For example, lesions or inactivation of the basolateral nuclei block both the acquisition and expression of fear conditioning (LeDoux et al., 1990; Helmstetter & Bellgowan, 1994; Campeau & Davis, 1995; Wilensky et al., 1999). Similarly, stimulation of the CeA elicits all the CRs characteristic of fear conditioning in the absence any training (Iwata et al., 1987; Kapp et al., 1982). Likewise, lesions of the CeA block all CRs normally produced by fear conditioning to both visual and auditory CSs (Campeau & Davis, 1995; Goosens & Maren, 2001; Hitchcock & Davis, 1986, 1991; Young & Leaton, 1996).

The mechanisms proposed to underlie the role of the amygala in fear conditioning and other paradigms advancing a similar "serial system" hypothesis of information processing have greatly added to our understanding of amygdalar functional organization and the neural substrates of experimental anxiety. Yet there is also considerable evidence from corroborating behavioural and anatomical studies suggesting that the CeA is not just an output pathway of the basolateral complex. For example, emotional responses characterized by freezing, potentiated startle, stress hormone release, and changes in heart

rate and blood pressure are induced when the CeA is activated and, in turn, stimulates the autonomic and hormonal systems via the brain stem or hypothalamus, respectively (Davis, 1998; Kapp et al., 1982; LeDoux et al., 1988). In agreement with these behavioural findings, projections have been shown to exist from the CeA to the hypothalamus, as well as to the periaqueductal grey (involved in vocalizations, startle, analgesia, and cardiovascular changes), parabrachial nucleus (responsible for pain mediation), and nucleus of the solitary tract (connected to the vagal system) in the brainstem (Behbehani, 1995; Rizvi et al., 1991; Gauriau & Bernard, 2002; Moga et al. 1990). Neuroanatomical studies have also shown the CeA to project to the midbrain, pons, and medulla, as well as the bed nucleus of the stria terminalis (BnSt; Veening et al., 1984; Dong et al., 2001). Importantly, the CeA sends a dense projection of neurons to cholinergic and monoaminergic systems including the cholinergic nucleus basalis, the noradrenergic locus coeruleus, the serotonergic raphe nucleus, and the dopaminergic substantia nigra (SN) and ventral tegmental area (VTA; Amaral et al., 1992; Davis & Whalen, 2001). Therefore, one way the CeA may exert control over fear and anxiety is by mediating the mesocortical and mesolimbic dopamine (DA) systems.

There is substantial experimental evidence implicating the involvement of dopaminergic activity in conditioned experimental anxiety. This includes studies examining the effects of both single acute administration of DA agonists and antagonists as well as the effect of repeated administration of psychostimulants in tests of conditioned fear (for review, see Pezze & Feldon, 2004). However, given the well-established role of the mesocorticolimbic DA system in mood regulation (eg. Laviolette, 2007; Stein et al., 2002), relatively little attention has been devoted to elucidating the

brain areas responsible for mediating these dopaminergic pathways. While the central DA-containing systems exhibit a complex organization, they generally comprise the mesostriatal, and the mesocortical and mesolimbic pathways, which link the DA cell-body region of the SN and the VTA, respectively, with their target sites (Haber and Fudge, 1997). Of the dopaminergic pathways originating in the VTA, the mesocortical DA system comprises projections linking DA-containing neurons in the VTA predominantly to the medial prefrontal cortex (mPFC), but also with the anterior cingulate and suprarhinal cortex. Conversely, the mesolimbic DA system is composed primarily of projections to the nucleus accumbens (NAcc), but also to the amygdala, bed nucleus of the stria terminalis (BnSt), olfactory tubercle, and lateral hypothalamus (LH). The specific role of dopaminergic mechanisms in emotionality is currently unknown. However, with respect to the potential interactions between the mesocorticolimbic DA system and the CeA, a pivotal study by Trulson and Preussler (1984) suggests that aversive stimuli modulate the activity of DA neurons in the VTA. Specifically, the stress associated with a conditioned emotional reaction paradigm significantly increased the discharge rate of these dopaminergic neurons in freely-moving cats. More recently, another study employing rabbits offered corroborating evidence for the modulation of VTA DA neurons by auditory stimuli previously paired with shock (Guarraci & Kapp, 1999). Thus, these findings indicate that the CeA may regulate aversively motivated behaviour via modulation of DA activity, in addition to other output pathways.

Although the specific circuitry underlying amygdala-dependent modulation of DA neurotransmission remains unknown, there has been considerable work investigating the role of the different amygdalar subregions in mediating the mesocorticolimbic DA

pathways. The body of evidence resulting from these studies also supports an alternate "parallel systems" hypothesis of amygdalar function by suggesting that the BLA and CeA exert control over appetitive and aversive learning by modulating the mesocorticolimbic DA system via parallel but separate pathways. For example, the BLA directly mediates the mesolimbic DA system by sending dense excitatory glutamatergic afferents to the NAcc (Kelley et al., 1982; Wright et al., 1996; Brog et al., 1993) which synapse in close proximity to mesolimbic DA varicosities originating in the VTA (Johnson et al., 1994). Additionally, the BLA directly regulates the mesocortical DA system (and consequently indirectly mediates the mesolimbic DA pathway) by providing excitatory afferents to the PFC (McDonald, 1992). The PFC also sends projections to the VTA (Carr & Sesack, 2000) and the VTA in turn sends dopaminergic projections to both the PFC and NAcc (Lindvall & Bjorklund, 1978). In contrast, the circuitry through which the CeA interacts with the aforementioned DA systems is less clear. Unlike the BLA, the CeA sends inhibitory GABAergic efferents to the vicinity of midbrain DA cell bodies (Fudge & Haber, 2000; Phillipson, 1979; Wallace et al., 1992) and autonomic sites (Krettek & Price, 1978; Swanson & Petrovich, 1998). Thus, the CeA may interact with the mesocorticolimbic DA pathways by providing direct GABAergic projections to the VTA (Gonzales & Chesselet, 1990; Wallace et al., 1992; Everitt et al., 1999). However, although efferents from the CeA terminate in the VTA, there is no direct evidence that they synapse in close proximity to DA-containing cell-bodies (Fudge & Haber, 2000; Gonzales & Chesselet, 1990). Interestingly, more recent studies using advanced tracing methodologies have failed to find a direct innervation of the VTA by the CeA (Zahm et al., 1999). Nevertheless, there are alternate routes through which the CeA may regulate

DA transmission in the PFC and NAcc via direct projections to either the BnSt (Zahm et al., 1999) or LH (Krettek & Price, 1978; Gonzales & Chesselet, 1990; Zahm et al., 1999; Fadel & Deutch, 2002), which in turn project to the VTA (Phillipson, 1979; Hosoya & Matsushita, 1981).

Given the wealth of experimental evidence implicating the amygdala in fear and anxiety, it is essential to delineate the roles of specific amygdalar subnuclei in these phenomena in order to advance current scientific theories of amygdalar function. In a parallel stream of research, accumulating research suggests that DA may be a key neuromodulator of experimental anxiety. Other behavioural and anatomical data even suggest that parallel but dissociable modulation of mesocorticolimbic DA transmission by the BLA and CeA may serve as the neurochemical bases of emotionality. However, in light of this substantial body of evidence, little is currently known about how the CeA and these DA systems interact because the few studies investigating CeA-dependant neural mechanisms underlying mesocorticolimbic DA transmission offer conflicting results. For example, in vivo studies suggest that brief CeA electrical activation has no effect on DA release in the NAcc (Howland et al., 2002), yet CeA inactivation has been shown to decrease DA efflux in the NAcc (Ahn & Phillips, 2003). Further post mortem in vitro and additional in vivo analyses, however, suggest that chemical CeA activation increases DA levels in both the NAcc and mPFC (Stalnaker & Berridge, 2003). Even more surprisingly, to our knowledge, no electrophysiological data currently exist delineating any specific effects of the CeA on parameters of VTA DA neuron activity. Thus, the goal of Chapter 2 was to address the question of how the CeA exerts regulatory control of the mesocortical and mesolimbic DA systems. The specific aim of the

experiment described in Chapter 2 was to use in vivo electrophysiological measures to directly assess the neurophysiological alterations occurring at the DA cell-body region of the VTA following either activation or inactivation of the CeA. A behavioural approach was implemented in the experiments presented in Chapter 3 to complement the electrophysiological findings of Chapter 2 and further investigate the neural bases of appetitively and aversely motivated learning. Experiment 1 and Experiment 2 in Chapter 3 employed an operant conflict paradigm known to be dependent on mesocortical DA transmission that simultaneously incorporates both rewarded and punished learning. In this series of experiments we investigated the effects of CeA inactivation, in a manner similar to that described in Chapter 2, on this form of experimental anxiety.

CHAPTER 2

THE CENTRAL NUCLEUS OF THE AMYGDALA SELECTIVELY REGULATES VARIOUS ELECTROPHYSIOLOGICAL PARAMETERS OF VENTRAL TEGMENTAL DOPAMINE NEURON ACTIVITY

2.1 INTRODUCTION

A plethora of evidence from neurophysiological studies supports and strengthens the hypotheses proposed by neuroanatomical research surrounding the extensive interactions between the amygdala and the mesocorticolimbic DA system. However, there have been very few studies that have conducted a direct analysis of the nature of these interactions. The control of DA release by the BLA is relatively well-detailed. However, the specific function of the main output nucleus of the amygdala, the CeA, in DA release at the terminal regions of these DA pathways is unclear. Furthermore, the mediation by the CeA of DA cell-body activity remains to be investigated.

The CeA sends projections to the vicinity of VTA DA cell-bodies (Fudge & Haber, 2000; Phillipson, 1979; Wallace et al., 1992) that densely innervate the mesocorticolimbic DA system (Lindvall & Bjorkland, 1978). Corroborating evidence shows that lesions of the CeA block fear, or experimental anxiety, in many experimental paradigms. In light of these correlative data, a critical in vitro study by Davis and colleagues (1994) examined the effect of CeA lesions on mPFC DA metabolism in response to various anxiety-inducing paradigms. CeA lesions were shown to block mPFC increases in the DA metabolite homovanillic acid that normally accompany footshock; in addition, lesions of the CeA also attenuated an increase in DA turnover, measured by DOPAC/DA ratios, following novelty stress. CeA lesions administered either pre- or

post-training in a conditioned stress paradigm also block the increase in mPFC DA

utilization (Goldstein et al., 1996), implicating the CeA in afferent control of the

mesocortical DA system, and extending previous work suggesting that lesioning this

nucleus blocks activation of mPFC DA in response to novelty and footshock (Davis et al.,

1994).

Direct experimental support from behavioural studies for the role of the CeA in

DA transmission is, with a few noteworthy exceptions, lacking. However, indirect

evidence for afferent modulation of accumbal DA by the CeA may be gleaned from

studies using CeA lesions to assess behaviour mediated by NAcc DA. For example, CeA

lesions disrupt the potentiation of conditioned reinforcement by intra-accumbens

amphetamine administration (Robledo et al., 1996). In this study, rats were trained to

associate a light/tone compound stimulus with the availability of a sucrose solution prior

to receiving CeA lesions. Lesions subsequently blocked a potentiation of responding for

this conditioned reinforcer following NAcc amphetamine infusions, a phenomenon that is

dependant on mesoaccumbens DA transmission (Taylor & Robbins, 1986). Therefore,

this study suggests a potential neurophysiological mechanism by which the CeA may

mediate instrumental behaviour via exerting control over accumbal DA efflux.

Recent in vivo experiments have delineated a prominent, although conflicting,

role of the CeA in the regulation of dopaminergic systems originating in the VTA. For

example, Howland and colleagues (2002) demonstrated that a brief electrical stimulation

of the CeA had no effect on accumbal DA efflux in freely moving rats. In contrast,

similar stimulation of the BLA did evoke an increase in NAcc DA efflux. However, the

same group later demonstrated that inactivation of the CeA but not the BLA, decreased

basal levels of DA efflux in the NAcc (although not in the mPFC). CeA inactivation also attenuated feeding-evoked increases in DA release in both areas, and affected feeding-related behaviours (Ahn & Phillips, 2003). Thus, the CeA appears to exert an influence over basal and feeding-evoked extracellular DA levels. However, brief electrical stimulation of this nucleus does not appear to cause a measurable increase in DA extracellular levels in the NAcc.

In addition to the above mentioned findings, another study reported that chemical activation of the CeA (but not of the BLA) with the glutamatergic agonist AMPA elicited a significant increase in DOPAC/DA levels in both terminal regions of the mesocorticolimbic DA system (Stalnaker & Berridge, 2003). Furthermore, when infused into the CeA of sleeping rats, AMPA also elicited a rapid transition to waking, and in vivo microdialysis confirmed that AMPA into the CeA, but not the BLA, increased extracellular mPFC DA levels. These data are in line with the findings of Ahn and Phillips (2003) but contrast with those of Howland and colleagues (2002). Although the data appear inconsistent, their discrepancy may be due to methodological differences. Specifically, Ahn and Phillips (2003) and Stalnaker and Berridge (2003) employed pharmacological manipulations to inactivate and activate the CeA, respectively; in contrast, Howland and colleagues used electrical stimulation (2002). Chemical activation (and inactivation) of neural structures results in prolonged neurotransmission (or cessation of transmission) at the terminal regions of afferent pathways; however, electrical stimulation leads to rapid and intense, albeit short-lived, neural activity. Therefore, the possibility cannot be ruled out that electrical stimulation of the CeA may

also elicit short-lasting changes in DA efflux that were not detected by the microdialysis technique used to quantify accumbal DA release (Howland et al., 2002).

One approach that may reconcile the differences observed by the aforementioned studies examining the role of the CeA in mesocorticolimbic DA release is to examine the activity of the DA neurons themselves. However, there have been few studies that have conducted neurophysiological recordings from VTA DA neurons following amygdalar manipulations. Maeda and Mogenson (1981) reported that a large majority of presumed dopaminergic neurons in the VTA responded to stimulation of either the CeA or BLA by increasing or decreasing their firing rate. The onset latencies for activation and suppression were either short or long, suggesting that the amygdala can regulate DA neuron activity through both mono- and poly-synaptic pathways

It is important to note that subcortical regions may modulate a number of other parameters of DA neuron activity in addition to brief excitatory and inhibitory changes in firing rate. For example, a pivotal study used simultaneous extracellular single-unit recordings and in vivo microdialysis to investigate the relationship between DA neuron activity and tonic, or extrasynaptic, DA. In that study, the authors found a significant correlation between the number of spontaneously active DA neurons (population activity) in the substantia nigra and DA efflux in the striatum (Moore et al., 1998). These data provided evidence that the tonic extracellular pool of DA in the striatum (Grace, 1991) depends partly on the number of DA cells that are active and on their firing rate. In addition, it is well established that burst firing of DA neurons is related to increased DA release in the striatum (Nissbrandt et al., 1994). Taken together, these findings indicate that tonic levels of extracellular DA are maintained by the overall population activity of

DA neurons, with additional synaptic, or phasic, changes as a result of DA cell bursting. More generally, they also strongly suggest a relationship between the changes observed in experiments examining events occurring at the level of the cell-bodies and those investigating changes at the axon terminals in the mesocortical and mesolimbic DA systems. More recently, Floresco and colleagues (2003) reported that an increase in population activity or bursting of VTA DA cells following the manipulation of two separate afferent systems differentially influenced tonic and phasic DA release in the NAcc. Manipulations that increased the number of spontaneously active neurons resulted in increased DA efflux, whereas those that selectively increased bursting did not; yet when DA reuptake was blocked, increased bursting now also increased accumbal DA efflux. These results confirm that correlative changes are in fact occurring between specific aspects of DA neuron activity in the VTA and levels of DA release in the NAcc.

In light of the inconsistent findings of Howland and colleagues (2002) and Ahn and Phillips (2003), and the data from Stalnaker and Berridge (2003) assessing the regulatory role of the CeA in the mesocorticolimbic DA system's terminal regions (NAcc and mPFC), the present experiment sought to investigate the neurophysiological alterations that occur at the level of the VTA DA cell-bodies following either CeA activation or inactivation. Increased DA release in the NAcc following the manipulation of afferent pathways to the VTA has recently been found to elicit corresponding, but selective, changes in both DA efflux in the NAcc and specific electrophysiological properties of VTA DA neurons (Floresco et al., 2003). Therefore, various neural structures exerting afferent control over the mesocorticolimbic DA pathway and DA release may do so via differentially affecting various parameters of VTA DA neuron

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activity. To investigate this hypothesis, the current goal was to elucidate the mechanisms underlying the effects observed by other studies at the axon terminals of the mesocorticolimbic DA system by examining the role the CeA plays in regulating various electrophysiological parameters of the VTA DA neurons themselves. Accordingly, the current study examined the modulation of VTA DA neuron (i) population activity, (ii) firing rate, and (iii) bursting by either activation or inactivation of the CeA to obtain insight into the mechanisms by which the CeA may regulate VTA DA neuron activity.

2.2 METHODS

2.2.1 Subjects

Male Sprague-Dawley rats (University of British Columbia Animal Care Center) weighing between 275 g and 350 g were used. Prior to the experiment, rats were group housed in hanging wire mesh cages (maximum 4 per cage) in a temperature-controlled (21 +/-1 °C) colony room maintained on a 12hr:12hr light-dark schedule. They were allowed to free-feed and were also given ad libitum access to water. The treatment of animals and all experimental testing was in concordance with the ethical standards of the Canadian Council for Animal Care and the Animal Care Committee of the University of British Columbia.

2.2.2 Surgery

At a minimum of one week following arrival to the facilities, rats were deeply anesthetized with chloral hydrate (400mg/kg; ip) and placed in a stereotaxic apparatus with the incisor bar set at -3.3 mm. Core body temperature was monitored by a rectal probe and maintained at 35 °C by a thermostatically controlled heating pad. Supplemental anesthetic was administered throughout the course of the experiment via a

tail vein catheter. In all surgical preparations, the scalp was incised, the connective tissue encasing the skull was removed, and holes were drilled in the skull overlying the VTA and the CeA. In addition, the dura mater encasing the area of the brain overlying the VTA was removed. All rats were implanted with stainless steel 23-gauge guide cannulae directed to a location ~0.8 mm dorsal to the CeA (Anterior-Posterior [AP], -2.2 mm from bregma; Medial-Lateral [ML], +4.0 mm from midline; Dorsal-Ventral [DV], -7.5 mm from dura). The locations of the surgical placements for all brain regions were according to coordinates derived from the neuroanatomical atlas of Paxinos and Watson (1998).

2.2.3 Identification of Dopaminergic Neurons and Extracellular Recording Protocol

Extracellular recording microelectrodes (2.0 mm borosilicate glass capillary tubing, ~1 um tip diameter, 4-10 MΩ impedance) were constructed using a microelectrode puller (Narishige, Tokyo, Japan). The tips of the electrodes were broken back against a glass rod to ~1 μm tip diameter and filled with 2 M NaCl containing 2% Pontamine sky blue dye. The in vivo impedance of the microelectrodes ranged from 5 to 10 MΩ. The electrode signal was amplified and filtered (50-2000 Hz) using an X-Cell3+ microelectrode amplifier (Fredric Haur Co., Bowdin, ME). Action potential data were acquired, discriminated from noise, stored, and analyzed using Spike 2 software (CED, Cambridge, UK) running on an Intel-based personal computer with a data acquisition board interface (micro 1401 mk II, CED). Ten minutes following microinfusion of either vehicle, bicuculline, or the baclofen/muscimol cocktail, a recording electrode was lowered into the VTA (ipsilateral to guide cannula and stimulating electrodes; AP, -5.3 mm from bregma; ML +1.0 mm from midline; DV, 6.5-8.5 mm ventral from brain surface) via a hydraulic microdrive and a cell-searching procedure was implemented.

Within the VTA, 6-9 vertical passes of the recording electrode were made through the DA cell-body region (separated by 200 μm), and spontaneously active DA neurons were identified using established electrophysiological criteria described in detail elsewhere (Grace & Bunney, 1983). Specific DA cell waveforms include a segment-somatodendritic break in the initial positive phase, a biphasic (positive-negative) or triphasic (positive-negative-positive) waveform with duration of 2.0-4.0 ms, and emission of a characteristic low-pitched popping sound from the audio monitor. DA cells generally fire action potentials in a slow, irregular pattern that occasionally alternates with bursts of spike activity (2-10 spikes), with a progressive increase in interspike intervals and a decrease in spike magnitude during a burst (Grace & Bunney, 1983). All spontaneously active DA neurons were isolated and their activity was recorded for a minimum of 120 s. The electrophysiological properties of these neurons were subsequently assessed by sampling three parameters of activity: (1) the number of spontaneously active DA neurons per electrode track, (2) the average firing rate of these neurons, and (3) their bursting activity. The first measure was the number of spontaneously active DA neurons recorded per electrode track (i.e. population activity). This index of activity has been shown to reliably quantify DA neuron activity (West & Grace, 2000), and has been a reliable measure across studies (Bunney & Grace, 1978; Chiodo & Bunney, 1983). Moreover, pharmacological treatments that either increase or decrease population activity have been shown to elicit an increase or decrease, respectively, of DA efflux measured at the axon terminals (Floresco et al., 2003; Moore et al., 1998). For measures of bursting activity, the onset of a burst was defined as two spikes with an interspike interval of <80 ms, and the termination of a spike was defined as two spikes with an interspike interval

>160 ms (Grace & Bunney, 1983). The percentage of spikes in bursts was calculated by dividing the number of spikes occurring in bursts by the total number of spikes occurring in the same period of time.

2.2.4 Pharmacological Manipulations

A $GABA_A$/$GABA_B$ agonist cocktail of muscimol hydrobromide and baclofen hydrochloride, respectively (125 ng each; Sigma Aldrich Inc, St. Louis, MO), and the $GABA_A$ antagonist bicuculline (100 µg; Sigma Aldrich Inc, St. Louis, MO) were freshly prepared in saline and were injected into a 30-gauge injection cannula. The injection cannula was inserted into the CeA guide cannula, extending 0.8 mm below the tip of the guide. Subsequently, it was connected with PE tubing to a gastight 10 µl syringe attached to an infusion pump. Pharmacological agents were infused no sooner than 30 min following surgery at an infusion volume of 0.5 µl over 135 s by a microsyringe pump. The CeA was inactivated by infusion of the $GABA_A$/$GABA_B$ agonist cocktail, and activated by administration of the $GABA_A$ antagonist bicuculline. The dosages were selected from previous studies showing these agents to be active when infused into various brain regions known to affect DA neuron firing (Floresco et al., 2003, Kitamura et al., 2001; Samson & Chappell, 2001; Milner & Mogenson, 1988). The control group consisted of 7 rats that received microinfusions of saline into the CeA.

2.2.5 Histology

At the end of each experiment, the stimulating sites were marked using ejection of current from the tip of the stimulating electrodes (100 µA constant current for 10-20 s). The recording site was then marked using iontophoretic application of Pontamine sky blue dye from the tip of the recording electrode (50 µA constant current for 30-40 min).

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Following the marking of recording sites with dye, rats were sacrificed via an overdose of chloral hydrate injected into the tail vein. Brains were removed and fixed in a 4% formalin solution containing 0.1% potassium ferricyanide for at least 24 hours. The tissue was then frozen and sectioned into 50 μm coronal slices, mounted, and stained with cresyl violet. Histological determination of recording electrode and cannula infusion sites was enabled by the cresyl violet staining. Furthermore, placement verification was complemented with reference to the atlas of Paxinos and Watson (1998). Rats with recording electrodes lying outside of the VTA were excluded from further statistical analysis. Rats with cannula placements that did not fall within the CeA were also excluded from the study.

2.2.6 Statistical Analysis

The number of spontaneously active DA neurons observed per electrode track, the basal firing rates, and the burst firing of all spontaneously active DA neurons were calculated as an average value for each rat. They were then analyzed using 3 separate one-way analysis of variance tests (ANOVAs) with treatment group as the between-subjects factor. Given the recent results obtained by studies investigating the role of the CeA in regulating the mesocorticolimbic DA system (Ahn & Phillips, 2003; Stalnaker & Berridge, 2003; Howland et al., 2002), a priori hypotheses regarding the influence of the present CeA manipulations on VTA DA neuron activity justified the use of one-tailed Dunnett's tests for multiple comparisons. Analyses of the distribution of firing rates and burst firing of all DA neurons across all rats in different treatment groups were assessed using Kolmogrov-Smirnov two-sample tests.

2.3 RESULTS

2.3.1 Histology

Recording electrodes were verified to lie primarily within the VTA with reference to the neuroanatomical atlas of Paxinos and Watson (1998). All animals with electrode placements outside the DA cell-body containing region of the VTA were excluded from statistical analysis. All rats included in the analyses had infusions localized within the CeA (Figure 2.1). Rats with cannulae placements ventral to the amygdaloid complex or encroaching upon the basal or lateral nuclei of the amygdala (BLA) were also excluded. Following histological analysis, a total of 7 rats receiving a vehicle control, 6 rats receiving a muscimol/baclofen cocktail, and 6 rats receiving bicuculline were included in further analyses.

2.3.2 Effects of CeA Activation on VTA DA Neuron Activity

Rats that received control infusions of saline in the CeA (n=7 rats, 70 neurons) exhibited an average of 1.20+/- 0.13 spontaneously active VTA DA neurons per electrode track, which fired at an average rate of 3.91 +/- 0.15 Hz with 26.73% +/- 4.5 of action potentials fired in bursts. These results are consistent with previous findings (Floresco et al., 2003; Floresco et al., 2001), and are presented in Figure 2.2A-C (white bars). Analysis of the number of spontaneously active DA neurons per electrode track for all treatment groups revealed a significant effect of treatment [$F_{(2, 16)} = 11.23$, $p<0.01$]. A significant effect was also observed with respect to the number of action potentials fired in a burst-mode [$F_{(2, 16)} = 3.91$, $p<0.05$]; interestingly, however, these effects occurred without a significant main effect on the average firing rates of these cells [$F_{(2, 16)} = 2.66$, $p>0.1$].

Multiple comparisons with Dunnett's tests revealed that activation of the CeA by an infusion of bicuculline (n=6 rats, 87 neurons) resulted in a significant ($p<0.05$) increase in VTA DA neuron population activity relative to vehicle treatments. This is reflected as an increase in the number of spontaneously active neurons observed per electrode track (Figure 2.2A, black bar). Although no significant effect of treatment was found on the average firing rate of these cells, infusions of bicuculline also resulted in an increase in their average firing rate (Figure 2.2B, black bar). Furthermore, a Dunnett's test confirmed that activation of the CeA with bicuculline produced a significant ($p<0.05$) increase in the bursting activity of VTA DA neurons relative to control treatments. This analysis is presented in Figure 2.2C (black bar), and reflects an increase in the number of spikes (i.e. action potentials) fired in bursts.

2.3.3 Effects of CeA Inactivation on VTA DA Neuron Activity

In contrast to the effects of bicuculline, Dunnett's post-hoc tests revealed that inactivation of the CeA via infusions of muscimol/baclofen (n=6 rats, 39 neurons) had the opposite effect, causing a significant ($p<0.05$) decrease in the population activity of VTA DA neurons relative to control treatments. This analysis is presented in Figure 2.2A (gray bar), and reflects a decrease in the number of spontaneously active DA neurons encountered per electrode track. Moreover, this effect occurred without a significant change in either the mean firing rate ($p>0.05$) or number of action potentials fired in bursts ($p>0.05$). Surprisingly however, infusions of muscimol/baclofen caused an, albeit non-significant, increase ($p = 0.118$) in the mean firing rate of DA neurons (Figure 2.2B, gray bar). Inactivation of the CeA also resulted in a non-significant increase in bursting activity that, nonetheless, approached significance ($p=0.066$; Figure 2.2C, gray bar).

Further independent analyses of the distributions of DA neurons as a function of firing rate and number of spikes fired in bursts revealed that infusions of muscimol/baclofen did not affect the proportion of DA neurons that displayed activity at substantially higher firing rates but did increase the proportion of cells that fired >50% of their action potentials in a burst mode relative to control treatments (Kolmogorov-Smirniv, p = 0.119 and p<0.002, respectively; Figures 2.3 and 2.4). Overall, the present data reveal that CeA activation and inactivation dissociably modulate various parameters of DA neuron activity.

Each animal received one infusion of vehicle, bicuculline, or muscimol/baclofen into the CeA prior to the beginning of each experiment. Therefore, it was important to determine whether the observed effects over 9 electrode tracks (~2 hrs) were a direct result of the treatments. To ascertain whether CeA activation or inactivation caused an increase or decrease, respectively, in population activity for the duration of each experiment, separate analyses were conducted to confirm that there were no differences in the number of spontaneously active DA neurons encountered across all 9 electrode tracks following bicuculline or muscimol/baclofen administration (F= 0.44, n.s., and F=1.05, n.s., respectively). These analyses verified that the present infusions were active for the entire length of the experiments and thus directly affected this parameter of DA neuron activity. Furthermore, these analyses suggest that the infusions also affected the mean firing rate and burst-firing mode of activity throughout the entire course of each experiment.

2.4 DISCUSSION

The results of the current experiment demonstrated that activation of the CeA via pharmacological antagonism of $GABA_A$ receptors resulted in a significant increase in the number of spontaneously active neurons (population activity) observed per electrode track in the A10 DA cell-body region of the VTA. Additionally, CeA activation also resulted in a significant increase in the bursting activity of these neurons. Similarly, activation of the CeA produced a non-significant increase in the average firing rate of these neurons. Alternately, pharmacological inactivation of the CeA via administration of a cocktail of agonists to both the $GABA_A$ and $GABA_B$ receptors produced the converse effect and resulted in a significant reduction in the population activity of A10 dopaminergic neurons. While this treatment did not result in any significant alterations in the burst activity of these neurons, or their firing rate, inactivation of the CeA did result in a surprising non-significant elevation of both of these variables. Thus, the current data suggest that the CeA elicits a tonic regulation of the population activity of dopaminergic neurons in the VTA, while having differential effects on the firing rate and burst activity of these neurons. These are the first electrophysiological data to demonstrate that the CeA elicits differential regulation of distinct physiological parameters of dopaminergic neuron activity in the VTA.

The neuroanatomical mechanism by which the CeA exerts regulation of population activity of DA-containing neurons in the VTA has yet to be elucidated, but the current data suggest that this modulation is excitatory in nature. Several studies have demonstrated that the CeA sends projections that terminate in the vicinity of the VTA (Fudge & Haber, 2000; Phillipson, 1979), suggesting that a direct monosynaptic

connection between the CeA and the VTA could mediate their communication. However, to date, no studies have explicitly demonstrated that these projections directly impinge upon the cell bodies of dopaminergic neurons (Fudge & Haber, 2000; Gonzales & Chesselet, 1990). Furthermore, the afferent projections from the CeA to the VTA are of a GABAergic phenotype (Amaral et al., 1992; Everitt et al., 1999); thus, if these inhibitory afferents synapsed onto VTA dopaminergic cell bodies, the CeA would function to inhibit midbrain DA neuron activity, instead of increasing it. Thus, the most parsimonious account of these data would be that the CeA extends GABAergic afferents that synapse in the VTA onto local inhibitory GABAergic neurons, which in turn would release local inhibitory tone of dopaminergic neurons in the VTA and result in a net increase in population activity and subsequent DA efflux at terminal regions (Ahn and Phillips, 2003; Stalnaker & Berridge, 2003). Alternately, several recent studies using advanced tracing methodologies have failed to even find a direct innervation of the VTA by the CeA (Zahm et al., 1999). This suggests that communication between these structures could be due to a polysynaptic connection likely relayed through an intermediary structure, such as the BNST (Zahm et al., 1999) or LH (Krettek & Price, 1978; Amaral et al., 1992; Gonzales & Chesselet, 1990; Zahm et al., 1999; Fadel & Deutch, 2002), which in turn projects to the VTA (Phillipson, 1979; Hosoya & Matsushita, 1981). Through this pathway, the CeA would project a GABAergic afferent to the BNST or the LH, which would inhibit a subsequent GABAergic projection from these structures and, again, result in a reduction of inhibitory tone on VTA dopaminergic neurons and a net increase in A10 DA neuron activity. Further studies employing tracer

techniques to define pathways and immunohistochemical studies to define neuronal phenotypes will be required to fully understand the nature of this connectivity.

In addition to the significant increase in population activity, pharmacological activation of the CeA also resulted in a concomitant increase in the burst activity, and a near significant increase in the firing rate, of dopaminergic neurons within the VTA. If this CeA-mediated phenomenon is due to a reduction in local inhibitory tone on dopaminergic cell bodies, then an increase in the firing rate and bursting activity of these neurons is a logical consequence that is in line with the increase in population activity. Conversely, while pharmacological inactivation of the CeA elicited a reduction in the population activity of dopaminergic neurons in the VTA, there was an unexpected concurrent, but non-significant, increase in the firing rate and burst activity of these spontaneously active neurons. While this may seem inherently contradictory, two putative explanations may be offered for this effect. First, the increases in firing rate and burst activity following the reduction in population activity could be a compensatory response; due to the overall reduction in the number of spontaneously active neurons, the cells that continued to fire may have done so with a more intense firing rate and increased bursting activity. On the other hand, it is possible that the CeA regulates the activity of a specific subtype of neuron in the VTA which, under normal conditions, exhibits a lower level of firing rate and burst activity. If this is the neurophysiological mechanism underlying the observed effects, inactivation of the CeA reduces activity of slower firing DA neurons; this in turn suggests that the remaining neurons that were recorded from were those that generally exhibit higher levels of firing and bursting. Support for this latter hypothesis comes from analyses of distributions of DA neurons as a function of

both firing rate and bursting activity (Figures 2.3; 2.4), which revealed a reduced number of neurons firing in the 2-4 Hz range.

The release of DA at the terminal regions of the mesocorticolimbic DA system is strongly correlated with distinct parameters of DA neuron activity within the DA cell-bodies in the VTA, particularly within the NAcc (Floresco et al., 2003). Specifically, DA neurons exhibit two forms of synaptic release patterns, which are referred to as tonic and phasic (Grace, 1991). Tonic DA levels occur when these cells fire in a slow, single-spike mode, and refer to steady-state DA release into the synapse that is ongoing and provides basal dopaminergic transmission. Tonic DA release is thought to be primarily mediated by population activity, or changes in the number of spontaneously active DA neurons within the VTA (Grace, 1991). Phasic, or synaptic, levels of DA refer to a much larger release of DA that rapidly increases DA signaling but in turn accelerates DA-reuptake and thus exhibits a brief synaptic half-life. Phasic DA release is primarily mediated by a bursting pattern of activity at the cell-body region, when the neurons fire in a fast, irregular, multi-spike manner (Grace, 1991). In a pivotal study linking events at the cell-body region to those occurring at the terminal regions of the mesolimbic DA system, Floresco and colleagues (2003) activated two separate afferent pathways to the VTA and showed that tonic and phasic levels of DA in the NAcc are differentially controlled by distinct firing properties of DA neurons in the VTA. Inhibition of GABAergic input from the ventral pallidum increased DA population activity and was associated with an increase in accumbal DA efflux. Conversely, activation of inputs from the pedunculopontine tegmental nucleus increased burst-firing of these cells but did not affect population activity or DA release under basal conditions; however, when DA-

reuptake was blocked, NAcc DA release was increased by 3-4 fold. In line with these data suggesting that multiple afferent pathways to the VTA selectively regulate (i) DA neuron firing parameters in the VTA DA cell-body region and (ii) DA efflux in one of its terminal regions (NAcc), the current data argue that the CeA mediates the tonic DA release that corresponds to increased population activity of VTA DA neurons. Presumably, increased CeA activity corresponds to an increase in steady-state DA release into the synapse, while reductions in CeA activity correspond to a decline in this ongoing, basal transmission. The current data also imply that increased CeA activity would also increase phasic dopaminergic release, as increased burst activity was elicited by this experimental manipulation. Furthermore, the current observations of a near-significance in bursting activity following CeA inactivation also suggest that while tonic DA release is reduced there may be a higher frequency of phasic DA release.

Given the correlation between events at the cell-body and those at the axon terminals (Grace, 1991; Floresco et al., 2003), the current study agrees with the contention that changes in neuronal activity within the DA cell-body region of the VTA are associated with alterations in DA release. Thus, it is of direct relevance to compare the findings of the current electrophysiological study with those of microdialysis studies examining the role of the CeA in DA efflux in the mesocorticolimbic DA system. The first study on this topic demonstrated that electrical stimulation of the CeA had no effect on DA release within the NAcc (Howland et al., 2002), which would appear in contrast to the current data. However, two subsequent studies presented opposing data. Specifically, Ahn and Phillips (2003) demonstrated that chemical inactivation of the CeA resulted in a reduction in both basal and feeding-evoked DA efflux within NAcc, although not within

the PFC. Similarly, Stalnaker and Berridge (2003) showed that pharmacological activation of the CeA induced an increase in DA release in the mPFC, as well as the core and shell of the NAcc in vivo, and an increase in DA metabolite content in vitro. Thus, in conjunction with these latter two studies, the current data reveal that inactivation of the CeA results in a reduction in population activity of dopaminergic neurons in the VTA and DA release in the NAcc; consistently, increased activity of CeA output neurons evokes an increase in population activity and bursting activity, which in turn corresponds to an increase in synaptic dopaminergic content in the mPFC and NAcc. The lack of concordance of this hypothetical model with the study of Howland and colleagues (2002) could be due to methodological differences. Specifically, the latter two studies, which support the current data, utilized microdialysis to assess DA efflux following pharmacological activation and inactivation of the CeA. In contrast, whereas the inconsistent study also performed microdialysis, it employed electrical stimulation of the CeA to elicit DA release. Electrical stimulation delivers a train of stimulations that induces a rapid and intense, but transient, increase in firing of GABAergic projection neurons, while pharmacological treatments result in prolonged alterations in the chemical microenvironment of the soma of CeA output neurons. Thus, electrical stimulation may have elicited short-lived changes in NAcc DA efflux that, due to their brevity, were not detected by the microdialysis procedure employed (Howland et al., 2002). Alternately, pharmacological modulation likely induces a more sustained modulation of neuronal firing that evokes changes in dopaminergic transmission within the detection threshold of microdialysis. Despite the results of the aforementioned discrepant study, the current data do support previous microdialysis investigations of the role of the CeA in

mesocorticolimbic DA release (Ahn & Phillips, 2003; Stalnaker & Berridge, 2003). Importantly, the current activation and inactivation of the CeA and subsequent investigation of neuronal events at the DA cell-body region of the VTA offer the first electrophysiological data of its kind and extend the previous studies by suggesting that CeA activation drives DA efflux within the mesocorticolimbic DA system in a distinct manner. This hypothesis is also in agreement with several behavioural studies which also suggest that communication between the CeA and the NAcc occurs via a CeA-mediated increase in VTA neuronal activity (Hall et al., 2001).

Figure 2.1: Histology figures depicting the infusion locations of saline, the GABA$_A$ receptor antagonist bicuculline, or a cocktail of the GABA$_A$ and GABA$_B$ receptor agonists muscimol and baclofen, respectively, into the central nucleus of the amygdala.

Figure 2.2: The effect of infusion of saline (VEH; white bars), the $GABA_A$ receptor antagonist bicuculline (BIC; black bars) or a cocktail of the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen, respectively, (BM; gray bars) into the central nucleus of the amygdala on A) the number of spontaneously active dopamine neurons per electrode track, as well as both the B) firing rate and C) the bursting activity, of identified dopamine neurons within the ventral tegmental area. Significant differences ($p < 0.05$) relative to saline treated animals denoted by *.

A)

B)

C)
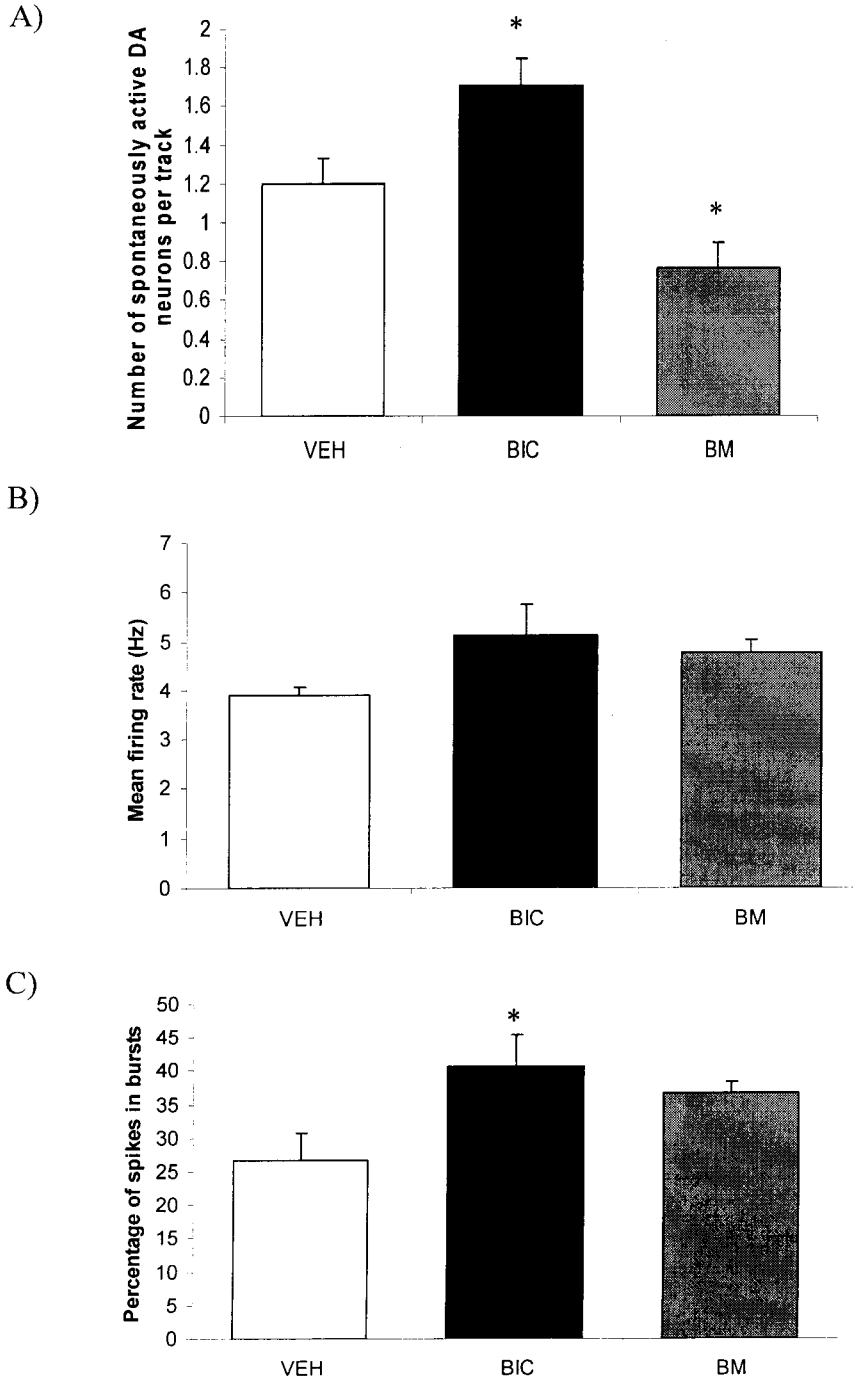
Figure 2.3: The proportion of firing rates of identified dopamine neurons in the ventral tegmental area following infusion of either saline (upper panel) or a cocktail of the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen, respectively, (lower panel) into the central nucleus of the amygdala.

Saline

Muscimol/Baclofen
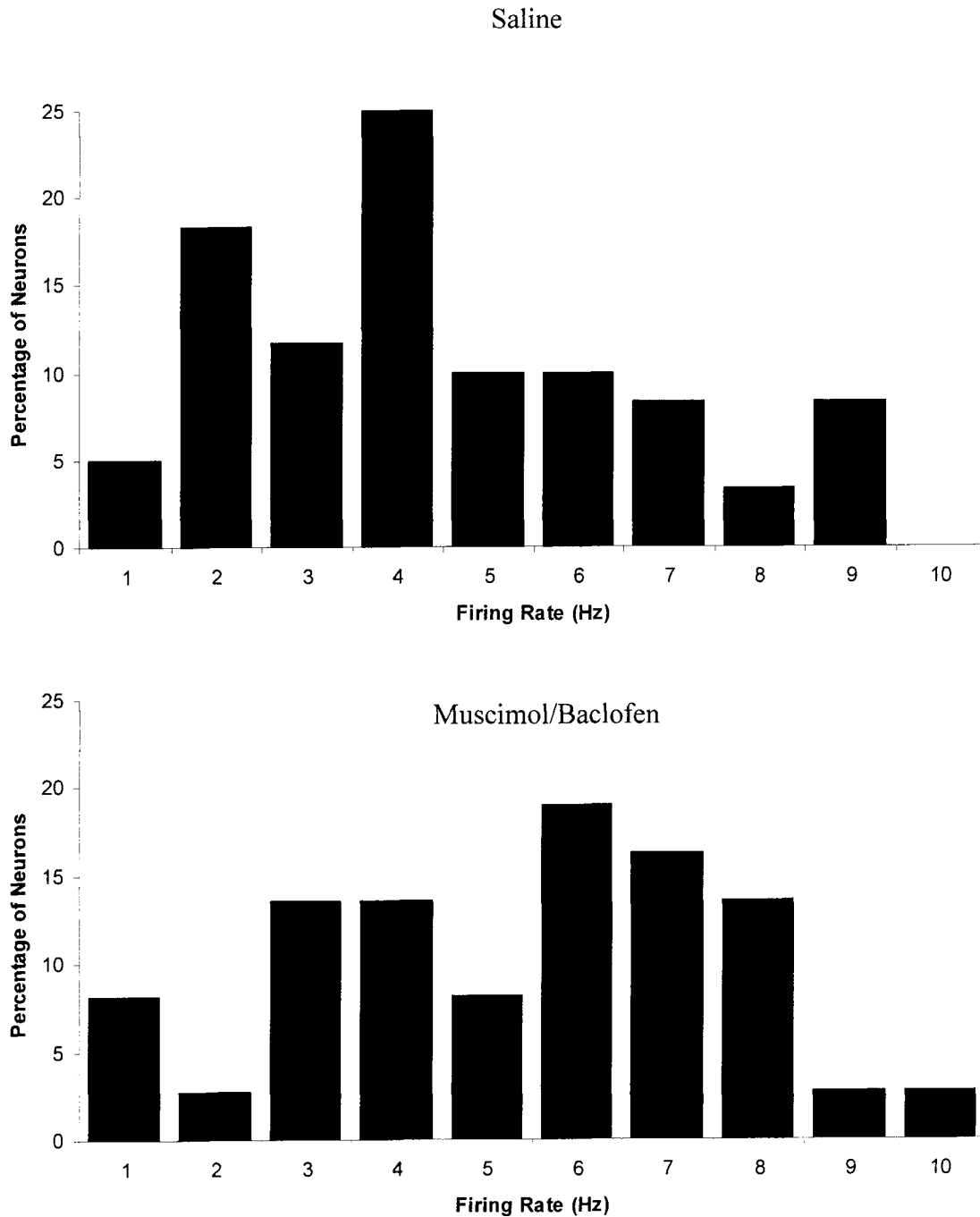
Figure 2.4: The proportion of dopamine neurons in the ventral tegmental area that fired action potentials in a burst mode following infusion of either saline (upper panel) or a cocktail of the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen, respectively, (lower panel) in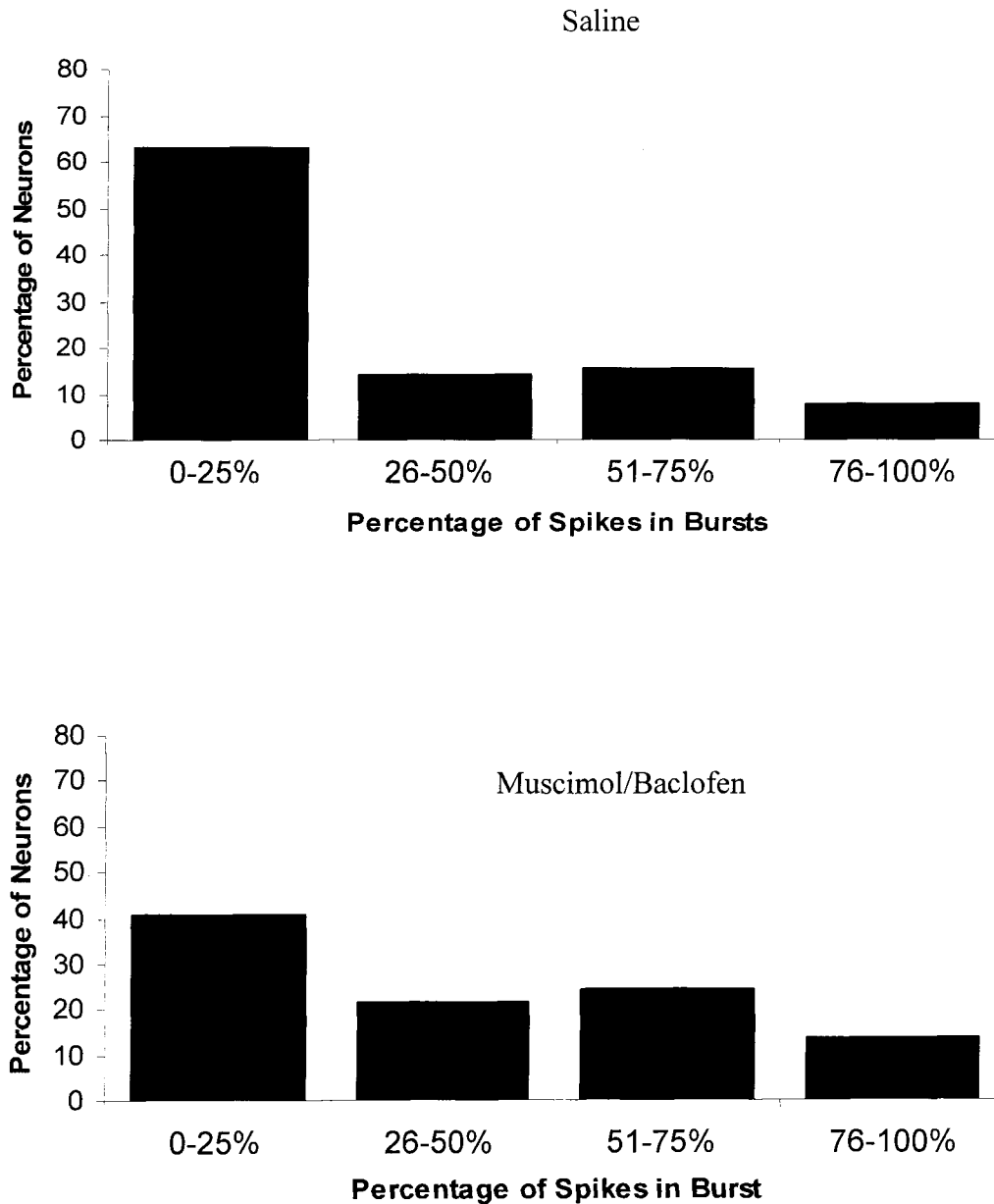 the central nucleus of the amygdala. Infusion of baclofen and muscimol resulted in a significant increase in the proportion of cells that fired >50% of their action potentials in a burst mode ($p < 0.02$).

Saline

Percentage of Spikes in Bursts

Muscimol/Baclofen

Percentage of Spikes in Burst

CHAPTER 3

THE CENTRAL NUCLEUS OF THE AMYGDALA DIFFERENTIALLY

REGULATES INSTRUMENTAL RESPONDING IN A

CONFLICT TEST OF EXPERIMENTAL ANXIETY

3.1 INTRODUCTION

The amygdalar complex is well established to be a critical brain region underlying

fear and anxiety, as well as appetitively motivated behaviours. Evidence from

neuroanatomical and neurochemical studies, including the results described in Chapter 2,

strongly suggests that the neural circuits by which the CeA may regulate appetitive and

aversive learning include interactions with the mesocortical and mesolimbic DA systems.

Given these data, our contention is that popular fear conditioning paradigms may offer an

overly simplistic view of the neural mechanisms underlying the more complex forms of

anxiety subserved by these types of learning. Given that hypotheses proposed by clinical

researchers suggest that anxiety disorders may result from a pathological bias away from

pleasant emotions (mediated by an appetitive system) and a progressive bias towards

anxiety-associated stimuli (mediated by an aversive system), conflict models may

provide a useful tool for investigating the neural correlates of experimental anxiety.

Conflict procedures typically utilize subjects that are trained over time to

suppress a learned response for reinforcement. Similar to experimental models of anxiety

investigating unconditioned behaviours, such as the elevated plus maze or open field,

punishment-based conflict paradigms have also been consistently used to identify many

anxiolytic agents. The basic task was introduced by Geller and Seifter (1960) and

employs food-deprived rats that are initially trained to lever press for a food reward and

are then exposed daily to a "conflict" component where responding becomes inhibited by electric shocks over time. In this situation, the tendency of rats to seek out a positive stimulus (food reinforcement) is countered by the impulse to avoid it due to learned fear (electric shock). The conflict is inherent in that the response has become both conditioned and punished. Anxiolytic properties are attributed to drugs that selectively increase punished responses in the presence of shocks relative to unpunished responses in their absence. Accordingly, benzodiazepines and barbiturates were initially shown to exert these anxiolyic properties, and many more potential anxiolytic agents have been characterized by the Geller-Seifter test (Millan, 2003).

Given the lengthy training period required by the Geller-Seifter model, Vogel and colleagues (1971) developed a novel conflict protocol where 48 h water-deprived rats receive a three min test session in which drinking is punished by a shock delivered through the spout of the bottle every 20 licks. Anxiolytic properties using the Vogel conflict test are reflected in a drug-induced increase in the number of shocks received and have the primary advantage of being easily assessed without the long training protocol used by the Geller-Seifter test. Since its introduction, the Vogel conflict test has been extensively employed to characterize the monoaminergic mechanisms (for reviews, see Millan, 2003; Millan and Brocco, 2003) implicated in the development and treatment of anxiety (Belzung & Griebel, 2001; Lesch, 2001). Data on DA "D_1-like" receptors (D_1, D_5) and the Vogel test is, to our knowledge, unavailable. However, the prototypical DA "D_2-like" (D_2, D_3) receptor agonists apomorphine (APO), albeit a DA D_1 and D_2 agonist, and 3-(3-hydroxyphenyl)-N-propylpiperidine (3-PPP) have all significantly increased punished responding in this task (Hjorth et al., 1986, 1987). Although these drugs do not

distinguish DA D_2 from D_3 and D_4 receptors (eg. Millan et al., 2002), Millan and

colleagues (2004) recently replicated these results with a more selective D_2/D_3 receptor

agonist, S3 2504, which in turn were blocked with selective antagonists at these

receptors. These studies suggest that activation of DA D2 receptors underlies the

mediation of experimental anxiety in the Vogel test; however, other DA D2-like receptor

antagonists have either no effect or also exhibit anxiolytic properties (Siemiatkowski et

al., 2004; Rogoz et al., 2000) in this conflict test. Notably, the anxiolytic actions of (-)-

PPP, a partial agonist that preferentially stimulates presynaptic DA D2 receptors (Hjorth

et al., 1987), suggest that DA D2 autoreceptors in the VTA may underlie the effects

elicited by this compound in the Vogel conflict test.

In contrast to the wealth of data available on the effects of peripheral

administration of DA drugs in the Vogel test, there have been few studies examining the

effects of local manipulations of the mesocorticolimbic DA system. Lesions of the PFC,

for example, disinhibit punished drinking (Yamashita et al., 1989), and selective

destruction of DA terminals in the mPFC also significantly increases punished drinking

(Ravard et al., 1989; Ravard et al., 1990). On the other hand, stimulation and lesion

studies have clearly demonstrated a crucial role for the amygdala in the Vogel conflict

paradigm (Kopchia et al., 1992; Moller et al., 1994, 1997; Shibata et al., 1989; Yamashita

et al., 1989). More specifically, early studies report a reduction in experimental anxiety

following both lesions of the CeA (Yamashita et al., 1989) and injections of anti-anxiety

drugs into the CeA (Shibata et al., 1989). Most recently, Moller and colleagues (1997)

produced bilateral lesions of the CeA with microinjections of ibotenic acid and also

found a robust release of punished drinking in the Vogel test. Importantly, evidence has

also emerged suggesting that the immediate early gene c-fos, a marker of neuronal

activation, is also induced in the CeA of animals subjected to the Vogel procedure

(Moller et al., 1994). These data offer direct evidence implicating the CeA in this task by

showing that neuronal activation in the CeA accompanies the anxiety-producing conflict

situation.

Although the Vogel conflict test remains of unquestionable value, it does not

include a non-punished session. In contrast, the Geller-Seifter conflict test, by including

both punished and non-punished sessions, allows a more direct assessment of the neural

mechanisms mediating specific conflict situations compared to a potential delineation of

general motor and motivated processes. Similar to the effects observed in the Vogel test,

the effects of peripheral administration of DA receptor drugs on performance in the

Geller-Seifter task have also included anxiolytic, anxiogenic, and no effects following

injections of general DA receptor ligands (Lazareno, 1979; Geller & Seifter, 1960; Tye et

al., 1979).

In comparison with the inconsistent results obtained by peripheral manipulations,

the effects of local mesocorticolimbic DA system manipulations on the Geller-Seifter

behavioural repertoire are clearer. In a direct investigation of the role of the mesocortical

DA system in the behavioural consequences of a conflict test, Broersen and colleagues

(1995) infused either the DA receptor agonist APO or antagonist cis-flupenthixol (FLU)

directly into the mPFC in a modified version of the Geller-Seifter test, and its variant, the

extinction of conflict test. Whereas local infusions of APO into the mPFC further

inhibited responding suppressed by punishment (a pro-conflict effect), FLU increased

responding during the conflict component of both tests. These results suggest that the

PFC and its dopaminergic innervation play a key role in the anxiety-related behavioural changes accompanying performance on this task, and offer novel insight into the behavioural significance of the mesocortical DA system in conflict tests.

With respect to the present study, there have been no experiments investigating the role of the amygdala in the Geller-Seifter version of this conflict task. Given the converging evidence from neuroanatomical and neurochemical studies, including the electrophysiological data revealed in Chapter 2, a theory encompassing a critical role for the CeA in modulating the mesocorticolimbic DA system is emerging. Moreover, the complementary data from Broersen and colleagues (1995) suggest a rather selective involvement of the mesocortical and mesolimbic DA systems in the experimental anxiety produced by the Geller-Seifter test of conflict. However, in light of the fact that the CeA has been implicated as a significant mediator of the behavioural repertoire observed in the Vogel conflict test (Kopchia et al., 1992; Moller et al., 1994, 1997; Shibata et al., 1989; Yamashita et al., 1989), the role of this amygdalar nucleus in the operant Geller-Seifter paradigm is currently unknown.

To date, the specific role of the mesolimbic DA system in tests of conflict have not been thoroughly investigated. However, NAcc DA, and its motivational mechanisms, may also play a significant role in these types of tasks, although they are largely dependant on the work requirements of the test. For example, lever-pressing schedules with minimal work requirements such as a fixed ratio (FR) 1, where every press is rewarded, are insensitive to the effects of NAcc DA depletions (Salamone et al., 1995; Aberman & Salamone, 1999; Ishiwari et al., 2004). This suggests that the positively reinforced responding in tasks such as the operant Geller-Seifter conflict test may not be

the process dependant on accumbal DA. However, reinforcement schedules requiring increased work, such as operant conditioning schedules with high ratios, are greatly impaired by NAcc DA depletions. Investigations of DA depletion on a wide range of ratio schedules including FR1, FR4, FR5, FR16, and FR64 indicate that this manipulation impairs lever-pressing at all ratios greater than FR1 (Salamone et al. 2001; Ishiwari et al., 2004); however, responding on FR4 and FR5 schedules exhibits the most rapid recovery of function (Aberman & Salamone, 1999; Salamone et al., 1993; Ishiwari et al., 2004). Moreover, when variable interval (VI) schedules are used, tandem VI/FR schedules are required to suppress response rates following NAcc DA depletions (Correa et al., 2002; Mingote et al., 2005). These data suggest that accumbal DA is not involved in primary motivation for food per se, but in helping animals overcome work requirements to obtain food. The mesolimbic DA system, therefore, may be the integral part of the limbic and forebrain circuitry that enables the animal to exert effort to constantly make difficult decisions, such as those involving simultaneous appetitive and aversive motivation.

The primary aim of Chapter 3 was to provide insight into the role of the CeA in a conflict situation which may more closely resemble human anxiety and is dependant on mesocortical DA transmission (Broersen et al., 1995). Given the dense connectivity between the CeA and the mesocorticolimbic DA pathways, and an additional suggested role of the mesolimbic DA system in conflict situations, the significance of the CeA was assessed by characterizing its behavioural significance in a modified Geller-Seifter conflict test incorporating a relatively high effort requirement (tandem VI15s/FR5 ratio). Ultimately, however, the goal of the current study was to utilize a behavioural paradigm involving simultaneously appetitive and aversive motivated learning to more thoroughly

investigate Chapter 2's aim of delineating the CeA's role in exerting afferent control over the mesocorticolimbic DA system.

3.2 METHODS

3.2.1 Subjects

Male Long-Evans rats (Charles River Laboratories, Montreal, QC) weighing between 275 g and 350 g at the beginning of the experiment were used. Rats were group-housed in hanging wire mesh cages (maximum 4 per cage) upon arrival to the facilities and given ad libitum access to food and water. Immediately following surgery, all rats were housed individually in opaque plastic cages in a temperature-controlled (21 +/-1 °C) colony room on a 12hr: 12hr light dark schedule. After surgery, rats were immediately food-deprived and restricted to 85% of their free-feeding weight, but were given free access to water throughout the experiment. All experimental procedures were conducted in accordance with the Canadian Council of Animal Care guidelines and were approved by the Animal Care Committee of the University of British Columbia.

3.2.2 Surgery

Approximately one week following arrival to the facilities, rats were anesthetized (IP) with 100 mg/kg ketamine hydrochloride and 7 mg/kg xylazine, placed into a stereotaxic apparatus, and were prepared for surgery using aseptic procedures. The scalp was incised, the connective tissue overlying the skull was removed, and 23-gauge, stainless-steel guide cannulae were bilaterally implanted into the CeA ([AP], -2.2 mm from bregma; ML, +/-4.0 mm from midline; DV, -7.5 mm from dura). The location of surgical placements for the CeA was according to coordinates derived from the neuroanatomical atlas of Paxinos and Watson (1998). The guide cannulae were

permanently held in place by four steel screws and dental acrylic cement. Stainless steel obdurators (30-gauge) flush with the end of the guide cannulae were inserted into the guides to prevent occlusion by ensuring they remained free of debris, and remained in place for the duration of the experiment with the exception of microinfusions. Immediately following all surgical preparations, antibiotic ointment was applied to the incision site to facilitate healing and prevent infection. All rats were given seven days to recover from surgery prior to commencing behavioural testing, and during which animals were immediately transferred to individual housing, food-restricted to 85% of their free-feeding weight, and handled daily for 5 mins.

3.2.3 Apparatus

Experiments took place in eight operant chambers (30.5 x 24 x 21 cm; Med-Associates; St. Albans, VT) enclosed in ventilated, sound-attenuating boxes. All boxes were equipped with a fan to aid in ventilation and the attenuation of extraneous noise. In each chamber, two retractable levers were fitted equidistant from a food tray, situated in the bottom-center of the front panel, where food reinforcement (45 mg; Bioserv, Frenchtown, NJ) was delivered via a food-pellet dispenser. Two identical stimulus lights (100 mA; 2.5 cm in diameter) were located above each lever. A houselight (100 mA) mounted on the top-center of the wall opposite the levers was utilized to illuminate each chamber. Auditory stimuli were delivered via a speaker connected to a programmable audio generator (ANL-926; Med-Associates) located in the top-left corner of the wall opposite the levers. The floor consisted of 20, 5mm grids spaced 1.6cm apart that were connected to a shock generator/scrambler through which footshocks could be delivered. Four infrared photobeams (one mounted on each of the chamber's four sides) were used

to detect locomotor activity. Each chamber was connected to a personal computer located in an adjacent room via an interface (Med-Associates). All experimental contingencies and data acquisition were programmed utilizing MED-PC IV and recorded by an IBM® personal computer.

3.2.4 Conflict Training Procedure

Following a seven day recovery period from surgery, all rats began the first day of training on a conflict protocol similar to those derived by Geller and Seifter (1960) and Tye and colleagues (1979). Food-deprived rats were initially trained to press the left lever for sugar pellets (Bioserv; Frenchtown, NJ). During 15 min training sessions, a tandem variable interval 15 s fixed ratio 5 (VI15"/FR5) schedule was implemented, i.e., the fifth response after a mean interval of 15 s was reinforced. Throughout the experiment, sessions commenced with the presentation of the lever, ~60-120 s after the rat was placed in the chamber.

Once the animal reliably pressed the lever, the training procedure was adjusted so that each 15 min session consisted of three 5 min components. During the first and last "Reward" periods, the chamber was illuminated by the houselight and the tandem VI15"/FR5 schedule of reinforcement was utilized. During the middle "Conflict" period (signaled by the houselight off and illumination of a stimulus light above the lever) food was presented on a fixed ratio 1 (FR1) schedule (one pellet for every press) and 0.5 s footshocks (coinciding with the presentation of another stimulus light) were simultaneously delivered on a random ratio 2 schedule (RR2; Fig. 3.1). Training on the conflict paradigm was conducted daily and shock intensities were first set at 0.05 mA and individually adjusted in increments of 0.025 mA; final shock intensities in the present

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series of experiment ranged from 0.7 mA to 1.3 mA in Experiment 1. Once an individual

rat achieved criterion performance of receiving less than ten footshocks per session over

three consecutive days, rats were tested on this conflict test (Experiment 1) and

subsequently on the extinction of conflict test (Experiment 2) following pharmacological

manipulations.

3.2.5 Microinfusion and Conflict Test Procedure (Experiment 1)

After lever pressing had stabilized over three consecutive days, rats were

randomly counterbalanced to receive microinfusions of either vehicle (saline) or a

$GABA_A/GABA_B$ agonist cocktail of muscimol hydrobromide and baclofen

hydrochloride, respectively (75 ng each, Sigma Aldrich Inc, St. Louis, MO) into the CeA.

Drugs were freshly dissolved in saline and mixed together immediately before

administration. Prior to initiation of conflict testing, the obdurators were removed from

the guide cannulae and 30-gauge stainless steel infusion cannulae were lowered

bilaterally to the sites of injection, located 0.8 mm ventral to the end of the guide

cannulae. The infusion cannulae were connected by PE-20 tubing to gastight Hamilton

syringes and a volume of 0.5 µL of vehicle or muscimol/baclofen cocktail was delivered

to the freely moving rats over 72 s via a microsyringe pump. A 60 s diffusion period was

allowed before infusion cannulae were removed. Upon completion of the microinfusion,

each rat was placed back into its home cage for an additional ten minutes before initiating

the conflict test session.

Each rat received one bilateral infusion of saline vehicle and one bilateral infusion

of the muscimol/baclofen cocktail prior to undergoing two conflict test sessions that were

separated by at least three consecutive days at the aforementioned criterion. A within-

subjects design was implemented whereby half of the rats received saline before the first

conflict test and muscimol/baclofen before the second test; accordingly, the remaining

rats received the reverse order. The order of infusions was counterbalanced according to

the number of shocks received by each rat during the conflict training session on the day

prior to the first conflict test.

3.2.6 Extinction of Conflict Test Procedure (Experiment 2)

Following the second conflict test session, each rat from Experiment 1 was re-

trained on the same conflict training procedure described above until again achieving

criterion performance of less than ten footshocks per session over three consecutive days.

On the following day, rats received microinfusions of either saline or the muscimol and

baclofen cocktail into the CeA. The microinfusion procedure used in this experiment was

identical to that in Experiment 1. Extinction of conflict sessions were identical to those

implemented in Experiment 1 with the exception that the shock generators were turned

off and the rats did not receive any footshocks during the "Conflict" component of the

test session.

Each rat received one bilateral infusion of saline vehicle and one bilateral infusion

of the muscimol/baclofen cocktail prior to undergoing extinction of conflict tests. Again,

a within-subjects design was implemented whereby half of the rats received saline before

the first extinction of conflict test and muscimol/baclofen before the second test;

accordingly, the remaining rats received the reverse order. The order of infusions was

counterbalanced based on the order of administration in Experiment 1 such that each

animal received either vehicle or the muscimol/baclofen cocktail first, and the remaining

treatment second, in both the conflict test (Experiment 1) and the extinction of conflict

test (Experiment 2). Test sessions were separated by at least three days to re-establish the criterion of less than ten footshocks over three consecutive conflict training sessions.

3.2.7 Histology

Following the completion of all behavioural testing, rats were euthanized in a carbon dioxide chamber. Subsequently, brains were removed and fixed in a 4% formalin solution for at least 24 hours. The tissue was then frozen, sectioned into 50 μm coronal slices, and stained with cresyl violet to enable histological examination of cannula placements. Placements were verified with reference to the neuroanatomical atlas of Paxinos and Watson (1998). Rats with cannulae placements outside of the CeA were excluded from data analysis.

3.2.8 Statistical Analysis

Responding on levers during Reward 1, Conflict, and Reward 2 sessions during both conflict (Experiment 1) and extinction of conflict tests (Experiment 2) was analyzed using paired samples t-tests. Paired samples t-tests were also used for analyses of various other data acquired in the present series of experiments.

3.3 RESULTS

3.3.1 Histology

The location of infusions into the CeA (Experiment 1 and 2, respectively) are presented in Figure 3.2. Placements were verified with reference to the neuroanatomical atlas of Paxinos and Watson (1998). All animals with placements ventral to the amygdaloid complex or encroaching the basolateral nuclei of the amygdala (BLA) were excluded from statistical analyses. Following histological analysis, a total of 9 rats were included in the analyses.

3.3.2 Experiment 1: Conflict Test

By the end of training on the conflict procedure prior to the first infusion, all animals were responding robustly on the lever for food reinforcement (overall mean lever presses = 762.11 +/- 299.71). Animals required an average of 26 days to achieve criterion performance of less than ten footshocks per session for three consecutive days (overall mean lever presses during Conflict session on day prior to first infusion = 13.33 +/- 8.62; overall mean footshocks received on day prior to first infusion = 5.33+/- 2.29). Both groups (i.e.; those that would receive saline or muscimol/baclofen infusions first) were evenly matched for overall lever responding on the last day of conflict training immediately prior to the first infusion. Different rates of lever pressing were generated by the reinforcement and punishment contingencies that were in effect during different components of the conflict procedure, but levels of unpunished responding during Reward were consistently high, while lever responding was suppressed by punishment during Conflict training. As such, no significant between-group differences were found on collective rates of lever pressing over 15 min training sessions [t (8) = 0.465, n.s.] or during their 5 min Reward 1 [t (8) = 0.713, n.s.], Conflict [t (8) = 0.836, n.s.], and Reward 2 [t (8) = 0.142, n.s.] components.

Rats received counterbalanced infusions of baclofen/muscimol and saline on separate test days. Analysis of the lever pressing data showed that there were no significant main effects of injection order, or interactions with order and any of the within-subjects variables (All F's < 2.63, n.s.). This confirmed that there was no treatment order effect by suggesting that animals receiving saline or muscimol/baclofen prior to the first conflict test session responded similarly to those receiving saline or

muscimol/baclofen, respectively, prior to the second. This validated the collapse of these groups and the use of paired samples t-tests for further analyses.

Figure 3.3A, B, and C display the overall number of lever presses made during the three phases of the conflict tests following saline and muscimol/baclofen infusions into the CeA. Analysis of these data revealed significant treatment effects during the Reward 1 [t (8) = 4.305, p<0.01; Figure 3.3A], Conflict [t (8) = 4.036, p<0.01; Figure 3.3B], and Reward 2 [t (8) = 9.835, p<0.001; Figure 3.3C] components of the conflict test. Rats responded more during the Conflict session (p<0.01) following infusions of muscimol/baclofen into the CeA relative to saline. Surprisingly however, rats responded less during the Reward 1 (p<0.01) and Reward 2 (p<0.001) sessions when they received infusions of baclofen/muscimol, indicating dissociable roles of the CeA in the different components of the conflict test.

It was also of interest to examine nosepoking between saline and muscimol/baclofen treated animals on test days to ascertain whether this manipulation also affected Pavlovian approach behaviour. There was no significant effect of treatment on approach to the food receptacle [t (8) = 1.386, n.s]. A paired samples t-test was also used to compare locomotion on conflict test days in order to assess whether any main effects seen as a result of CeA inactivation could be attributed to changes in locomotor ability. Similarly, infusions of muscimol/baclofen did not alter locomotor activity relative to control treatments [t (8) = 1.536, n.s.].

3.3.3 Experiment 2: Extinction of Conflict

As with Experiment 1, all animals vigorously responded on the lever on the last day of conflict training prior to the first extinction of conflict test (overall mean lever

presses = 894.78 +/- 454.34). Following the final conflict test (Experiment 1), animals

required an average of 5 days to stabilize on a level of responding that resulted in fewer

than ten footshocks per session (overall mean lever presses during Conflict session on

day prior to first extinction of conflict test = 6.89 +/- 5.90; overall mean footshocks

received on day prior to first extinction of conflict test = 3.78 +/- 2.59). Although

different rates of lever responding were generated by the reinforcement and punishment

contingencies that were in effect during different components of the conflict procedure,

overall levels of unpunished responding during Reward were consistently high while

response rates were suppressed by punishment during Conflict. No significant between-

group differences were found prior to testing on collective rates of lever pressing over 15

min training sessions [t (8) = 0.103, n.s.] or during their 5 min Reward 1 [t (8) = 0.041,

n.s.], Conflict [t (8) = 0.614, n.s.], and Reward 2 [t (8) = 0.219, n.s.] components. Thus,

the same order of counterbalancing was used as in Experiment 1.

As in Experiment 1, rats received counterbalanced infusions of muscimol/

baclofen and saline on separate extinction test days. Analysis of the lever pressing data

again revealed no significant main or interaction effects of injection order (All F's < 5.32,

n.s.), suggesting that animals receiving saline or muscimol/baclofen prior to the first

extinction test session responded similarly to those receiving saline or muscimol/baclofen

prior to the second. Moreover, this validated the collapse of these groups and the use of

paired samples t-tests for further analyses.

Figure 3.4A, B, and C display the overall number of lever presses made during

the three phases of the extinction of conflict tests, which were identical to those used in

Experiment 1 with the exception that no footshocks were delivered during the Conflict

period, following saline and muscimol/baclofen infusions into the CeA. Paired samples t-tests revealed significant differences between treatment conditions during the Reward 1 [t (8) = 3.192, p<0.05; Figure 3.4A], Conflict [t (8) = 3.843, p<0.01; Figure 3.4B], and Reward 2 [t (8) = 4.629, p<0.01; Figure 3.4C] components of the extinction of conflict test. Omission of footshocks during extinction of conflict tests resulted in a clear disinhibition of lever responding during the Conflict session compared to when footshocks were delivered (Experiment 1). However, inactivation of the CeA resulted in a further increase in responding during the Conflict session (p<0.01) compared to saline treatments. Again, rats responded less during the Reward 1 (p<0.01) and Reward 2 (p<0.001) sessions when they received infusions of muscimol/baclofen, suggesting a similar dissociation to that observed in Experiment 1 of the role of the CeA in the different components of the conflict test.

Inherent in the present analysis was also a necessity to assess nosepoking behaviour to elucidate whether CeA inactivation affected extinction of a Pavlovian approach to the food receptacle. There was no significant effect of treatment on approach to the food receptacle [t (8) = 1.307, n.s]. Similarly, infusions of muscimol/baclofen did not alter locomotion [t (8) = 1.889, n.s.] relative to control treatments. This confirmed that the main effects of CeA inactivation observed during the extinction of conflict test could not be attributed to impairments in locomotor ability. Collectively, these data suggest that the CeA plays a robust regulatory role in behaviour during both the conflict and extinction of conflict tests, and, moreover, that it modulates the motivational and anxiety-like behaviors assessed by the various components (Reward 1 and 2, and Conflict, respectively) of these tests in a distinctly dissociable manner.

3.4 DISCUSSION

The results from the present series of experiments demonstrated that the CeA selectively influences instrumental responding for food under normal conditions and under conditions of experimental anxiety. Animals were initially trained to lever press for sugar pellets under a tandem VI15s/ FR5 schedule (i.e., the fifth response after a mean interval of 15 seconds was reinforced).Once animals were reliably lever pressing, the procedure was adjusted to include three components. During the first and last "Reward" periods, the test chamber was illuminated and this tandem VI15s/FR5 schedule of food delivery was in effect; in contrast, during the middle "Conflict" period (signaled by the houselight off and a stimulus light on above the lever) food was presented on a FR1 schedule and footshocks were delivered simultaneously on a RR2 schedule. Under test conditions utilizing this modified Geller-Seifter conflict procedure (Experiment 1), pharmacological inactivation of the CeA via infusions of $GABA_A/GABA_B$ agonists resulted in a reduction of responding during the two "Reward" periods. Alternatively, CeA inactivation caused a robust release of the suppressed responding induced during training by the coupling of footshock with food reward. Collectively, these data suggest that the CeA plays distinct roles in mediating motivational responding under appetitive conditions and suppressing responding following exposure to aversive, or conflict-inducing, situations.

After animals were tested in the conflict test (Experiment 1), they were subjected to a variant of this paradigm, the extinction of conflict test (Experiment 2). These tests were identical to those used in Experiment 1 with the exception that the shock generators were turned off and the animals did not receive footshocks during the "Conflict" session.

Omission of footshocks in this test resulted in a significant disinhibition of lever pressing. Subsequently, pharmacological inactivation of the CeA facilitated this response as these animals performed a greater number of bar presses during the "Conflict" session of extinction testing relative to saline infused animals. Similar to the findings from conflict testing, inactivation of the CeA resulted in a significant reduction in lever pressing during the two "Reward" components of the extinction of conflict test. Collectively, the results from the "Conflict" session of Experiment 2 suggest that the CeA plays a specific role in behavioural suppression under aversive conditions (Experiment 1) and during tests in extinction. The data obtained under standard "Reward" conditions support and replicate the findings from Experiment 1, indicating a critical role of the CeA in regulating motivational salience for stimuli under increased demand, possibly via the regulation of A10 DA neuron activity within the VTA.

Interestingly, the results from Experiment 2 suggest that it may not be the footshock per se in Experiment 1 but the cues signaling the "Conflict" period (i.e. houselight off and stimulus light on above the lever) that mediate the effects of the CeA in this component of the task. Tests during Experiment 1 and Experiment 2 were conducted identically, with the exception that the footshocks were omitted in Experiment 2, allowing a quantification of the extinction process. The findings from testing during extinction suggest that the CeA mediates the effect of the cues associated with footshock, in contrast to the shocks alone, given that the "Conflict" component is signaled. The results show that the effects of CeA inactivation during regular "Conflict" (Experiment 1) may be due, at least in part, to a disruption in the cues signaling "Conflict" to suppress behaviour. The fact that CeA inactivation caused a robust release of suppressed

responding during "Conflict" in both Experiment 1 (with cues and shock) and Experiment 2 (with cues but no shock) suggests that the CeA mediates suppression of behavioural responding during conflict by interpreting the cues associated with footshock.

3.4.1 A Proposed Model of CeA-Mediated Responding During Aversive and Appetitive Conditions

Integrating these data with those demonstrated in Chapter 2, a functional model can be derived to explain how the CeA dissociably regulates these behavioural phenomena. Under basal conditions, the CeA presumably drives population activity and burst-firing of VTA dopaminergic neurons, which in turn results in both tonic and phasic DA release in the NAcc; the NAcc then encodes reward salience to specific stimuli. Following inactivation of the CeA, data from Chapter 2 show that there is a reduction in VTA DA neuron population activity as well as a corresponding decline in accumbal DA transmission (Ahn and Phillips, 2003). This, presumably, would result in a devaluing of normally appetitive stimuli such as the sugar pellets used in the current study. However, the CeA is also activated by aversive stimuli, such as footshock or cues associated with it, which increases mesocortical DA transmission and in turn results in an increase in experimental anxiety that may contribute to the suppression of responding for rewarding stimuli. In this situation, it must be assumed that the induction of experimental anxiety from increased mesocortical DA release overrides the increased reward salience that is encoded by the increased mesolimbic DA transmission, to result in a net suppression of behavioural responding for sugar pellets. Therefore, inactivation of the CeA would presumably abrogate the increase in mesocortical DA transmission, as well as the

subsequent increase in experimental anxiety, which in turn would disinhibit responding during the "Conflict" component of the test.

3.4.2 CeA-Mediated Responding During "Conflict" May Involve a PFC DA Mechanism

Within the aforementioned model, it is assumed that the mechanism by which CeA inactivation increases responding during the "Conflict" phase (with simultaneous food and footshock) is due to a reduction in experimental anxiety. While typically not considered a traditional regulator of anxiety, an accumulating body of preclinical data demonstrates that modulation of mesocortical DA transmission induces significant alterations in experimental anxiety where higher-order cognition, such as the introduction of conflict, is involved. Specifically, using the Geller-Seifter conflict test, it has been demonstrated that selective destruction of dopaminergic terminals in the mPFC results in a release of punished responding, which in turn may be viewed as a reduction in anxiety (Broersen et al., 1995), or as a failure to suppress inappropriate behaviours. Similarly, an increase in mPFC DA transmission is often documented in traditional fear conditioning paradigms (Yoshioka et al., 1996), and broad pharmacological antagonism of DA receptors in the mPFC can reduce the expression of freezing behaviors (Pezze et al., 2003). Studies examining unconditioned fear responses have also revealed an important role of mPFC DA receptors in driving the expression of anxiety-like behaviors (Shah et al., 2004). Importantly, it has even been demonstrated that pharmacological agents which either increase or decrease fear-like responses in an array of both conditioned and unconditioned paradigms elicit a corresponding increase and decrease, respectively, in mesocortical DA transmission (Tam and Roth, 1990). Thus, it is possible that in response

to footshock or the cues associated with it, the CeA is activated (Moller et al., 1994); this in turn increases mesocortical DA transmission and results in an increase in anxiety-like behaviour that ultimately results in a suppression of responding during shock exposure. When the CeA is taken off line, via pharmacological inactivation in the current study, the footshock and associated cues may not elicit an increase in mesocortical DA release; hence, there would be a resultant decrease in both shock-induced anxiety and subsequent inhibition of responding for sugar pellets. This model is directly supported by previous work demonstrating that local infusion of the non-specific DA receptor antagonist FLU resulted in a significant release of punished responding during the "Conflict" component of this task (Broersen et al., 1995). These results are identical to what was seen in the current study, in both Experiment 1 and Experiment 2, following inactivation of the CeA. Additional data collected by Broersen and colleagues (1995) following local infusions of the general DA receptor agonist APO indirectly support this model by offering direct evidence that activation of mPFC DA receptors inhibited punished responding (a pro-conflict effect) to a greater extent than that following saline treatment.

The increase in lever presses observed during extinction testing (Experiment 2) indicates that, under normal conditions, neuronal activity within the CeA mediates suppression of behaviour not only induced by footshock but also that induced by cues associated with footshock. It is likely that this effect is also mediated by CeA driven mesocortical DA transmission, given that local infusion of DA receptor agonists or antagonists into the mPFC during the extinction of conflict test disrupts and facilitates tests during extinction, respectively (Broersen et al., 1995). Specifically, these data extend the work of Broersen and colleagues (1995), who suggest that blockade of mPFC

DA receptors causes an increase in responding during the "Conflict" component of extinction testing (Broersen et al., 1995). Furthermore, they are in line with the suggestion that this enhancement of responding during extinction sessions (i.e. when only cues signaling shock, but not shock, are present) may reflect a decrease in anticipatory anxiety (Ketelaars & Bruinvels, 1989). The presence of the cues signaling the "Conflict" period may elicit CeA activation during extinction testing, which in turn enhances neurotransmission within the mesocortical DA system and results in the behavioural suppression of lever pressing.

An alternative explanation of the release of punished responding during the "Conflict" session following inactivation of the CeA is that this manipulation resulted in a disruption of the pairing of the aversive footshock cue with the lever pressing for sugar pellets. The CeA is known to function as a relay station between corticolimbic structures, processing external stimuli, and brainstem nuclei, which in turn elicit peripheral visceral responses to aversive stimuli such as increased cardiovascular output and adrenal hormone secretion (Davis, 1998; Kapp et al., 1982; LeDoux et al., 1988). Inactivation of the CeA could result in impaired activation of visceral responses, which in turn may reduce the aversive salience of the footshock (or cues signaling the "Conflict" period) and in turn disrupt the coupling of this cue to the lever pressing, resulting in an attenuation of response inhibition during the "Conflict" phase. However, the fact that local antagonism (and activation) of mPFC DA receptors (Broersen et al., 1995), produced effects similar to CeA inactivation suggests that the effects described here were mediated, at least in part, by a disruption in PFC DA transmission.

3.4.3 CeA-Mediated Responding During "Reward" Involves a Potential NAcc DA Mechanism

While the proposed model can account for how the CeA functions to inhibit responding under conflict conditions involving aversively-motivated learning, a separate mechanism must mediate the ability of the CeA to promote appetitively-motivated responding under non-stressful conditions. The current experiments demonstrated that inactivation of the CeA resulted in a reduction in lever pressing for sugar pellets under a tandem VI15s/FR5 schedule in both the conflict test (Experiment 1) and the extinction of conflict test (Experiment 2). In line with these findings, inactivation of the CeA has been shown to result in a reduction in both basal and feeding-evoked DA efflux in the NAcc as well as a reduction in free-feeding behavior (Ahn and Phillips, 2002). These data would indicate that under normal conditions, the CeA permissively contributes to the encoding of reward salience of cues, such as food, likely via regulation of accumbal DA transmission. In the current study, then, it is possible that inactivation of the CeA impaired DA transmission within the NAcc, and subsequently resulted in a devaluation of the sugar pellet reward, which was manifested as a reduction in responding under the "Reward" components of the conflict test. An extensive body of work has demonstrated that lever pressing for sugar pellets is directly related to accumbal DA transmission. However, under conditions of little work, such as a FR1 schedule where every lever press is rewarded, minimal DA transmission in the NAcc is required to maintain responding for rewards (Salamone et al., 1995; Aberman & Salamone, 1999; Ishiwari et al., 2004). However, as this schedule progressively increases in work demand, to a FR4 or FR5 (which allow the greatest recovery of function) or even to a FR16 and FR64 schedule, a

progressive increase in accumbal DA transmission is required to maintain responding (Salamone et al. 2001; Ishiwari et al., 2004). In other words, increased motivation to receive a reward is met by increasing DA transmission as a schedule progressively requires more work to receive the reward. This is in line with progressive ratio tasks, which gauge motivational drive by determining how much work an animal will perform to receive a reward, which in turn is dependent upon DA transmission within the NAcc (Hamill et al., 1999). The results of the current experiments lend direct evidence in support of this model. Furthermore, the current data suggest that the CeA may be a critical neural substrate in determining the regulation of accumbal DA transmission in response to increasing work demands. Specifically, inactivation of the CeA resulted in a reduction of responding for sugar pellets under a tandem VI15s/ FR5 schedule, which could be interpreted as a reduction in motivation for rewarding stimuli. Given the previously detailed neuroanatomical and neurophysiological circuit that has been proposed linking the CeA to the VTA, these data would suggest that, behaviourally, the CeA may contribute to increasing DA transmission under conditions of increased work, and a disruption of CeA activity will result in impaired motivated behaviour under normal conditions. This hypothesis is supported by previous research suggesting that the CeA-VTA-NAcc circuit is required to modulate performance-related alterations in reward (Hall et al., 2001; Corbit and Balleine, 2005). However, detailed work employing a range of fixed ratio schedules following inactivation of the CeA would be required to determine the extent to which the CeA is involved in regulating accumbal DA transmission, and subsequent motivational behaviour, in response to increasing demand to receive rewards.

Figure 3.1: Schematic representation of the conflict test. During the first and last "Reward" periods the chamber was illuminated by the houselight and the tandem VI15"/FR5 schedule of reinforcement was utilized. During the middle "Conflict" period (signaled by the houselight off and illumination of a stimulus light above the lever), food was presented on a continuous reinforcement schedule, and 0.5 s footshocks (coinciding with the presentation of another stimulus light) were simultaneously delivered on a RR2 schedule.

Component	Sugar Pellets	Foot Shocks	Visual Stimuli	Duration
Reward 1	VI-15/FR5	-	House Light	5 min
Conflict	FR1	RR2	Stimulus Light	5 min
Reward 2	VI15/FR5	-	House Light	5 min

Figure 3.2: Histology figures for Experiments 1 and 2 illustrating the infusion locations of saline and the GABA$_A$ and GABA$_B$ receptor agonist cocktail of, respectively, muscimol and baclofen into the central nucleus of the amygdala.

-2.12 mm   -2.30 mm   -2.56 mm
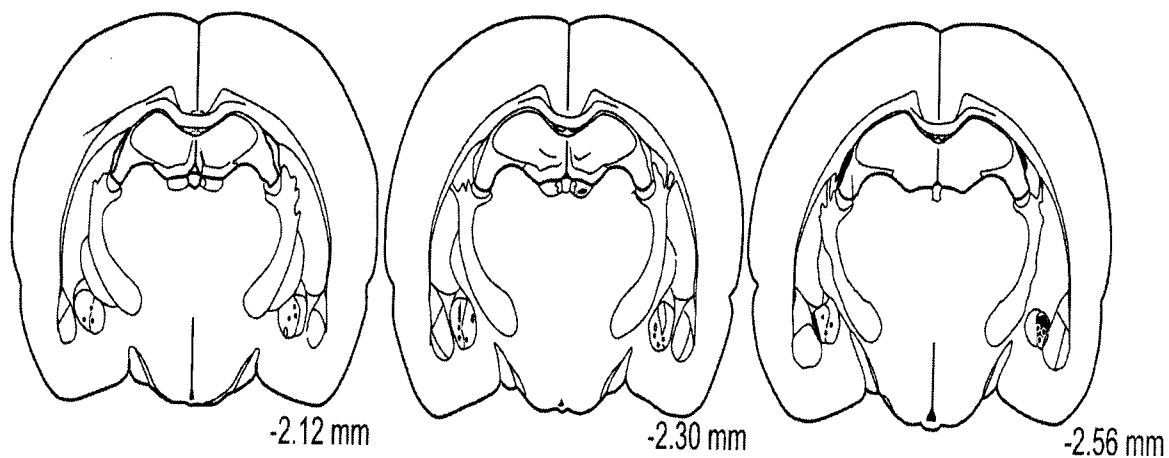
Figure 3.3: The effect of inactivation of the central nucleus of the amygdala, through infusion of the GABA$_A$ and GABA$_B$ receptor agonists muscimol and baclofen, respectively, (BM; grey bars) on lever pressing behaviour under conditions of A) Reward 1; B) Conflict and C) Reward 2 during the conflict task. Significant differences between infusions of saline (CON; white bars) and BM (p < 0.05) are denoted by *.
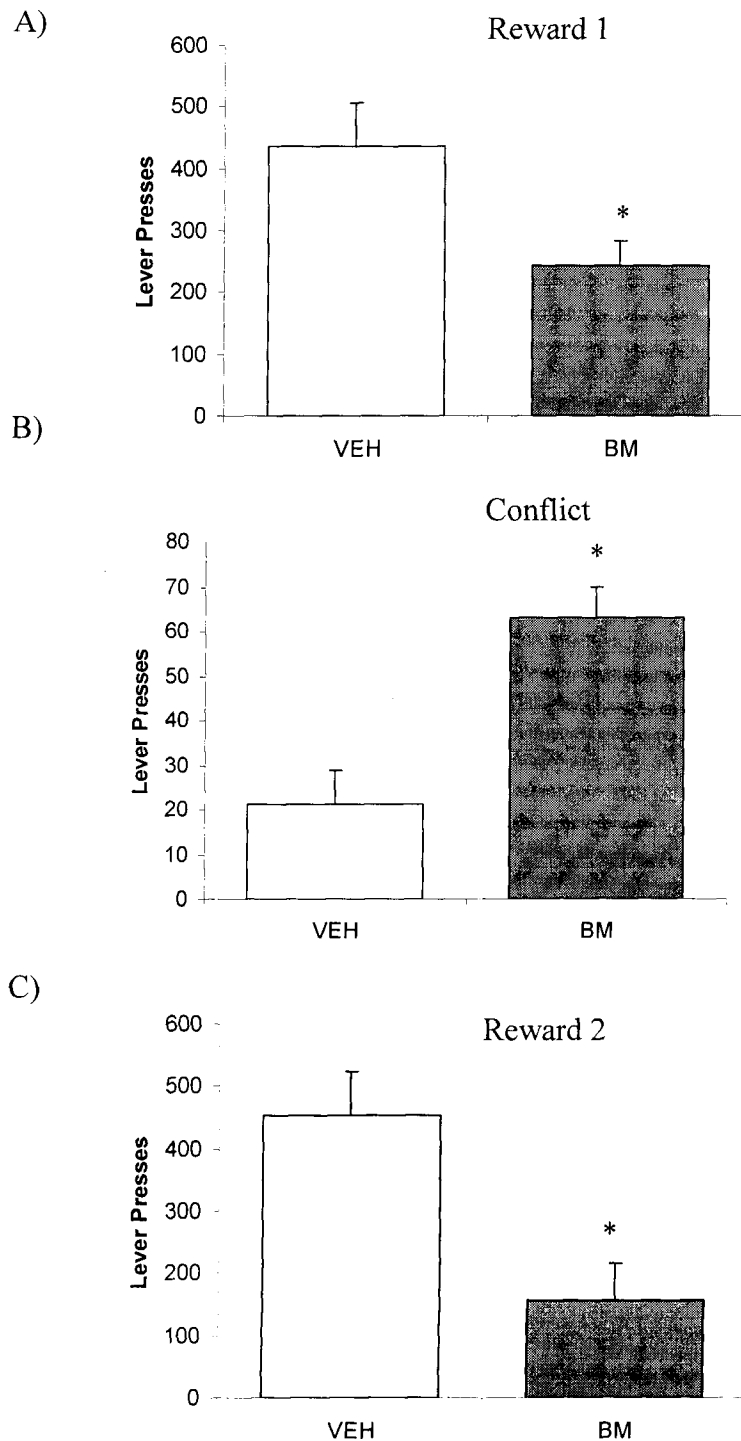
Figure 3.4: The effect of inactivation of the central nucleus of the amygdala via infusion of the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen, respectively, (BM; grey bars) on lever pressing behaviour under conditions of A) Reward 1; B) Conflict and C) Reward 2 during extinction of the conflict task. Significant differences between infusions of saline (CON; white bars) and BM ($p < 0.05$) are denoted by *.
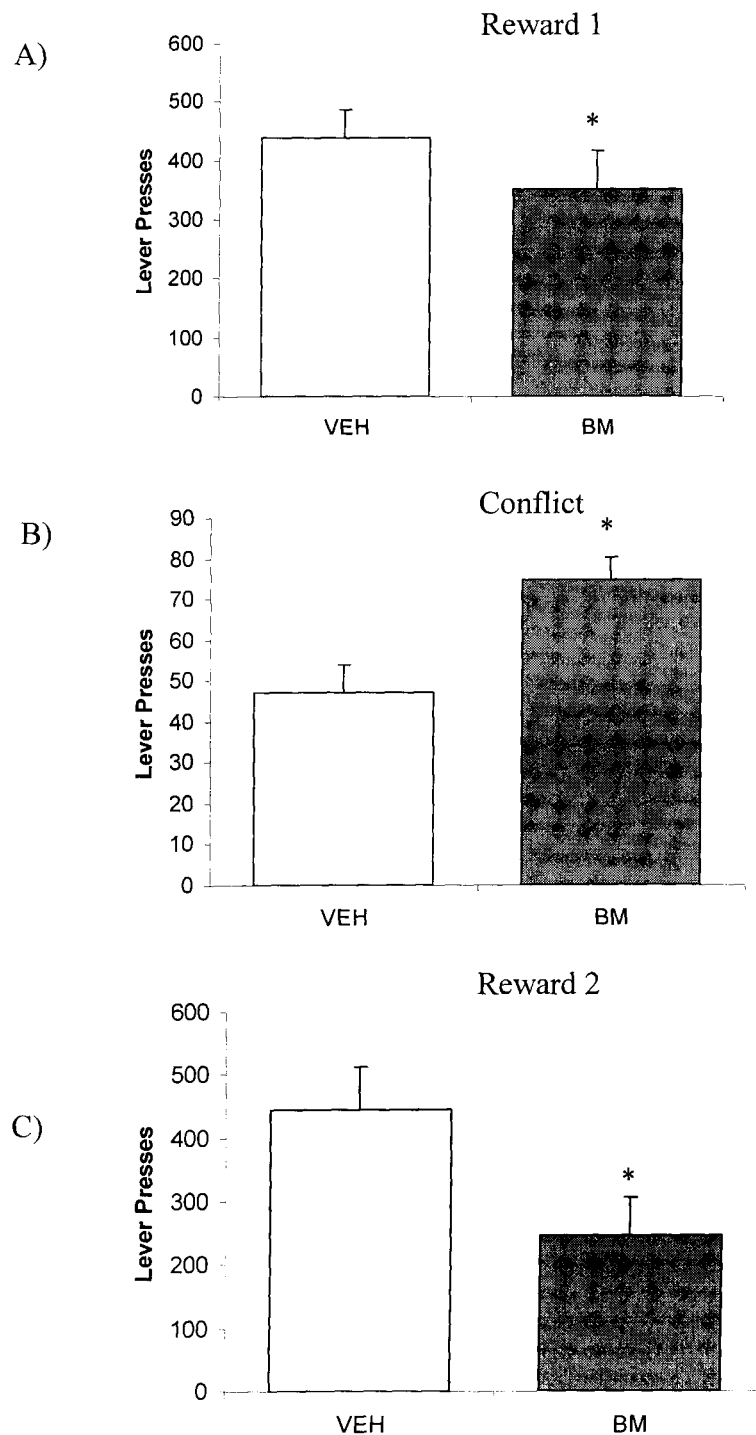
CHAPTER 4

GENERAL DISCUSSION

Collectively, the data from the experiments depicted in Chapter 2 and Chapter 3 offer corroborating evidence from both electrophysiological and behavioural perspectives for the role of the CeA in regulating the mesocorticolimbic DA pathways originating in the VTA. Moreover, the current results may provide a neural mechanism by which anxiety-provoking stimuli that alter neuronal activity within the CeA alter higher-order cognition such as appetitive and aversively motivated learning and behaviour.

Chapter 2 describes an experiment that directly assessed the role of the CeA in the neurophysiological alterations occurring at the VTA DA cell-body region, in order to gain a more thorough understanding of the effects observed by other investigators at the axon terminals of the mesocorticolimbic DA system. The results of this experiment suggest that the CeA elicits a tonic, excitatory regulation of the population activity of VTA dopaminergic neurons, while having differential effects on the firing rate and bursting activity of these cells. Specifically, activation of the CeA caused an increase in the population activity of VTA DA neurons and an increase in the proportion of action potentials these neurons fired in bursts, whereas CeA inactivation resulted in a decrease in their population activity. These are the first data to show that the CeA elicits differential regulation of distinct parameters of DA neuron activity within the VTA. Ultimately, the results depicted in Chapter 2 suggest that increased CeA activation increases the slow, single-spike mode of neuronal firing that ultimately results in tonic, or steady-state, DA release at the axon terminals of the mesocortical and mesolimbic DA

systems. Conversely, these data imply that reductions in CeA activation lead to a decrease in this ongoing, basal rate of neural transmission and release.

To lend further insight into this hypothesized role for the CeA in exerting afferent control over the mesocorticolimbic DA system, the experiments described in Chapter 3 employed a mesocortical DA-dependant operant conflict paradigm that simultaneously incorporates both appetitive and aversive components. The aim of this series of experiments was to assess the effects of CeA inactivation, in a manner similar to that described in Chapter 2, on a form of experimental anxiety that may more closely model human anxiety. Taken together with the data presented in Chapter 2, the results offered in Chapter 3 suggest a neural mechanism for the role of the CeA in selectively regulating responding for food reward under both normal conditions and under times of anxiety. Specifically, during both the conflict test (Experiment 1) and under extinction conditions (Experiment 2), CeA inactivation decreased lever-pressing during non-punished, rewarded periods of the task but robustly released punished responding during the component where simultaneous reward and punishment were delivered. Importantly, CeA inactivation increased behavioural responding during the "Conflict" session in both Experiment 1 (cues and shocks) and Experiment 2 (cues but no shocks). This suggests that the CeA mediates the behavioural repertoire seen during situations involving conflict by interpreting specific cues that signal, or are associated with, the aversive stimulus.

Under normal conditions the CeA mediates the population (and bursting) activity of VTA DA neurons (Chapter 2), which causes an increase in mesolimbic DA neurotransmission and, ultimately, DA efflux in the NAcc. With a reduction in CeA activation, there is a reduction in the population activity of the mesolimbic DA system

cell-bodies and, presumably, there is a resultant decrease in accumbal DA release. Given that NAcc DA transmission is directly related to increasing work demands in order to receive a reward, under normal conditions the CeA may contribute to this motivational phenomenon via accumbal DA. However, when the CeA is inactivated, there may be a reduced motivation to work for appetitive stimuli evidenced by the decrease in responding in Experiment 1 and Experiment 2 (Chapter 3), as a result of decreased NAcc DA efflux. Thus, the CeA may be a key component of the neural circuitry regulating NAcc DA transmission in response to increasing work demands. In addition, the CeA is concurrently activated by aversive stimuli, such as foot shock, which also leads to an increase in VTA DA neuron population (and burst) activity and, ultimately, increased mesocortical DA neurotransmission. This presumably results in increased experimental anxiety in tests of conflict, and the suppression of lever pressing observed in both the training and control treatments. In contrast, inactivation of the CeA would be expected to decrease DA activity in terminal regions such as the mPFC (Chapter 2). This in turn would cause an overall reduction in experimental anxiety and the robust disinhibition of responding observed during conflict testing, an effect even more pronounced in extinction of conflict tests (Experiments 1 and 2; Chapter 3).

4.1 Concluding Remarks

Using a systems approach to investigate how the CeA mediates the mesocorticolimbic DA system may offer novel insight into the neurophysiological mechanism by which one component of the amygdala exerts afferent control over a complex form of experimental anxiety. The amygdala is well-established to be the key neural substrate underlying anxiety, and a growing body of research is also implicating

the DA systems originating in the VTA as key neuromodulators of anxious states. By delineating the role of the CeA in various parameters of VTA DA neuron activity as well as in an operant paradigm incorporating both appetitive and aversive learning, this line of research may provide insight into the pathophysiological alterations underlying anxiety. Ultimately, the data from Chapter 2 and Chapter 3 may also aid in the pursuit of developing novel pharmacotherapeutic interventions for the treatment of anxiety disorders.

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