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A NOVEL INDIRECT PATHWAY OF CRF INNERVATION TO PERIFORNICAL OREXIN NEURONS IS RELAYED THROUGH THE LATERAL SEPTUM

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by

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to examine the thesis of Timothy D. Skog
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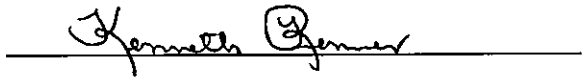

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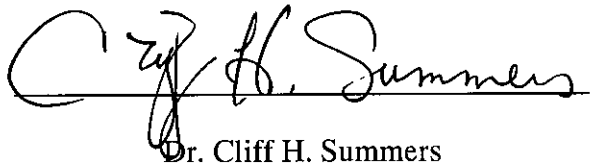

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ABSTRACT

A Novel Indirect Pathway of CRF Innervation to Perifornical Orexin Neurons is Relayed Through the Lateral Septum

Timothy D. Skog

Director: Patrick J. Ronan, PhD

Orexin (Orx) and corticotropin releasing factor (CRF) play integral, sometimes parallel, roles in a host of arousal/stress responses. These neuromodulators have been implicated in a variety of stress-induced psychiatric disorders including addiction and affective disorders. Previous work has indicated that these systems interact and regulate each other—perhaps providing a feed-forward mechanism for enhancing and fine-tuning stress responses. This is implied by the fact that orexinergic neurons in the hypothalamus receive innervation from many CRF-rich neuronal fields including the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeA). We sought to clarify if these CRF afferent pathways include CRF neurons and to determine whether these inputs have any topographical organization. To accomplish this, we used a combination of neuronal tracing methods and immunohistochemistry to visualize the distribution and anatomy of these systems in various brain regions. We provide further evidence that CRF neurons in these regions specifically project to Orx neuron fields, as well as describe a novel circuit pathway for indirect CRF innervation of orexinergic neurons in the hypothalamus through the lateral septum. There appears to be specific topographic distribution of inputs supporting the hypothesis that perifornical Orx neurons preferentially contribute to stress and anxiety responses.

KEYWORDS: Orexin, CRF, Immunohistochemistry, Stress, Anxiety

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

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CHAPTER ONE

Introduction

Background

The global stress response of an organism consists of a complex array of specific physiological processes or general mechanisms such as the hypothalamic-pituitary adrenal axis (HPA) endocrine response, and behaviors mediated by a variety of systems. The central corticotropin-releasing factor (CRF) and orexin/hypocretin (Orx) systems play key roles (D. H. Arendt et al., 2013; Binder & Nemeroff, 2010; Krahn, Gosnell, Grace, & Levine, 1986; P. J. Ronan & Summers, 2011). The neuroendocrine messengers CRF and Orx are important players individually in stress/arousal responses in organisms and are thought to sometimes work in tandem to produce physiological, emotional, and behavioral responses to stressful stimuli. In addition, CRF and Orx have been implicated in a range of stress-induced behaviors and psychiatric disorders including anxiety, addiction, depression, and sleep disorders (Banki, Bissette, Arato, O'Connor, & Nemeroff, 1987; Bhatnagar & Dallman, 1998; Binder & Nemeroff, 2010; Borgland, Ungless, & Bonci, 2010; Callahan, Tschetter, & Ronan, 2013; Chemelli et al., 1999; Galard, Catalan, Castellanos, & Gallart, 2002; Hartline, Owens, & Nemeroff, 1996; Holsboer & Ising, 2008; Lin et al., 1999; Nemeroff et al., 1984; P. J. Ronan & Summers, 2011; Thannickal et al., 2000). There is evidence that these systems interact and regulate each other—perhaps providing a feed-forward mechanism for enhanced stress responses (Winsky-Sommerer et al., 2004).

Numerous lines of evidence have shown that these neuropeptide systems mutually signal one another (Achua, 2014; Harris & Aston-Jones, 2006; Ida et al., 2000; Sakamoto, Yamada, & Ueta, 2004; Winsky-Sommerer et al., 2004). Previous work from this laboratory as well as others has provided clear anatomical evidence of CRF receptors on Orx neurons and the reciprocal—Orx innervation and Orx receptors on CRF neurons (Achua, 2014; D. H. Arendt, Brudvig, J.J., Kalkman, K.L., Callahan, L.B., Summers, C.H. and Ronan, P.J., 2012; Borgland et al., 2010; P.J. Ronan, Skog, Brudvig, Callahan, & Summers, 2016; Sakamoto et al., 2004; Winsky-Sommerer et al., 2004). Further, Orx neurons have been found to receive inputs from many CRF-rich neuronal fields including the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST), and the central nucleus of the amygdala (CeA) (Yoshida, McCormack, Espana, Crocker, & Scammell, 2006), but it is unclear if there are specific CRF projections from these regions.

In order to build on this earlier work and to clarify if these afferent pathways include CRF neurons, we used a combination of neuronal tracing methods and immunohistochemistry to visualize the anatomical relationships and colocalization of these two systems. We focus on the regions described by Yoshida et al. (2006), examining the PVN, BNST, and CeA. Our results reveal a novel, indirect pathway of CRF innervation via multiple synapses to perifornical hypothalamic orexinergic neurons through the lateral septum. In addition, there appears to be topographic organization of CRF inputs to different regions of the hypothalamus that supports the hypothesis that perifornical Orx neurons preferentially contribute to stress and anxiety responses.

Orexin/Hypocretin

Orexin/hypocretin is a neuropeptide messenger derived from prepro-orexin and is produced in the perifornical and lateral regions of the hypothalamus (Yoshida et al., 2006). Perikarya in the rat brain are mainly localized in the perifornical nucleus of the hypothalamus (PeF) and lateral hypothalamic area (LH) (Chen, Dun, Kwok, Dun, & Chang, 1999; Cutler et al., 1999; de Lecea et al., 1998; Nixon & Smale, 2007; Peyron et al., 1998; Sakurai et al., 1998). There are two main types of orexin produced in the hypothalamus: the 33-amino acid peptide Orexin-A (Orx_A) and 28-amino acid peptide Orexin-B (Orx_B) (hypocretin-1 and 2, respectively), which are derived from the same precursor protein prepro-orexin (de Lecea et al., 1998; Langmead et al., 2004; Mieda & Yanagisawa, 2002; Mondal, Nakazato, Date, Murakami, Yanagisawa, et al., 1999; Sakurai et al., 1998).

Initially, this neuropeptide was discovered and reported by two independent laboratories within the same month in 1998. This discovery was made almost simultaneously by the two independent labs (de Lecea et al., 1998; Sakurai et al., 1998). The peptide was named "hypocretin" by de Lecea et al. (1998) due to the area of its production (the hypothalamus) and its resemblance to secretin. Sakurai et al. (1998) dubbed the peptide "orexin" from the Greek work *orexis* meaning appetite because of its supposed role in feeding behavior, based on the known role of the LH in feeding. After this discovery, research groups raced to discover the function of this neuropeptide. Initial phenotype experiments, which knocked out an orphan-receptor ligand (Orx), resulted in the development of narcolepsy, the function of Orx began to be elucidated. The function of Orx in modulating circadian rhythm was later described (Chemelli et al., 1999; Lin et

al., 1999; Thannickal et al., 2000). Since then, Orx has been implicated in the regulation of general activity, body temperature, drinking and feeding behaviors, stress states, and addiction (D. H. Arendt et al., 2013; Berthoud, Patterson, Sutton, Morrison, & Zheng, 2005; Edwards et al., 1999; Estabrooke et al., 2001; Hagan et al., 1999; Harris, Wimmer, & Aston-Jones, 2005; Hungs et al., 2001; Kotz, Teske, Levine, & Wang, 2002; Kunii et al., 1999; Mondal, Nakazato, Date, Murakami, Yanagisawa, et al., 1999; Nixon & Smale, 2007; Piper, Upton, Smith, & Hunter, 2000).

The discovery of Orx contributions to sleep-wake cycles spurred a furor of further research. Individuals with narcolepsy exhibited an 85-95% decrease in Orx cells in the hypothalamus; melanin-concentrating hormone cells, on the other hand, exhibited no reduction in number (Thannickal et al., 2000). Similarly, mutation of Orexin receptor-2 (Orx_2) in canines led to the development of narcolepsy, again indicating the major role of Orx in sleep modulation (Lin et al., 1999). This conclusion is supported by the finding of a narcoleptic phenotype similar to humans in mice, in which a common narcolepsy medication (modafinil) as activated Orx neurons in the hypothalamus (Chemelli et al., 1999). These studies elucidate a role of Orx in sleep-wake cycles and further describes deep pathologies stemming from Orx system dysfunction.

In addition to its role in modulating sleep and arousal states, Orx_1 has been found to modulate feeding behavior as well. Studies examining the expression of the Orx peptides in the brain suggest a role in feeding. Rats in the fasting state exhibit an increase in Orx levels in the LH (Mondal, Nakazato, Date, Murakami, Yanagisawa, et al., 1999). Similarly, intercerebroventricular administration of Orx facilitated an increase in drinking in rats (Kunii et al., 1999).

Orexin has been indicated to be involved in depression pathologies as well. An increased number of Orx-positive neurons is observed in rats exhibiting depressive behavior, compared to control animals (Mikrouli, Wortwein, Soylu, Mathe, & Petersen, 2011). The levels of Orx_A in the cerebrospinal fluid (CSF) of depressed patients is lower than controls (Brundin, Bjorkqvist, Petersen, & Traskman-Bendz, 2007). The diurnal cycle of Orx_A levels exhibited significantly reduced amplitudes in patients with depression compared to controls (Salomon et al., 2003). Animals exhibiting depressive behavior exhibit significantly greater Orx expression in the hippocampus, but decreased expression in the amygdala (D. H. Arendt et al., 2013).

Addiction and reward seeking are other behaviors that Orx is implicated to play a role in. Activation of LH Orx neurons is linked to managing preferences for stimuli related to drug and food reward (Harris et al., 2005). After causing extinction of reward-seeking behavior, chemical activation of LH Orx neurons reinstates reward-seeking behavior (Harris et al., 2005). Antagonists of Orx_A manage to block this reinstatement effect of LH activation (Harris et al., 2005). Administration of psychostimulants cause an increase in Orx neuron activation (DiLeone, Georgescu, & Nestler, 2003). Expression of μ-opioid receptors has been observed on LH Orx neurons, and these also respond to chronic administration of morphine (Georgescu et al., 2003). Finally, intracerebroventricular administration of Orx_A leads to reinstatement of drug-seeking behavior in rats (Georgescu et al., 2003).

The role of Orx in stress states has been supported by several studies. Intracerebroventricular infusion of Orx_A into CRF-containing neurons in the PVN and CeA show increased Fos-like immunoreactivity after 90 minutes (Sakamoto et al., 2004).

After 20 minutes of immobilized stress and cold exposure at 4 degrees Celsius for 30 minutes, Orx_A-containing neurons had increased Fos-like immunoreactivity (Sakamoto et al., 2004). An increase in anxiety associated behaviors is observed after intracerebroventricular injection of Orx_A in mice (Suzuki, Beuckmann, Shikata, Ogura, & Sawai, 2005). Elevated levels of Orx have been observed in the CSF of anxious patients compared to non-anxious patients (Johnson et al., 2010).

From cells located exclusively in the LH, orexinergic neurons have extensive projections throughout the rat brain. Apart from projections within the hypothalamus, Orx axons project to areas including the locus coeruleus, raphé nuclei, periaqueductal central gray, paraventricular hypothalamic nucleus, arcuate nucleus, lining of the third ventricle, layers 1-6 of the cortex, the hippocampus, somatosensory nuclei, amygdala, claustrum, bed nucleus of the stria terminalis, septum, and the substantia nigra (Chen et al., 1999; Cutler et al., 1999; Date et al., 1999; Mondal, Nakazato, Date, Murakami, Hanada, et al., 1999; Nixon & Smale, 2007; Peyron et al., 1998; Saper, Scammell, & Lu, 2005). Brain regions connected to arousal and reward, such as the nucleus accumbens and the ventral tegmental area also receive significant input from Orx neurons (Peyron et al., 1998). Together these studies provide anatomical evidence to support a significant role of Orx in arousal states as well as drug-seeking behavior (Aston-Jones, Smith, Moorman, & Richardson, 2009; Aston-Jones et al., 2010; Carr & Kalivas, 2006; Harris & Aston-Jones, 2006; Harris et al., 2005; Mahler, Smith, Moorman, Sartor, & Aston-Jones, 2012). Efferent projections of orexinergic neurons are also implied to possess a functional topographical distribution between lateral and medial sections of the

hypothalamus (Gonzalez, Jensen, Fugger, & Burdakov, 2012; Harris & Aston-Jones, 2006).

The Orx peptides, Orx_A and Orx_B, bind to two types of G_q-protein-coupled receptors (GPCR): Orexin-1 (Orx_1) and Orexin-2 (Orx_2) receptors (Mieda & Yanagisawa, 2002; Sakurai et al., 1998). While Orx_A and Orx_B both bind equally well to Orx_2 with high affinity, Orx_A binds Orx_1 with 30 to 100 times more affinity than Orx_B (Sakurai et al., 1998; Smart et al., 1999), while the affinities of Orx_A for Orx_1 and Orx_2 receptors are equally high (Sakurai et al., 1998). The distribution of these two receptors are quite distinct. The mRNA for Orx_1 is expressed in many regions of the brain, particularly the prefrontal and infralimbic cortices, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamic nucleus, dorsal raphé nucleus, and the locus coeruleus (Kilduff & de Lecea, 2001; Marcus et al., 2001; Trivedi, Yu, MacNeil, Van der Ploeg, & Guan, 1998). Distribution of Orx_2 mRNA is localized to the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphé nuclei, and many hypothalamic nuclei, including the tuberomammillary nucleus, dorsomedial nucleus, paraventricular nucleus, and ventral premammillary nucleus (Kilduff & de Lecea, 2001; Lu, Bagnol, Burke, Akil, & Watson, 2000; Marcus et al., 2001; Trivedi et al., 1998). The differential distribution and selectivity of these receptors, has led to the suggestion that their roles are also distinct within the central nervous system (D. H. Arendt et al., 2013; Nixon & Smale, 2007).

There are additional hypotheses for distinct functional roles of Orx_A and Orx_B. For example, there is evidence for a greater involvement of Orx_A than Orx_B in encouraging feeding behavior (Edwards et al., 1999; Kunii et al., 1999; Sakurai et al.,

1998). On the other hand, while Orx_B has been found to play a lesser role in feeding behavior, it contributes more to arousal responses than Orx_A (Bayer et al., 2002; Brown, Sergeeva, Eriksson, & Haas, 2002; Edwards et al., 1999; Kunii et al., 1999; Sakurai et al., 1998). This differential activation may play an important physiological function in response to stressful stimuli (Cai et al., 2001; Mondal, Nakazato, Date, Murakami, Hanada, et al., 1999). The contribution of Orx neurons to the state of arousal links Orx activity to anxiety and stress responses in an organism (D. H. Arendt et al., 2013; Hagan et al., 1999; Harris & Aston-Jones, 2006; Ida et al., 2000; Sakamoto et al., 2004).

Corticotropin-Releasing Factor

Corticotropin-releasing factor (CRF), also referred to as corticotropin-releasing hormone or corticoliberin, acts in the brain to initiate a signaling cascade via the HPA axis that results in a global stress response (Binder & Nemeroff, 2010; Krahn et al., 1986; P. J. Ronan & Summers, 2011). In addition, this hormone also has extensive extrahypothalamic distribution and function. It is posited that many stress-related psychiatric disorders, such as depression, anxiety, post-traumatic stress disorder (PTSD), or addiction, are related to alterations of the CRF system in response to consistent intensely stressful events (Banki et al., 1987; Bhatnagar & Dallman, 1998; Binder & Nemeroff, 2010; Borgland et al., 2010; Callahan et al., 2013; P. J. Ronan & Summers, 2011).

The 41-amino acid peptide CRF signals a variety of neural and endocrine responses by binding to two different class B1 GPCR receptor types in the brain: corticotropin releasing factor receptor-1 (CRF_1) and corticotropin releasing factor

receptor-2 (CRF_2) (Hauger, Risbrough, Brauns, & Dautzenberg, 2006). Of the two receptors, CRF_1 is the more abundant CRF receptor and acts as the primary signal transduction pathway for hormonal and central CRF. Consequently, CRF_1 mRNA is highly expressed in regions such as the cerebral cortex, hippocampus, amygdala, raphé nuclei, sensory relay nuclei, and the cerebellum (Van Pett et al., 2000). On the other hand, CRF_2 is localized primarily in subcortical structures, including the lateral septal nucleus, the ventromedial hypothalamic nucleus, and the choroid plexus. The CRF_2 receptor was found to be expressed in moderate levels in the olfactory bulb, amygdaloid nuclei, the paraventricular and suraoptic nuclei of the hypothalamus, the inferior colliculus, cerebellum, and the raphé nuclei (Chalmers, Lovenberg, & De Souza, 1995). Additionally, CRF_2 was found in the BNST, the hippocampal formation, and anterior and lateral hypothalamic areas (D. H. Arendt, Brudvig, J.J., Kalkman, K.L., Callahan, L.B., Summers, C.H. and Ronan, P.J., 2012; Chalmers et al., 1995). The CRF_2 receptor is also bound by other members of the CRF family of peptides (urotensins, sauvagine, and urocortins), urocortin (UCn_1), and especially UCn_2 and UCn_3.

The expression of CRF receptors is observed in a wide range of vertebrate and invertebrate species including fish (Arai, Assil, & Abou-Samra, 2001; Cardoso, Power, Elgar, & Clark, 2003; Pohl, Darlison, Clarke, Lederis, & Richter, 2001), amphibians (Dautzenberg, Dietrich, Palchaudhuri, & Spiess, 1997), chickens (Yu, Xie, & Abou-Samra, 1996), rats (Chang, Pearse, O'Connell, & Rosenfeld, 1993; Lovenberg, Chalmers, Liu, & De Souza, 1995; Perrin, Donaldson, Chen, Lewis, & Vale, 1993), sheep (D. A. Myers, Trinh, & Myers, 1998), and humans (Kostich, Chen, Sperle, & Largent, 1998; Liaw et al., 1996; Valdenaire, Giller, Breu, Gottowik, & Kilpatrick, 1997; Vita et al.,

1993). This wide range of expression indicates that the CRF-like system of stress integration is very highly conserved (P. J. Ronan & Summers, 2011).

The CRF system has been implicated in a wide range of stress-induced psychiatric disorders (Banki et al., 1987; Bhatnagar & Dallman, 1998; Binder & Nemeroff, 2010; Borgland et al., 2010; Callahan et al., 2013; P. J. Ronan & Summers, 2011). In patients with clinical depression, elevated CRF levels in plasma (Galard et al., 2002) and cerebrospinal fluid (CSF) (Banki et al., 1987; Galard et al., 2002; Hartline et al., 1996; Nemeroff et al., 1984) were observed. Elevated CRF has also been found in patients with anxiety disorders (Holsboer & Ising, 2008). The condition post-traumatic stress disorder (PTSD) is another stress-induced psychiatric disorder linked to impairment of CRF signaling (de Kloet et al., 2006). Many studies have indicated the major role the CRF system plays in addiction relapse; additionally, the impairment or dysfunction of the HPA axis and CRF system lead to greater risk for the development of addictive behavior (Borgland et al., 2010; P. J. Ronan & Summers, 2011). The HPA-axis is activated by intracerebroventricular administration of Orx (Kuru et al., 2000; Russell et al., 2001).

Distribution of CRF expressing perikarya is more widespread than Orx, possibly implicating its involvement in a wider range of stress-induced behaviors and responses, but also indicating the difficulty of understanding the details of CRF circuits and their interactions with other brain signaling systems. The expression of CRF is highest in the paraventricular nucleus of the hypothalamus (PVN), where it subsequently travels to the pituitary gland and signals release of adrenocorticotropic hormone (ACTH) to signal release of cortisol/corticotropin from the adrenal cortex, and median eminence (Catt, Millan, Wynn, Mendelsohn, & Aguilera, 1987). However, CRF from the PVN projects to

other brain regions as well, such as the solitary nucleus (NTS). In addition, extrahypothalamic areas, such as the central nucleus amygdala (CeA) and bed nucleus of the stria terminalis (BNST), also express CRF (Catt et al., 1987; Merchenthaler, Vigh, Petrusz, & Schally, 1982; Millan, Jacobowitz, Hauger, Catt, & Aguilera, 1986).

The wide expression of CRF terminals and receptors throughout the CNS also indicates that CRF interacts with a number of other neural circuits, some well-characterized and some not. Some of these neural circuits include other neurotransmitter systems such as norepinephrine (NE) (Dunn & Swiergiel, 2008; Valentino, Foote, & Aston-Jones, 1983), serotonin (5-HT) (Lowry et al., 2008; Lowry, Johnson, Hay-Schmidt, Mikkelsen, & Shekhar, 2005; Valentino, Lucki, & Van Bockstaele, 2010; Waselus, Nazzaro, Valentino, & Van Bockstaele, 2009), and dopamine systems(DA) (Borgland et al., 2010; B. Wang et al., 2005; Wise & Morales, 2010). However, the interactions between the CRF system and the Orx system remain poorly understood.

Hypothalamus

The hypothalamus, particularly the lateral and perifornical areas, is sole region for production of the neuropeptide Orx (Yoshida et al., 2006). The hypothalamus regulates and maintains homeostatic processes, and integrates information from a variety of regions such as the forebrain, brainstem, spinal cord, and various intrinsic chemosensitive neurons to accomplish this (Lam et al., 2005; Miki et al., 2001; Williams et al., 2001). The hypothalamus receives afferent input from a variety of brain regions with the greatest inputs from areas which play a role in emotional and autonomic regulation such as the infralimbic cortex (IL), lateral septum (LS), and BNST (Yoshida et al., 2006).

Based on observations of differential function between areas of the orexin-producing region, the hypothalamus has been theoretically divided into 30 subregions; this study focuses on three subregions: the paraventricular nucleus of the hypothalamus (discussed later), the lateral hypothalamus (LH), which promotes feeding behavior and modulates reward projections to the ventral tegmental area (VTA), and the dorsomedial (DMH)/Perifornical (PeF) hypothalamus, which is related to stress and arousal responses, aversive motivation, and panic responses (Achua, 2014; Harris & Aston-Jones, 2006) (Figure 1). We examined the topographical organization of CRF innervation to Orx neurons in the hypothalamus to learn if distribution of CRF input parallels this division.

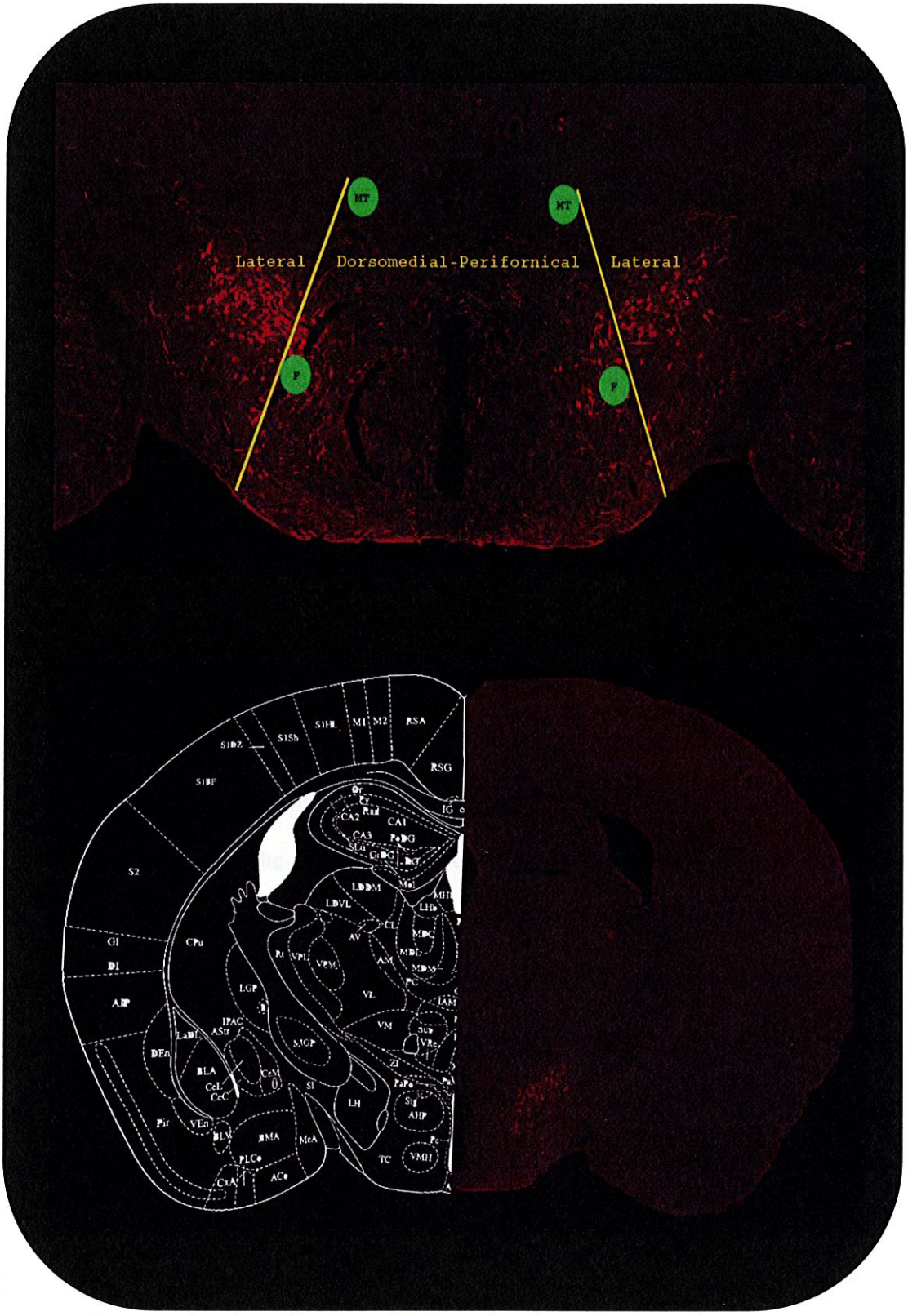

Figure 1 The theoretical division of functionally distinct sub regions of the hypothalamus. Medially is the dorsomedial/perifornical hypothalamus (DMH/PeF); the lateral portion is termed the lateral hypothalamus (LH)(D. H. Arendt et al., 2013).

Paraventricular Nucleus of the Hypothalamus

The paraventricular nucleus of the hypothalamus (PVN) participates in a host of actions, including regulating autonomic, neuroendocrine, and behavioral activities in the organism (Biag et al., 2012). The PVN is the primary region for synthesis and release of CRF for direct activation of the HPA axis and, subsequently, a global stress response (Korosi & Baram, 2008). This alone highlights the central role of the CRF in the PVN in the stress response. Additionally, the PVN, while not heavily innervated by CRF directly apart from local circuits, does innervate other brain regions with CRFergic projections, and receives indirect input from regions which receive CRF projections (Sawchenko et al., 1993).

Central Nucleus of the Amygdala

Outside of the PVN, the CeA has the highest concentration of CRF producing cells (Callahan et al., 2013; Catt et al., 1987; Millan et al., 1986). This area is the primary output region of the amygdala with CRF, GABA, and enkephalin projections that contribute to the control of the autonomic nervous system, particularly heart rate and blood pressure (LeDoux, Iwata, Cicchetti, & Reis, 1988; Veening, Swanson, & Sawchenko, 1984). The amygdala is a brain structure known to be involved in fear conditioning and the consolidation of emotional memory; more specifically, the amygdala is important in learning about both contextual and discrete stimuli, and has been implicated in its role in facilitating emotional memories (Phillips & LeDoux, 1992). The CeA plays a key role in the endocrine response to stressful stimuli (Callahan et al., 2013; Feldman, Conforti, & Weidenfeld, 1995; Gray & Bingaman, 1996; Herman & Cullinan, 1997; Roozendaal, Koolhaas, & Bohus, 1997). Many studies have shown that

lesions of the CeA lead to a decreased stress response due to a decrease in activation of the HPA-axis involving CRF (Beaulieu, Di Paolo, & Barden, 1986; Prewitt & Herman, 1994; Roozendaal, Koolhaas, & Bohus, 1991, 1992; Van de Kar, Piechowski, Rittenhouse, & Gray, 1991). More specifically, inhibition of CRF expression in the CeA leads to both decreased behavioral and endocrine responses to stressors (Callahan et al., 2013).

The CeA has a wide range of CRF projections to several different brain regions such as the LH, VTA, basolateral amygdala (BLA), periaqueductal gray, raphé nuclei, locus coeruleus, NTS, and the lateral BNST (Cummings, Elde, Ells, & Lindall, 1983; B. Myers & Greenwood-Van Meerveld, 2009; Rodaros, Caruana, Amir, & Stewart, 2007; Sakanaka, Shibasaki, & Lederis, 1986). Based on its interactions with the CRF system, the CeA is also implicated in addiction and withdrawal behaviors (P. J. Ronan & Summers, 2011).

There is evidence for the interaction between CRF and Orx, however the origin of CRF projections was not described (Winsky-Sommerer et al., 2004). The relationship of the CeA with stress and anxiety responses, as well as its participation in CRF signaling led us to hypothesize that it may be a region that also sends CRF projections to the PeF to contribute to the modulation of Orx in stress responses.

Bed Nucleus of the Stria Terminalis

An anatomical extension of the amygdala, the bed nucleus of the stria terminalis (BNST) is another region contains neurons that express significant amounts of CRF (Sink et al., 2013). The BNST and the CeA are intimately related anatomically, neurochemically, and cytoarchitecturally (Alheid, 1995). This limbic forebrain structure receives input from many brain regions such as the basolateral amygdala (BLA) and CeA, and projects to many other areas, most notably hypothalamic regions (Walker, Toufexis, & Davis, 2003).

The BNST contributes to CRF projections that produce fear and anxiety responses (Funk, Li, & Le, 2006; Kim et al., 2006; Ostrander et al., 2009; Shepard, Schulkin, & Myers, 2006; Sink et al., 2013; Walker et al., 2003). The BNST has also been implicated in unconditioned fear responses (Walker & Davis, 1997). However, this view has been refined and the role of the BNST has been proposed to function in the elicitation of learned behaviors in response to fear, rather than quick reflexes in response to specific fear-inducing stimuli (Walker et al., 2003). The BNST has also been implicated in the physiological responses associated with drug withdrawal, and plays a role in drug and alcohol relapse behavior (Aston-Jones, Delfs, Druhan, & Zhu, 1999; Delfs, Zhu, Druhan, & Aston-Jones, 2000; Erb & Stewart, 1999), which are all hallmark behaviors of CRF activity. These common behaviors imply a CRF connection to the LH as well.

Lateral Septum

The lateral septum (LS) has the highest expression of CRF_2 receptors in the brain (Chalmers et al., 1995). Injection of CRF_2 receptor antagonist into the lateral septum (LS) decreases stress-induced freezing behavior in rats (Bakshi, Smith-Roe, Newman, Grigoriadis, & Kalin, 2002). This behavioral link with CRF_2 receptors, along with evidence providing a link between Orx and CRF through the LS, indicates the contribution of the LS in this direct reciprocal interaction. This relationship is further defined to involve modulation of feeding behavior related to Orx and CRF (C. Wang & Kotz, 2002).

The LS is involved in widespread inhibition of various regions as well (Delgado, 1975), sending inhibitory signals throughout the brain. In addition, the LS also sends many excitatory signals throughout the brain. These signals highlight the LS function in regulation of a multitude of physiological processes including emotional behavior and memory (Thomas, 1988). The lateral septum is posited to modulate other brain regions involved in processing anxiogenic stimuli through suppression of adverse emotional states (Thomas, 1988; Yadin, Thomas, Grishkat, & Strickland, 1993). Many connections have been described passing through the LS relay center; for example, hippocampal and amygdalar systems are known to pass through the LS to signal cells in the BNST and make bidirectional connections with the prefrontal cortex (Canteras, Simerly, & Swanson, 1992; Chiba, 2000; Dong, Petrovich, & Swanson, 2001; Hoover & Vertes, 2007; Jones & Wilson, 2005; Thierry, Gioanni, Degenetais, & Glowinski, 2000).

The relationship between CRF and the LS has led to investigations into the role the LS plays in anxiety and stress responses. The effect of the LS on anxiety is related to

stress level (Henry, Vale, & Markou, 2006). The LS also is involved in the interaction between CRF and 5-HT release (Price & Lucki, 2001) and interacts with CRF in the learning processes (Koob & Bloom, 1985; Liang & Lee, 1988; Radulovic, Ruhmann, Liepold, & Spiess, 1999). Activation of CRF_2 receptors in the LS has been found to affect stress behaviors, such as anorexia (Bakshi, Newman, Smith-Roe, Jochman, & Kalin, 2007; C. Wang & Kotz, 2002).

This structure is known to be involved in the complex and redundant circuitries that are involved in creating stress responses that are known at this time (Tovote, Fadok, & Luthi, 2015) (Figure 2). Much of the circuitry underlying anxiety behaviors remains unknown. A long-range circuit is shown in Figure 2 indicating a connection between the LS and hypothalamus. We hypothesize that these connections may include Orx or CRF projections. This study further characterizes the role some of these cells in the LS possibly play in anxiety behavior.

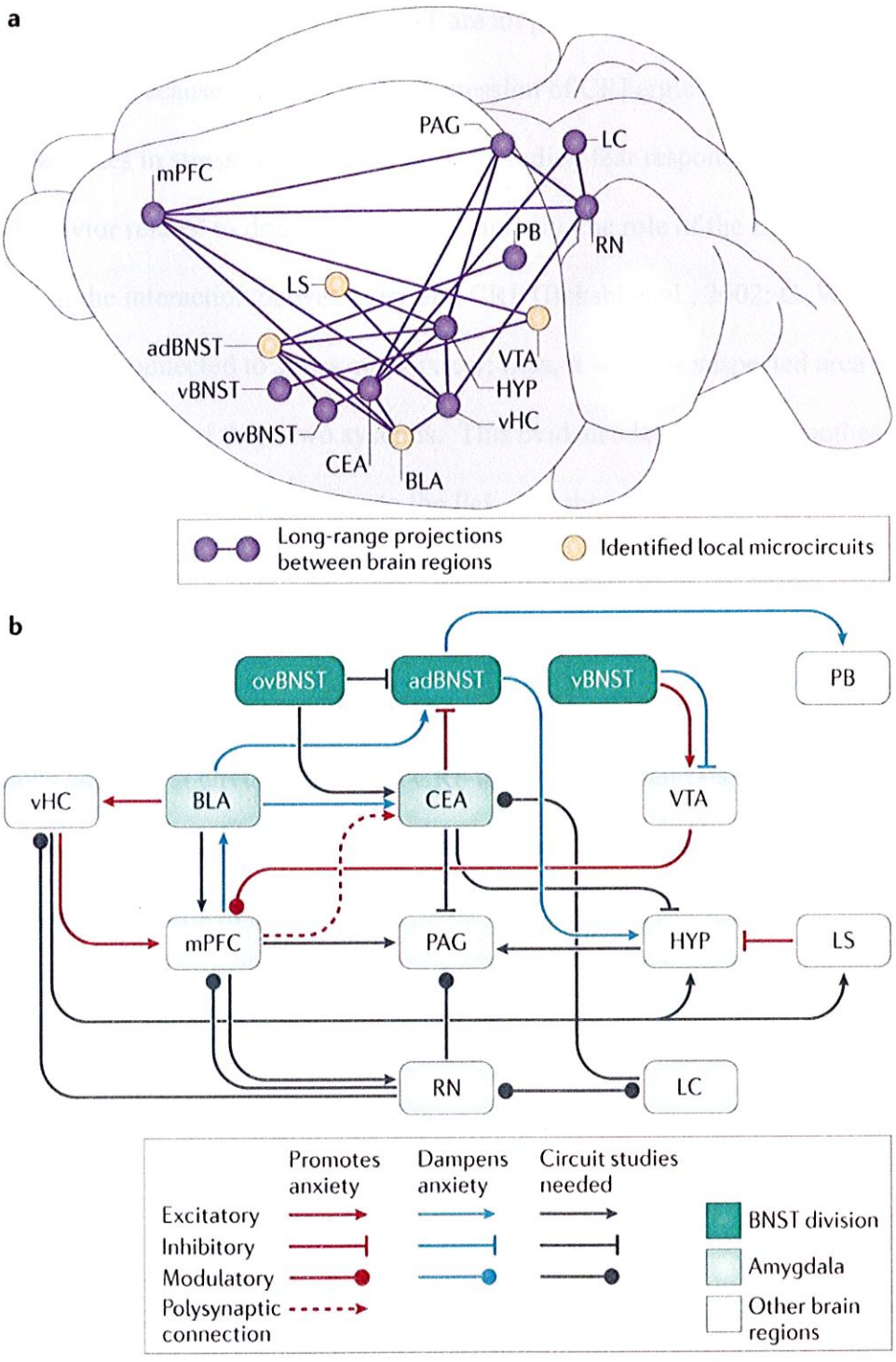

Figure 2 a) Anxiety states are mediated by local and long-range connections between multiple brain areas. b) Some regions that have major roles in anxiety mediate both anxiogenic and anxiolytic behavioral effects. Large parts of the anxiety network remain to be characterized in terms of cellular identity and functions as well as precise local and long-range connectivity using modern circuit based approaches (Tovote et al., 2015).

Together, the PVN, CeA, and BNST are all possible sites of CRF innervation to the PeF Orx field because of their known expression of CRFergic neurons, as well as their defined roles in stress/anxiety behaviors, including fear responses and reward-seeking behavior related to drug relapse. Additionally, the role of the LS has been implicated in the interaction between Orx and CRF (Bakshi et al., 2002; C. Wang & Kotz, 2002) and connected to stress and anxiety; thus, it is also a suspected area of anatomical interaction of these two systems. This evidence leads to the hypothesis that these areas may have CRF projections to the PeF, and thus contribute to the direct reciprocal interaction between CRF and Orx and play a role in the modulation of stress and anxiety behaviors. In this study, we examine these areas to elucidate their role, if any, in the regulation of Orx activity in stress responses. By describing the anatomical connections and neural circuitry between CRF and PeF Orx neurons, the relationship between the two systems can be further supported, and possible functional relationships implicated with anatomical evidence.

CHAPTER TWO

Materials and Methods

Animals and Handling

Procedures were performed on Sprague-Dawley rats (Envigo, 225-400g). Rats were kept in clear acrylic cages (24"x10") and were fed/watered *ad libitum* and kept on a regular 12:12 light: dark cycle. They were housed in pairs prior to injection. Following injection survival surgery, rats were housed individually for the recovery and tracing period. All experiments were performed in a manner that minimized suffering and the number of animals used, in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 20-23), and approved by the Institutional Animal Care and Use Committee of the University of South Dakota.

Intracranial Injections

Intracranial injections of 1.0% Fluorogold retrograde tracer (FG, Covidien Ltd.) into the PeF (Figure 3; AP: -3.14 mm, ML: ±1.32 mm, DV: 8.32 mm) were performed. Rats were deeply anesthetized with isoflurane and placed in a stereotaxic apparatus (Model 962 Dual Ultra Precise Small Animal Stereotaxic Instrument, Kopf Instruments, Tujunga, CA). Eye lubricant (Lacrilube) was applied after anesthetization to prevent irritation and drying. The scalp was shaved and cleaned with 4.0% w/v Chlorhexidine Gluconate (Mölnlycke Health Care) surface antiseptic. A single longitudinal incision was made on the scalp and the skin was retracted with sterile tissue clamps to reveal the superior skull surface. Bleeding was stemmed and the skull surface was cleared using 3% hydrogen peroxide and sterile gauze. Flat skull orientation was achieved using

dorsal/ventral measurements at bregma and lambda. A precise mark was made on the skull using a needle in an electrode holder arm with surgical ink. A hole was drilled using a precision drill (Foredom) mounted on a stereotactic arm equipped with a dental burr bit (0.7 mm) through the skull on the mark, just to the surface of the brain. With the aid of a stereomicroscope, the dura and any remaining bone disc was removed using a sterile 25-gauge hypodermic needle with the tip hooked by scraping on a sterile surface. A sterilized Hamilton syringe (2µl, Hamilton Co.; Reno, Nevada) was attached to a microinjector unit (Model 5000-5001, David Kopf Instruments, Tujunga, CA) which was fitted on the stereotactic frame. The syringe was zeroed on the surface of the brain with the aid of a stereomicroscope. The syringe was slowly lowered to the proper depth (DV: 8.32 mm) (Figure 4). Over the course of 10 minutes, 90 nl of FG was slowly and steadily injected into the PeF. After injection, the syringe was allowed to stay in place for another five minutes before being very slowly removed over the course of another five minutes. The hole was then filled with bone wax (World Precision Instruments, Inc., Sarasota, FL), and the incision sutured (5.0 Silk, Ethicon, Somervill, NJ). The animal was given ketorolac (5 mg/kg) and topical antibiotic (Neosporin) was applied to the incision.

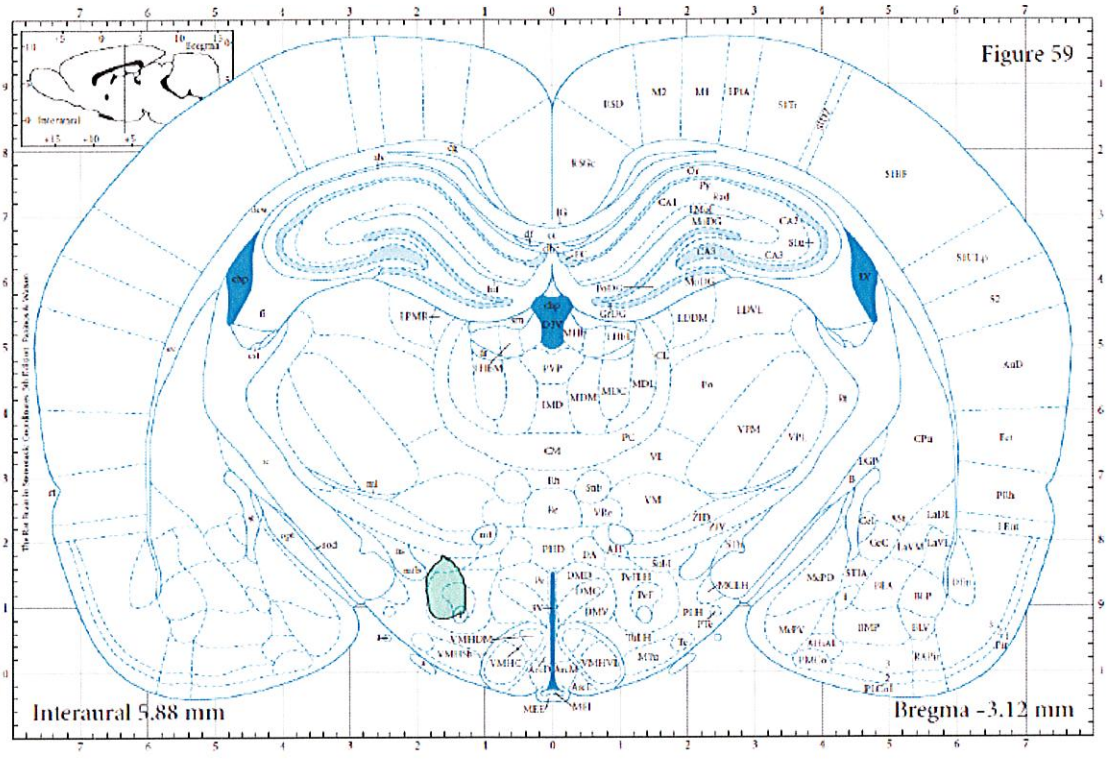

Figure 3 Injections of fluorogold were targeted to the PeF. The target area is outlined in this atlas image from a coronal section at -3.12 mm posterior to Bregma (Paxinos, 2004).

Immunohistochemistry

Immunostaining for CRF on brain sections containing the PeF, BNST, CeA, PVN, and LS were performed (Callahan et al., 2013). Seven days following the intracranial injection of FG, rats were deeply anesthetized with isoflurane and perfused transcardially with 300 ml of ice-cold saline (0.9%) with heparin (200 units/l) followed by 300 ml fresh, ice-cold 4% paraformaldehyde in 1X PBS using a gravity perfusion system. Brains were removed, post-fixed in 4% paraformaldehyde overnight (24 h) at 4°C, then cryoprotected in 30% sucrose in 1X PBS for up to 72 hours before being immersed in OCT compound (Tissue-Tek, Sakura), and frozen surrounded by powdered dry ice. Brains were sectioned at 35 µm on a cryostat microtome (blade -32°C, mount -

30°C; Thermo Scientific CryoStar NX70/NX50, Thermo Fischer Scientific; Kalamazoo, MI) and stored at 4°C in 1X PBS with 0.01% NaAzide (Sigma, St. Louis, MO). By visual comparison with a pictorial stereotaxic brain atlas (Paxinos, 1998), appropriate sections were chosen for examination of the bed nucleus of the stria terminalis (BNST, Bregma -0.26 mm), paraventricular nucleus (PVN, Bregma -1.6 mm), central nucleus of the amygdala (CeA, Bregma -1.8 mm), lateral hypothalamus (LH, Bregma -3.6 mm), and perifornical area of the hypothalamus (PeF, Bregma -3.6 mm). These sections were washed twice for 5 minutes each in 1X PBS, then placed in blocking buffer (3% Normal Goat Serum, 0.25% Triton X-100 in 1X PBS) for 60-90 min. Primary antibodies were diluted with blocking buffer (CRF 1:1000, rabbit anti-rat polyclonal cat# T-4037; Peninsula Labs, Belmont, CA; Orx 1:1000, rabbit anti-rat polyclonal cat# ab3704; Millipore, Temecula, CA). Sections were incubated overnight (~18 hours, [72 hours for CRF2] free floating) at RT with gentle agitation. The next day sections were rinsed three times for 3 minutes each in 1X PBS then incubated with fluorescently labeled secondary antibody (Cy3 or Cy5 Donkey anti-rabbit or Donkey anti-mouse IgG; Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in 1X PBS and incubated at RT in darkness with gentle agitation for 2-4 hours. Sections were washed in 1X PBS three times for 3 minutes each, mounted on poly-l-lysine coated and dried for 2 hours, incubated at 34°C. Slides were then washed in 1X PBS followed by an ethanol dehydration series (50, 75, 90, 100, 100% for 10 seconds each) and the xylene substitute Citrisolv (Fisher) 2 x 3 min before being cover slipped with DPX mountant (Fluka).

Microscopy

A Zeiss LSM 700 confocal microscope equipped with an Axiocam color camera was used to acquire digital images of immunofluorescence from 3-4 tissue sections containing the PeF, BNST, CeA, PVN, and LS from each animal.

CHAPTER THREE

Results

Analysis for Colocalization

To examine the circuitry and direct reciprocal interaction involved between PeF Orx cells and CRF neurons, the retrograde tracer fluorogold was injected into the PeF, then CRF neurons in several different regions were stained (see methods). This retrograde tracing and immunohistochemistry revealed expression of CRF neurons and their projections to Orx cells in the PeF. Using confocal microscopy, several areas known for expressing CRF were examined to analyze for colocalization of FG and CRF staining. CRFergic neurons also containing the retrograde tracer FG indicate CRF expressing neurons which project to the PeF. In addition to the lateral hypothalamus, three major CRF expressing regions were examined in this study, as well as the lateral septum because of its known expression of CRF_2 and functional role eliciting in stress and anxiety behaviors.

Lateral Hypothalamus

Sections containing the lateral hypothalamic region (LH) (Figure 4) were stained with Orx_A antibody. The resulting stain exhibits the existence of Orx perikarya within the LH, including axons and terminals (Figure 5), observable by the localized Orx stained cell bodies. In addition, the colocalization of FG and Orx staining demonstrates the inputs of LH neurons across the LH/DMH/PeF regions, including interneuron connections (Figure 5).

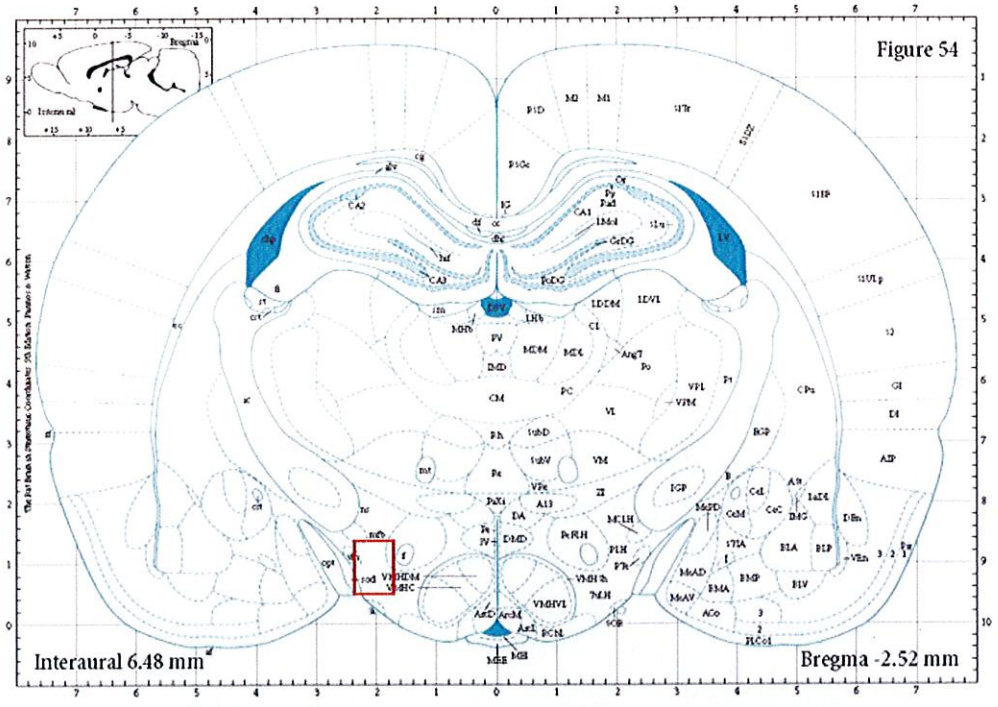

Figure 4 FG and Orx stained neuron spatial relationships were examined in the lateral hypothalamic region (Paxinos, 2004).

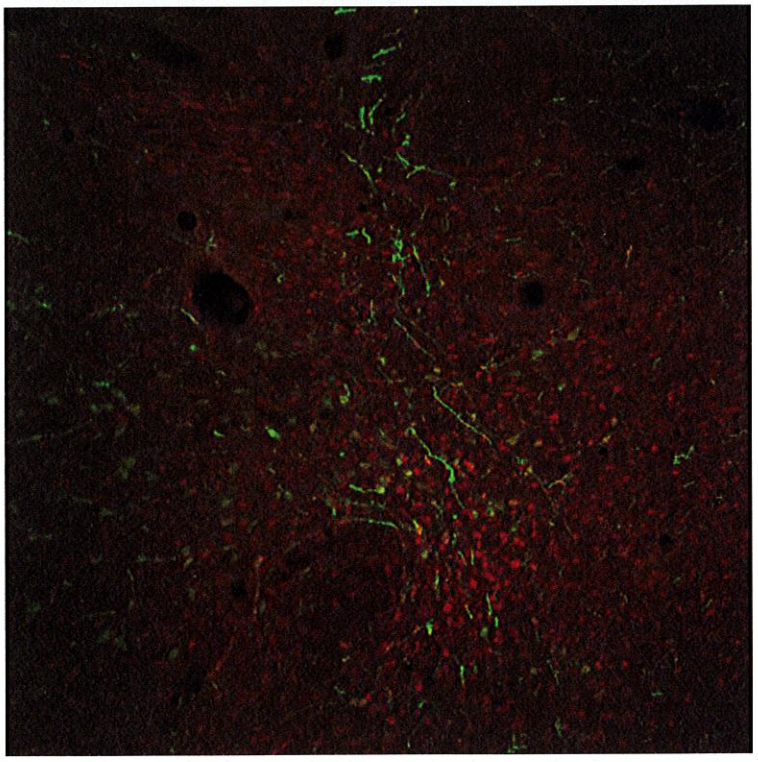

Figure 5 FG (red) and Orx (green) staining in the LH. This staining shows Orx perikarya, including axons and terminals, within the LH. Additionally, colocalization of FG and Orx identifies cross-talk within the LH/DMH/PeF, including interneuron connections.

These sections were also double stained for Orx and CRF expression, to further support the relationship between Orx and CRF within the hypothalamic region. The staining reveals CRF terminals at Orx cell bodies, as well as at interneuron synapses (Figure 6).

Figure 6 Orx (green) and CRF (red) expression in the LH/DMH/PeF shows CRF terminals at Orx cell bodies as well as at interneuron synapses. This anatomical relationship implies a functional cross-talk between CRF and Orx, and could indicate CRF-Orx coregulation.

Central Nucleus of the Amygdala

When examining CRF expression in sections containing the CeA (Figure 7), little colocalization of CRFergic neurons and FG stained neurons was observed. There is sparse staining from FG, indicating few cells projecting directly to the PeF region from the CeA (Figures 8 & 9).

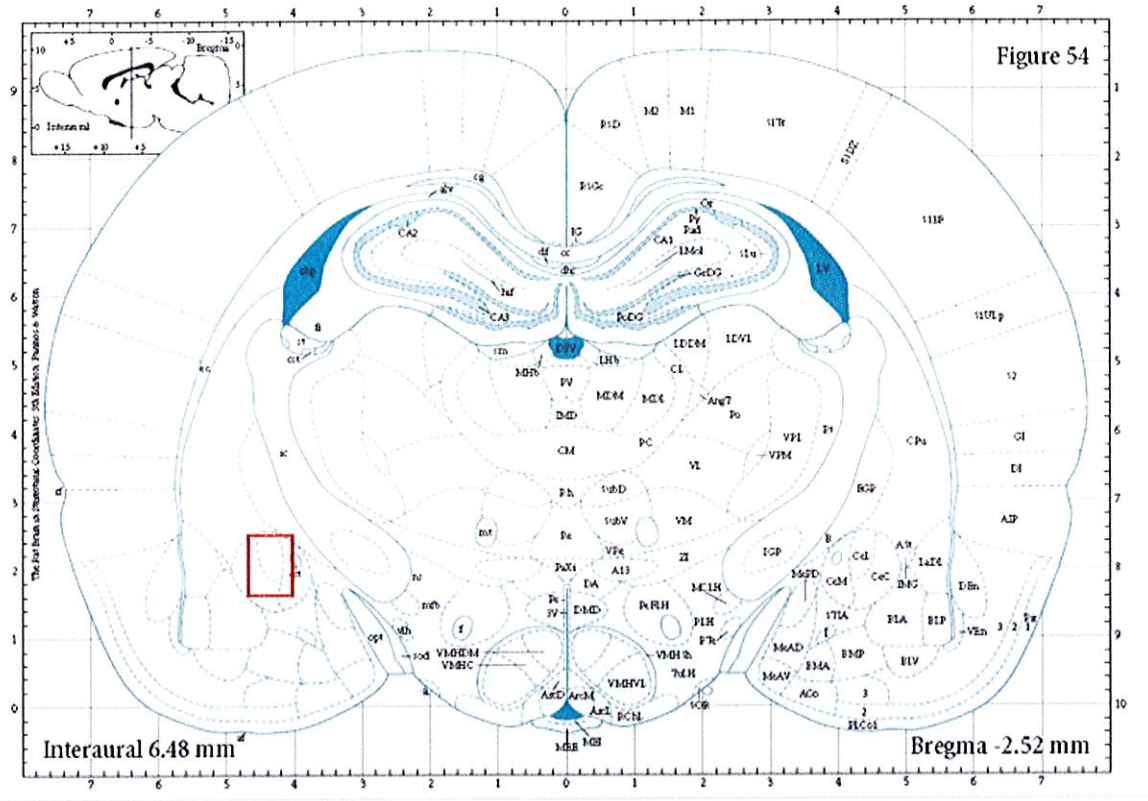

Figure 7 The Capsular (CeC), Lateral division (CeL), and Medial division (CeM) of the CeA are examined in the proceeding images (Paxinos, 2004).

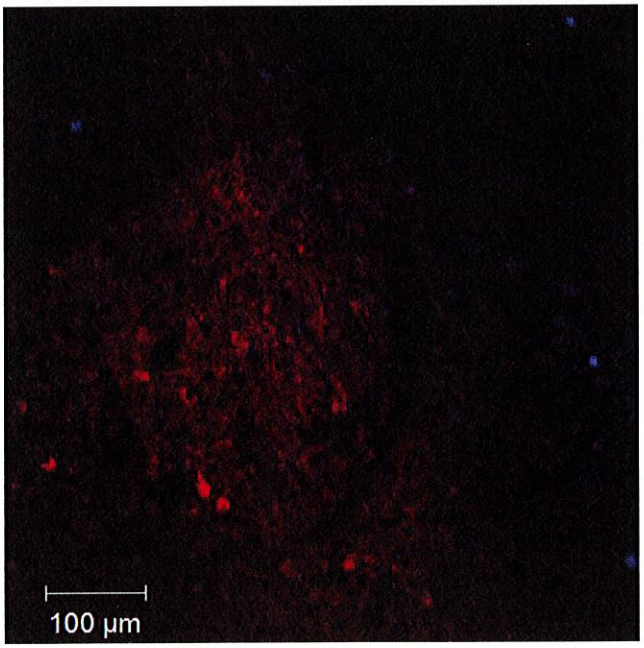

Figure 8 A magnified image of the CeA illustrates the sparse innervation to the PeF from the CeA, and no double-staining of FG (blue) and CRF (red) cells.

Within the ventral (inferior) portion of the CeA, many cell bodies and axons can be observed expressing CRF. However, within the cells stained with FG, the bodies are the only portion stained, and no projections of FG neurons seem to travel within the CeA (Figure 9). There is little colocalization of PeF projecting cells and CRFergic neurons within the CeA. However, the axon presence indicates communication of CRF neurons with others in the CeA.

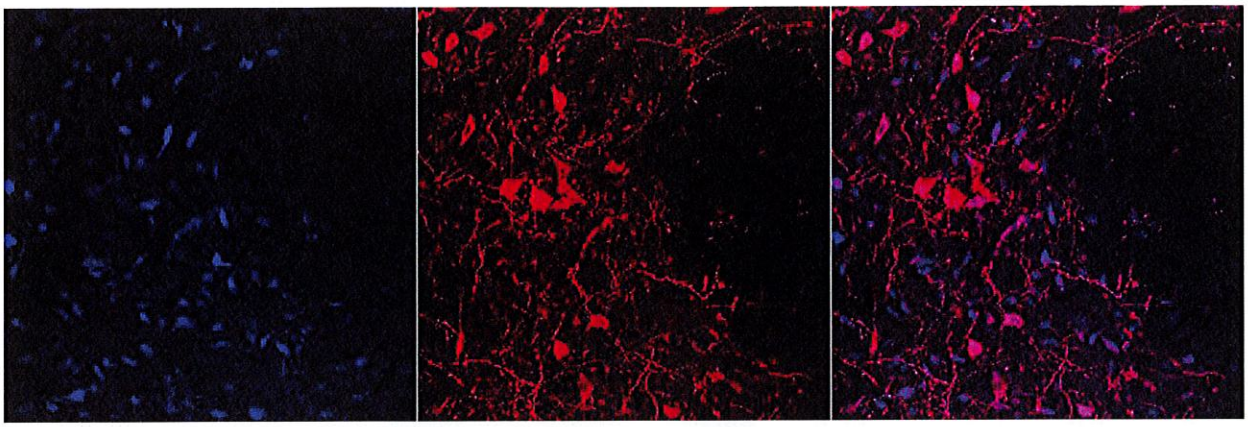

Figure 9 FG (blue) and CRF (red) staining reveals little overlap in CRF cells projecting to the PeF. Sparse staining of FG indicates very little innervation to the PeF from CeA CRF neurons.

Paraventricular Nucleus of the Hypothalamus

Sparse colocalization of CRF and FG staining is also observed in the PVN (Figure 10). Within the ventral and lateral portion of the PVN, double-staining is observed in CRF neurons. While more cells are double-stained in the PVN than the CeA, the amount is still very low compared to other regions (Figures 11 & 12). In addition, while many CRF cell bodies are observed in the PVN, few axon projections from cells are observed. The PVN may contribute to CRF control of PeF Orx neurons, however this contribution, anatomically, is apparently minor.

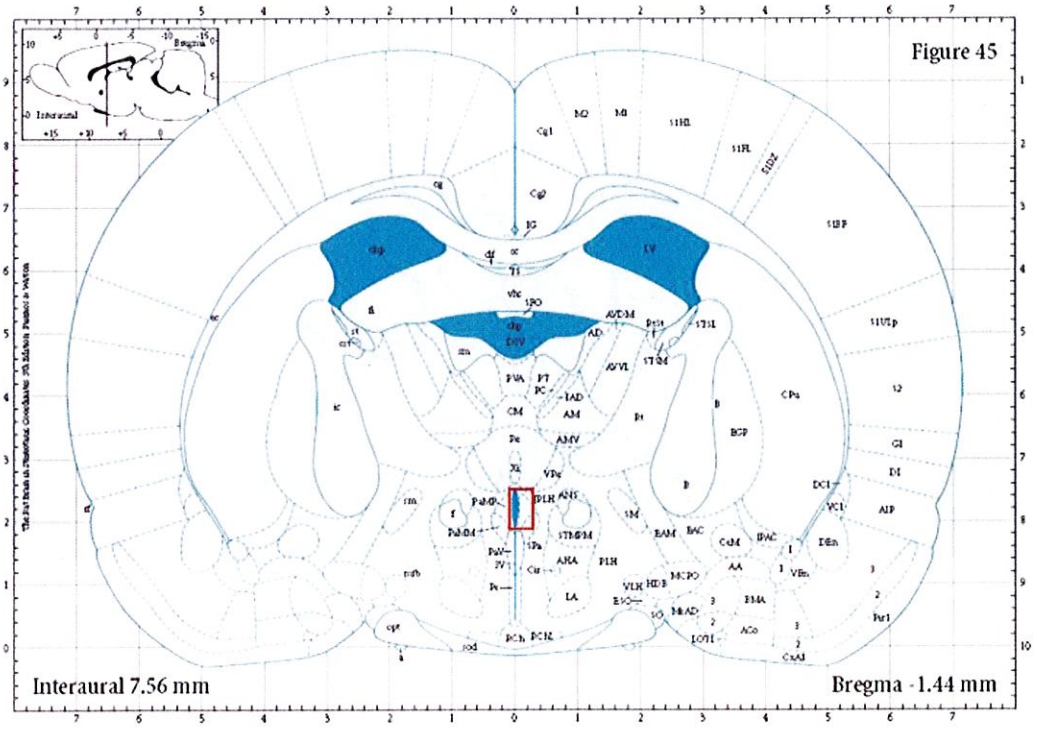

Figure 10 The Medial Magnocellular (PaMM) and Medial Parvicellular (PaMP) parts of the PVN are examined for CRF and Orx organization and topography (Paxinos, 2004).

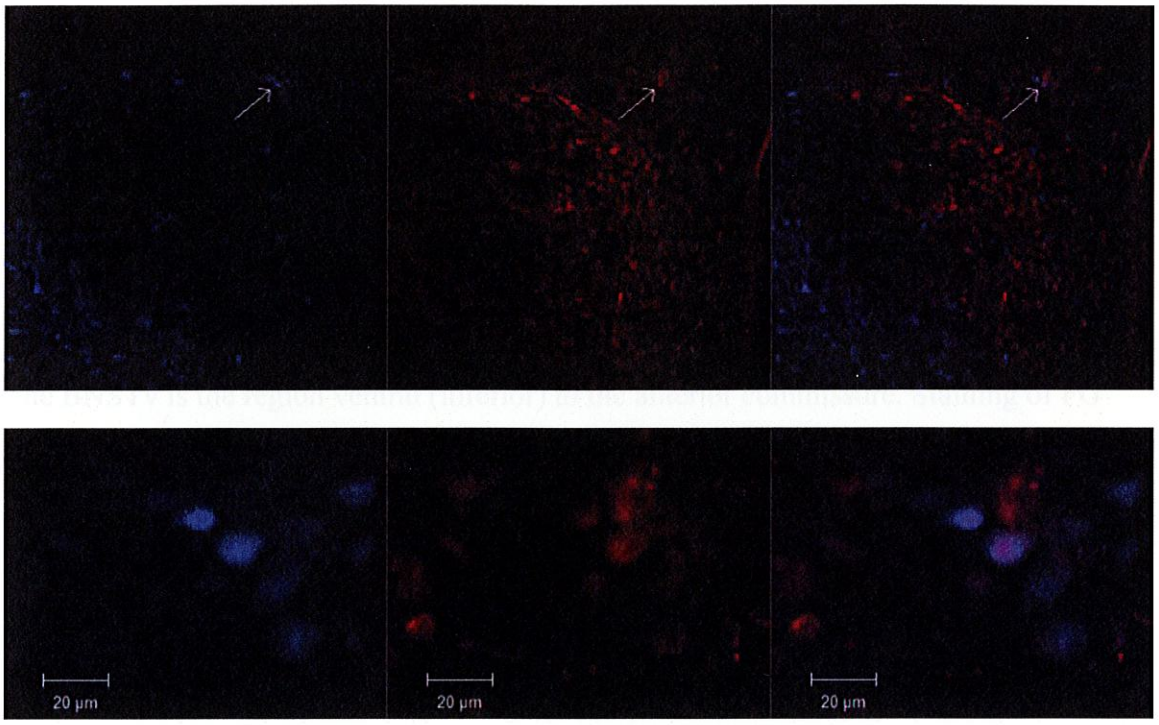

Figure 11 Double staining of FG (blue) and CRF (red) in a couple cells is visible in this area of the PVN.

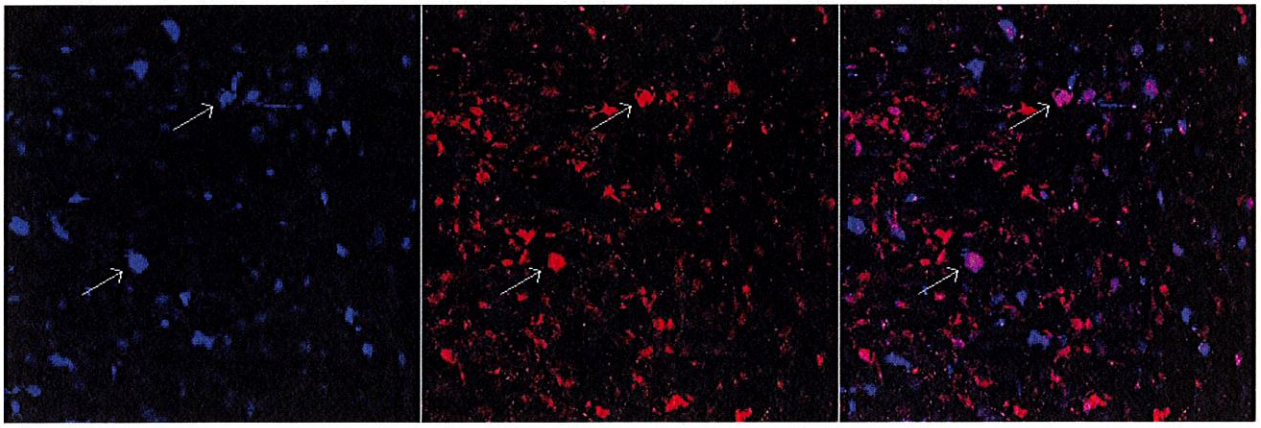

Figure 12 Double staining of FG (blue) and CRF (red) within several cells within the PVN.

Bed Nucleus of the Stria Terminalis

Within the BNST (Figure 13), colocalization was observed in larger amounts, particularly in the ventral BNST (BNSTv) (Figure 16). Both the dorsal (BNSTd) and ventral BNST (BNSTv) were examined for FG and CRF staining. The BNSTd is the region of the BNST dorsal (superior) to the anterior commissure, the dark spot pictured in Figure 14. The BNSTv is the region ventral (inferior) to the anterior commissure. Staining of FG was observed in similar amounts in both the BNSTd and BNSTv, however most double-staining, PeF FG staining plus BNST CRF staining, was observed in the BNSTv.

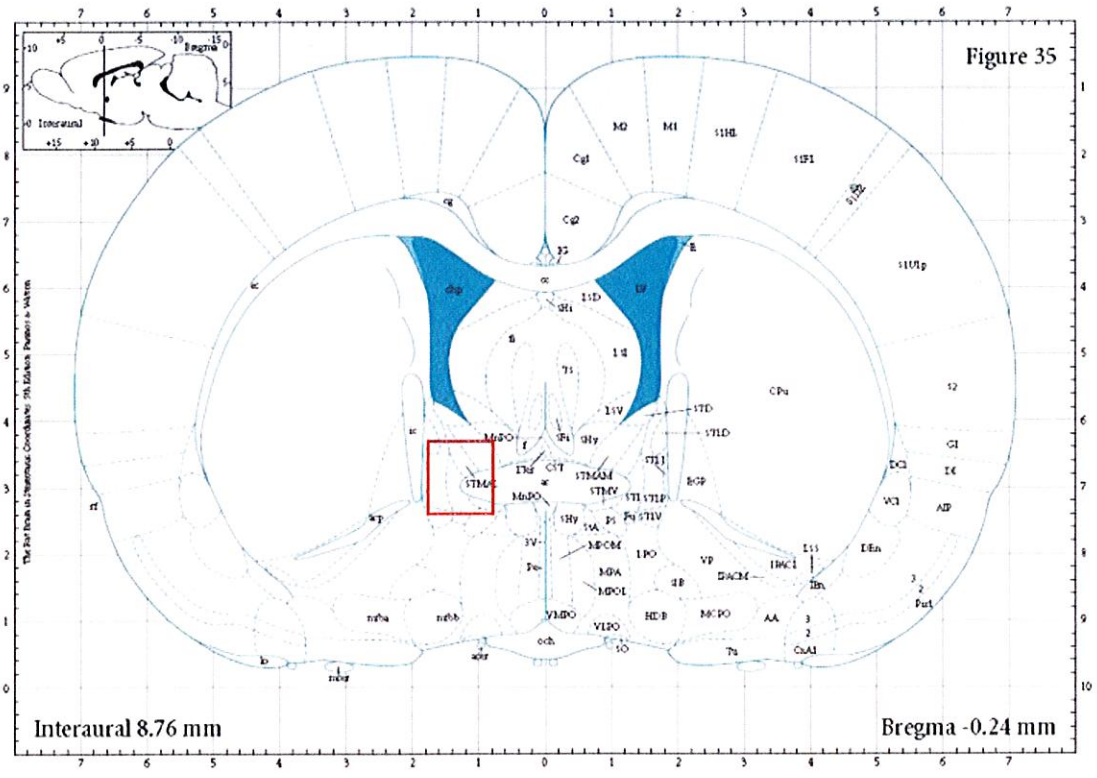

Figure 13 The dorsal (BNSTd) and ventral (BNSTv) regions of the BNST are examined in the proceeding images for CRF expression related to projections to the PeF .(Paxinos, 2004)

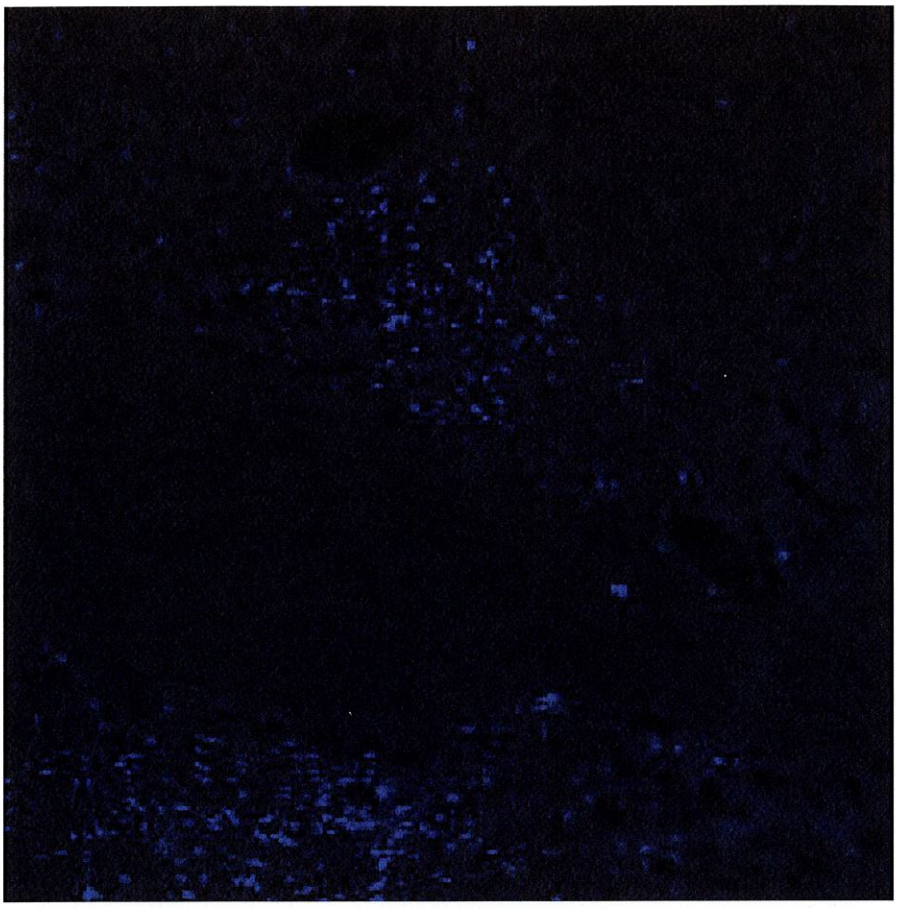

Figure 14 FG staining within the BNST. The dark area in the middle is the anterior commissure. There appears to be relatively equal staining in both the dorsal (BNSTd) and ventral BNST (BNSTv).

Within the BNSTd, significant CRF staining is observed (Figure 15); however, not much double-staining of CRF cells with FG is apparent in this region. Few cells exhibit the FG staining, and those few are spatially distinct from the dense population of CRFergic neurons in the BNSTd. Few axons and projections are stained as well, with only the cell bodies visible.

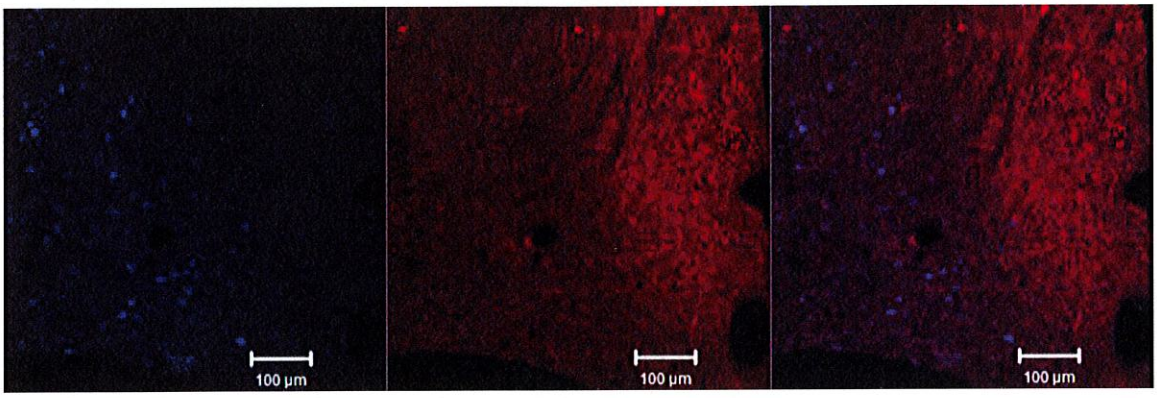

Figure 15 FG (blue) and CRF (red) staining within the dorsal BNST (BNSTd). While there is both FG and CRF cells stained in this region, there is little double-staining occurring.

The majority of CRF cells double-stained with FG appear in the BNSTv (Figure 16). In the BNSTv, the FG cells are interspersed within the dense population of CRFergic neurons, located close to the anterior commissure. While not many FG projections are visible, many CRF projections are present in the BNSTv.

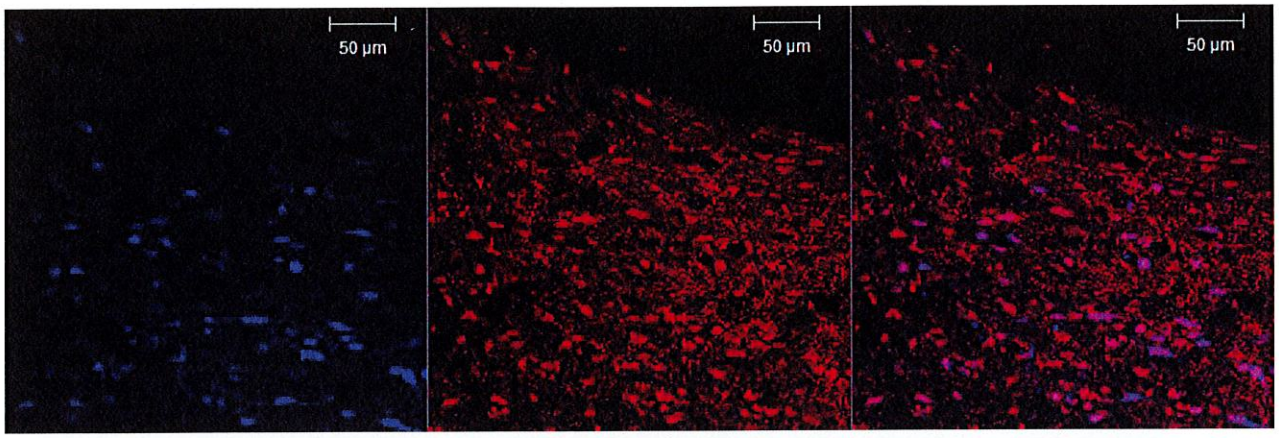

Figure 16 FG (blue) and CRF (red) staining within the ventral BNST (BNSTv). In this region of the BNST, many CRF cells are stained to indicate their projections to the PeF.

Lateral Septum

Most notably, we anatomically describes the indirect modulation by CRF cells on PeF Orx neurons through the LS (Figure 17). Within the LS, CRF terminals were observed to heavily populate cells stained with FG (Figures 18-20). This observation indicates a novel mechanism for CRF control of Orx activity in the hypothalamus through the LS.

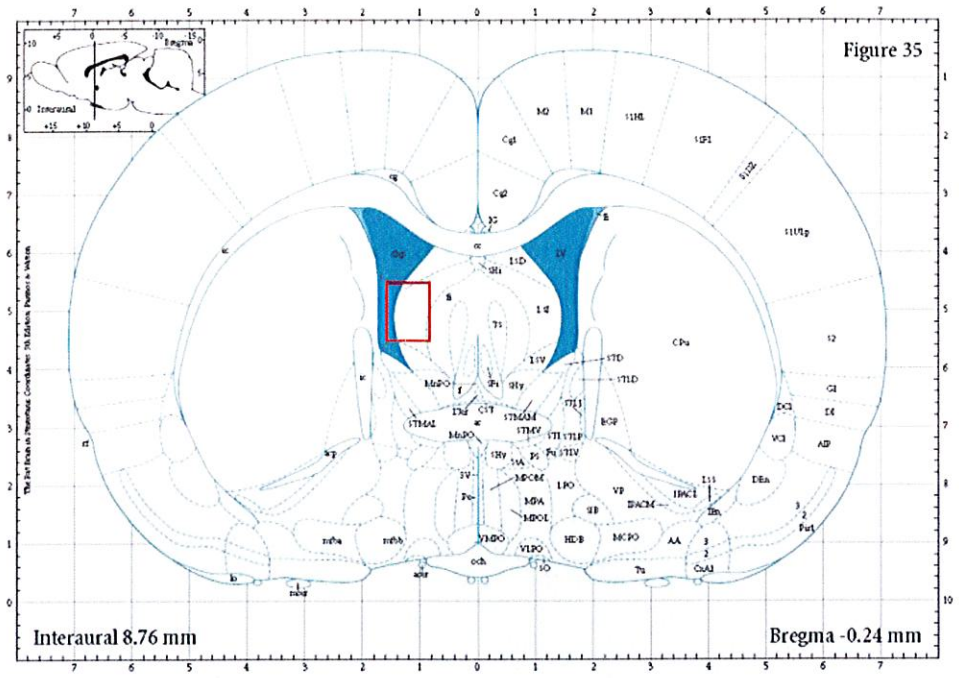

Figure 17 The intermediate part of the lateral septal nucleus (LSI) was examined for CRF expression and projection to the PeF in the following images (Paxinos, 2004).

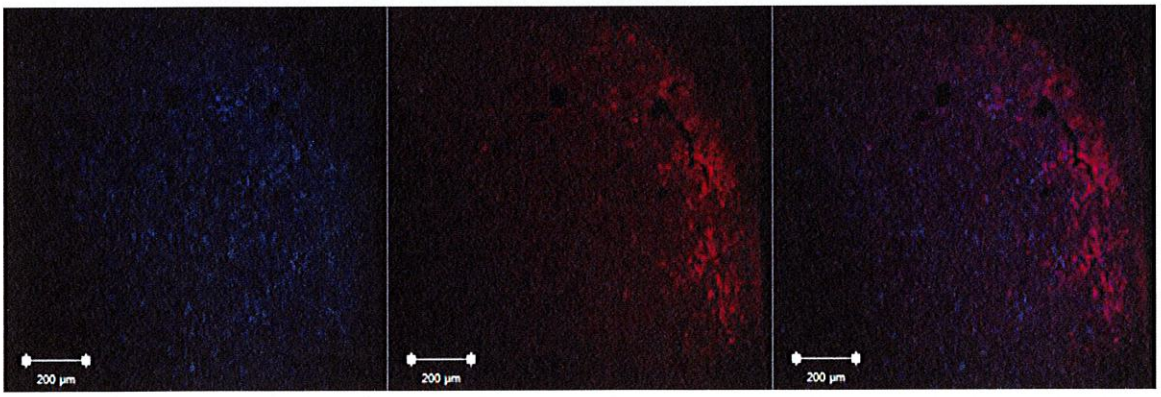

Figure 18 FG (blue) and CRF (red) double staining within the LS. The lateral ventricle is slightly observable on the right upper and lower corners of the image.

Dense innervation of FG stained cells by CRF projections is observed within the LS (Figure 19). While no CRFergic cell bodies are observed, a large number of terminals are present, with synapses on cell bodies and axons alike. In addition, many cell bodies in this region are stained by FG. In observing a single FG cell body, many terminals are observed (Figure 20).

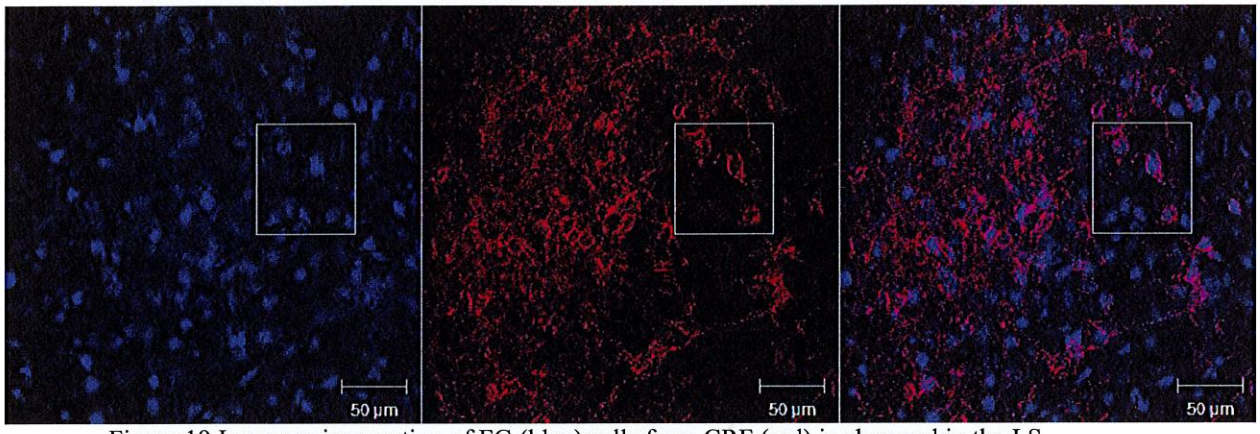

Figure 19 Immense innervation of FG (blue) cells from CRF (red) is observed in the LS.

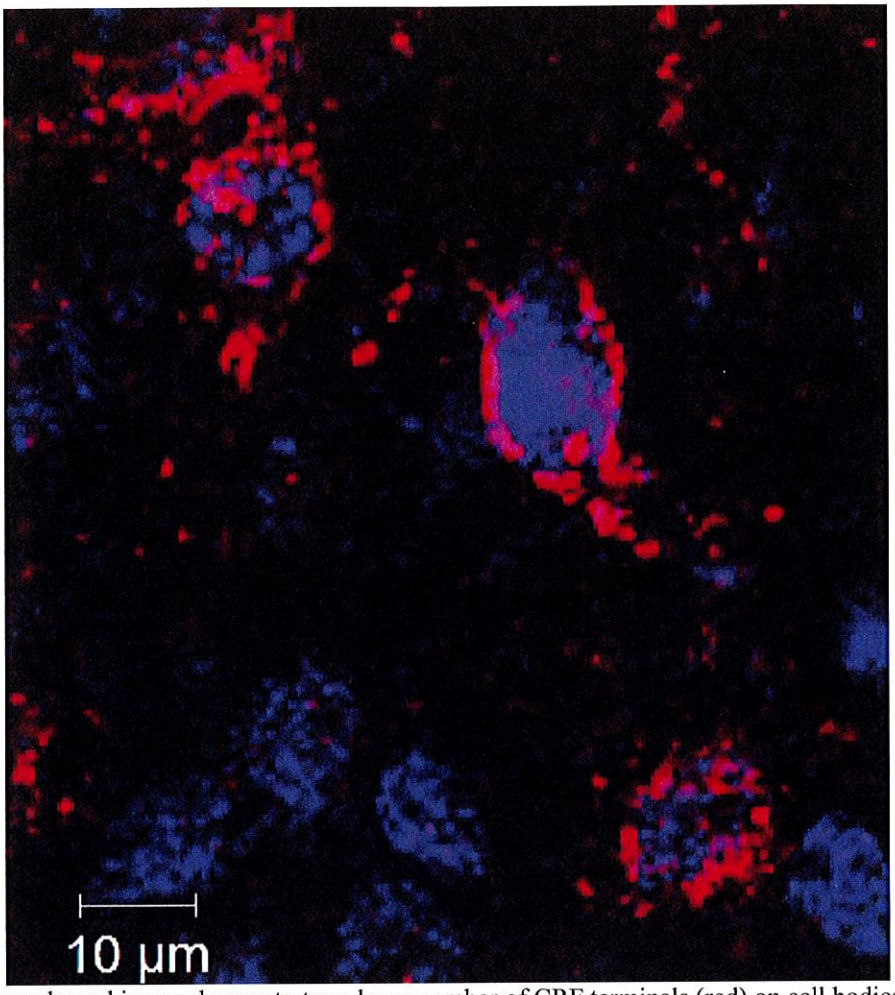

Figure 20 An enlarged image demonstrates a large number of CRF terminals (red) on cell bodies labeled with FG (blue) within the LS.

CHAPTER FOUR

Discussion and Conclusion

Discussion

Projections of CRF directly from the BNST and indirectly through the LS indicate a role of CRF on regulation of the Orx system, and vice versa. However, this direct modulation does not come from all CRF expressing regions, as the CeA and PVN exhibited very little colocalization of CRF expressing neurons and FG stained projection neurons. These results add to the anatomical work of Winskey-Sommerer (2004), which demonstrated CRF bouton structures at Orx cells, however, this study did not examine the origin of these CRF projections. Our results provide further anatomical evidence of the relationship between Orx and CRF cells within the hypothalamus and throughout the brain (Achua, 2014; Harris & Aston-Jones, 2006; Ida et al., 2000; Sakamoto et al., 2004; Winsky-Sommerer et al., 2004).

After injecting the retrograde tracer FG into the PeF, sections containing the LH/DMH/PeF were stained for both Orx and CRF and the staining was subsequently examined. This staining revealed specific Orx perikarya within the hypothalamus, including axons and terminals. However, these appear to be discrete from clusters of CRFergic cells, with CRF terminals at Orx cell bodies and at interneuron synapses. Additionally, within the distribution of both populations there were FG stained neurons. These results identify inputs to the LH/DMH/PeF, including interneuron connections, as well as provides anatomical evidence of CRF and Orx communication. The presence of CRF terminals at Orx cell bodies and interneuron synapses implies a functional

relationship, perhaps one of CRF-Orx coregulation, within the hypothalamus. These data further support anatomical and behavioral evidence provided in previous studies by this laboratory and others (Achua, 2014; Borgland et al., 2010; Harris & Aston-Jones, 2006; Ida et al., 2000; Sakamoto et al., 2004; Winsky-Sommerer et al., 2004).

Examination of CRF expression and cellular projections from the CeA to the PeF reveal little colocalization. With few neurons in the CeA fluorescing both FG and CRF, there appears to be little direct CRF input to the PeF from the CeA. This region, which contains dense populations of CRFergic neurons, exhibits few cells that project to the PeF. The role of the CeA in the CRF-Orx functional relationship is not diminished by these data, however. Activity of CRF neurons within the CeA is modulated by Orx, indicating Orx projections to these cells (Sakamoto et al., 2004). This is supported by results demonstrating many Orx fibers within the CeA, further indicating communication with Orx from the CeA (Winsky-Sommerer et al., 2004). Additionally, non-CRF cells did exhibit FG staining in the CeA, indicating the possibility of indirect CRF modulation of Orx; cells in the CeA expressing CRF could innervate these cells in the CeA stained with FG.

The same conclusion can be reached when examining the distribution of CRFergic neurons with PeF projection cells in the PVN. Within this region of the hypothalamus, there are dense populations of CRFergic neurons and those expressing FG staining. However, these two populations are anatomically discrete. A few cells do exhibit double-staining, indicating that a few CRFergic cells within the PVN do project to the PeF Orx neuronal field. While there is more double-staining than in the CeA, it is not a substantial amount. From this anatomical evidence, it can be concluded that the

contribution of the PVN to the direct coregulation of CRF and Orx is minimal through direct CRF projections. However, it has been observed that the PVN receives input from Orx cells which activates CRF neurons in this region (Sakamoto et al., 2004). Dense staining of Orx fibers has also been observed in the PVN, indicating the possibility of indirect mechanisms of CRF-Orx coregulation (Winsky-Sommerer et al., 2004). Non-CRF cells did show ample FG staining in the PVN, suggesting that there may be additional indirect connections from PVN CRF cells to Orx, or that the PVN influences Orx signaling, but not through CRF projections.

Of the several regions known to be involved in CRF activity, the BNST exhibited the greatest evidence for a connection with the PeF Orx field and the CRF cells. While the BNSTd exhibits both CRFergic neurons and FG stained cells, these two populations are topographically distinct. The PeF projecting neurons stained with FG remain medial to the dense CRFergic population. Previous studies examining CRF cell activity within the BNSTd have observed increased CRF cell activity in response to stress (Kim et al., 2006). With data from our study, it is possible that the role of Orx neurons outside the PeF contribute to the stress response; this does not, however, contradict the hypothesis that PeF Orx neurons preferentially contribute to stress responses. The neurons in the PeF field may still contribute to stress, but not through direct connections to the BNSTd. The role of Orx neurons outside the PeF demonstrates the highly redundant network of the mammalian brain.

Within the BNSTv, a large number of neurons stained for both CRF and FG is observed. The dense overlap indicates a relatively strong innervation of PeF cells by CRFergic neurons in the BNSTv, supporting a role for the BNSTv CRF cells in

modulation of the PeF Orx field. This is the first study to suggest an anatomical functional link between CRF and Orx through the BNST. Many studies have examined the role of CRF in the BNST, with results indicating BNST CRF neurons contributing to stress-induced drug relapse (Erb & Stewart, 1999), and hypotheses to functional topographic organization of CRF synthesizing cells in modulating stress (Choi et al., 2008). Studies examining the anatomical interactions of CRF and Orx have not discussed the BNST region (Winsky-Sommerer et al., 2004). Our results of colocalization of FG and CRF cells in the BNSTv expand on studies and provides anatomical evidence for direct reciprocal interaction between CRF and Orx occurring via the BNST.

While the BNSTv CRF cells are indicated to directly synapse onto PeF neurons, the LS exhibits an indirect pathway for CRF innervation of the PeF Orx field. Within the LS, many cells possessed the FG retrograde staining, and no CRFergic somi are visible. However, dense innervation of LS neurons is apparent from the large number of CRF terminals synapsing on both the cell bodies and axons of FG stained cells. These data indicate a strong innervation of neurons within the LS which project directly to the PeF. From this, it seems that CRF from another area exercises control of Orx activity via LS neurons.

The implications from these data leads us to examine 1) what receptors are involved in Orx-CRF cross-talk, 2) what type of non-CRF cells are innervated by Orx projections to CeA, PVN, BNST, and LS, and 3) what CRF population innervates LS for future research. Our interpretation of previous research combined with our anatomical results suggests that these receptors are CRF_2, as they are most expressed within the LS (Bakshi et al., 2007; Chalmers et al., 1995). Immunohistochemical methods or mRNA

expression could be used to obtain this data on CRF receptor expression of LS neurons. Second, the chemical profile of the neurons being activated by CRF should be ascertained. Learning what signaling molecules these cells express would indicate their functional role in modulating PeF cell activity. Third, investigation should be done to learn what CRF cells in which region are innervating these LS neurons; injection of a retrograde tracer into the LS would yield the answers to this question. Several regions could be culprit; in fact, the area responsible may be one examined in this study. While these regions may not have direct CRF input to the PeF, perhaps they have indirect input via the LS. Finally, to validate the anatomical evidence, functional relationships should be examined through behavioral experiments. Many studies already provide functional evidence for the involvement of the LS in stress and anxiety through CRF (Bakshi et al., 2007; Henry et al., 2006; Thomas, 1988; Yadin et al., 1993). Injection of CRF into the LS and subsequent stress-testing, as well as use of CRF antagonist with these models, will provide further evidence of the role of the LS in stress and anxiety.

Conclusion

The results of this study provide further anatomical evidence of cross-talk between CRF and Orx systems within the brain, and indicate a functional coregulatory relationship. There are anatomical projections of CRF neurons to the PeF Orx field from the BNSTv, and indirect modulation of these cells by CRF via neurons in the LS. This study adds to the literature of the involvement of both CRF and Orx playing parallel roles in stress and anxiety behaviors in an organism (Achua, 2014; D. H. Arendt et al., 2013; Binder & Nemeroff, 2010; Hagan et al., 1999; Harris & Aston-Jones, 2006; Ida et al.,

2000; Krahn et al., 1986; P. J. Ronan & Summers, 2011; Sakamoto et al., 2004; Winsky-Sommerer et al., 2004; Yoshida et al., 2006). Additionally, it indicates a role of the LS in connecting CRF and Orx systems for modulating the stress response, where several studies have shown the involvement of the LS in anxiety responses (Bakshi et al., 2007; Henry et al., 2006; Thomas, 1988; Yadin et al., 1993). However, function investigation is required to continue to elucidate the exact mechanisms and systems involved. This study provides clear anatomical evidence of the coregulation of CRF and Orx.

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