TONIC MODULATION OF CENTRAL STRESS CIRCUITS BY ENDOGENOUS OXYTOCIN

By

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To my parents
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<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
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<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<tr>
<td>AP</td>
<td>Action potential</td>
<td></td>
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<tr>
<td>A1R</td>
<td>Adenosine 1 receptor</td>
<td></td>
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<tr>
<td>BLA</td>
<td>Basolateral amygdala</td>
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<tr>
<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
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<tr>
<td>CeA</td>
<td>Central amygdala</td>
<td></td>
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<tr>
<td>CeL</td>
<td>Lateral division of CeA</td>
<td></td>
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<tr>
<td>CeM</td>
<td>Medial division of CeA</td>
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<tr>
<td>CORT</td>
<td>Corticosterone</td>
<td></td>
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<tr>
<td>CRF</td>
<td>Corticotrophin releasing factor</td>
<td></td>
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<tr>
<td>CRFR1</td>
<td>CRF receptor 1</td>
<td></td>
</tr>
<tr>
<td>CRFR2</td>
<td>CRF receptor 2</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
<td></td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamus-pituitary-adrenal</td>
<td></td>
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<tr>
<td>I_A</td>
<td>Fast transient outward potassium current</td>
<td></td>
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<tr>
<td>i.p.</td>
<td>Intra-peritoneal</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LTS</td>
<td>Low threshold spike</td>
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<tr>
<td>MNC</td>
<td>Magnocellular</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
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<td>OT</td>
<td>Oxytocin</td>
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OTR  Oxytocin receptor
PVN  Paraventricular nucleus of hypothalamus
PKC δ+  Protein kinase C delta positive
PKC δ−  Protein kinase C delta negative
pNa+  Plasma sodium
SON  Supraoptic nucleus of hypothalamus
VP  Vasopressin

**DRUGS**

APV  D-(-)-2-Amino-5-phosphonopentanoic acid; competitive NMDA receptor antagonist
CGP  CGP 55845; GABA\(_B\) receptor antagonist
DNQX  6,7-Dinitroquinoxaline-2,3-dione; AMPA and kainite receptor antagonist
GDP-\(\beta\)-S  Guanosine 5′-[\(\beta\)-thio]diphosphate; non-hydrolysable GDP analog
L-368  L-368,899 hydrochloride; non-peptide OTR antagonist
OTA  \((d(CH\_2)\_5 \^1, Tyr(Me)\_2, Thr\_4, Orn\_8, \text{des-Gly-NH}_2\_9)\)-Vasotocin; peptide OTR antagonist
PTX  Picrotoxin; GABA\(_A\) antagonist
TGOT  (Thr4,Gly7)-Oxytocin; selective OTR agonist
TTX  Tetrodotoxin; voltage-gated sodium channel blocker
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TONIC MODULATION OF CENTRAL STRESS CIRCUITS BY ENDOGENOUS OXYTOCIN

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Oxytocin (OT), a neuropeptide well characterized for its traditional roles in parturition and lactation has recently been implicated for its distinct central effects associated with regulation of mood and anxiety. With the increasing emphasis on intranasal oxytocin delivery as a plausible therapeutic intervention for neuropsychiatric conditions such as schizophrenia, anxiety and Autism Spectrum Disorder (ASD) it is imperative to elucidate the neurobiological factors that underlie the anxiolytic and prosocial effects of oxytocin in the brain. The overarching goal of this dissertation was to utilize peripheral salt loading model to drive sustained release of OT in the rodent brain to investigate how endogenous oxytocin can modulate stress-related circuitry in the hypothalamic paraventricular nucleus (PVN) and the central amygdala (CeA).

A plausible neural basis for oxytocin mediated stress reduction is via mitigation of the activation of corticotropin-releasing factor (CRF) producing neurons in the PVN. CRF is a key neuropeptide responsible for orchestrating the endocrine, autonomic and behavioral responses to stress. In Chapter 3, we utilized a cre-lox based mouse line to selectively target CRF neurons and used whole-cell electrophysiology to show that
peripheral salt loading tonically inhibits CRF neurons by activating oxytocin receptors (OTRs) independent of synaptic activity.

Intriguingly, preautonomic-like neurons in the PVN which are responsible for the maintenance of sympathetic tone displayed an opposite phenotype as discussed in Chapter 4. These neurons showed a prominent OTR-mediated GABA-A dependent inhibitory tone in the isotonic mice that was lost following 2.0 M NaCl injection. Together, our data support a paracrine mechanism by which endogenous OT can tonically impact parvocellular neurons in the PVN.

In Chapter 5, the synaptic effects of endogenous OT on CRF neurons in the CeA was investigated. Under basal conditions OT predominantly inhibited CRF neurons independent of GABAergic signaling or postsynaptic GPCRs. Peripheral salt loading unmasked a subset of CRF neurons that were tonically excited by OT via activation of postsynaptic GPCRs.

Collectively, the data presented in this dissertation demonstrate novel pathways through which OT impacts stress circuitry in both the PVN and the CeA and furthers our understanding of the neuromodulatory role of endogenous oxytocin.
There are a wide variety of anxiety disorders, including post-traumatic stress disorder, obsessive-compulsive disorder, and generalized anxiety disorder to state a few example. According to the National Institute of Mental Health (NIMH) anxiety-related disorders are among the most common mental disorders experienced by Americans. A dysregulated stress response leads to heightened anxiety levels that has been shown to be associated with neuroadaptive changes in stress-related limbic brain areas. The neural correlates underlying such maladaptive plasticity in stress systems in the limbic region is an ongoing investigation.

Neurohypophyseal neuropeptides like oxytocin (OT) and vasopressin (VP) have garnered significant attention as the key modulators of complex social behaviors (Neumann and Landgraf, 2012). Centrally released OT can attenuate hormonal responses to stress and fear within the hypothalamus and within the amygdala complex. OT can modulate complex social behaviors in humans, particularly during times of stress (Kirsch et al., 2005; Kosfeld, 2005) under healthy and pathological conditions (Hollander et al., 2007; Hollander et al., 2002). Its anxiolytic properties have been reported both in male and female rats. However, the mechanisms underlying the central actions of OT are not well understood. The focus of this brief literature review is to provide current knowledge about how centrally released OT may modulate neural activity and synaptic functions of the stress-related circuitry with regards to the central corticotrophin releasing factor (CRF) system.
The Central Oxytocinergic System

OT Synthesizing Magnocellular Neurons

OT is a highly conserved nonapeptide (Fig. 1-1) synthesized principally by magnocellular neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, from where it is transported to the posterior pituitary and directly released into the bloodstream. The OT gene encodes the pre-propeptide which is synthesized in the ribosomes of magnocellular neurons and cleaved in the endoplasmic reticulum. The mature peptide product and its carrier molecule neurophysin are stored in the axon terminals until the neuron is activated to release the peptide. Peripherally, OT is important for ovulation, parturition and lactation (Russell et al., 2003). OT neurons are also found in the parvocellular neurons of the PVN and suprachiasmatic nucleus, and in the bed nucleus of the stria terminalis (BNST), and they can project to extra hypothalamic regions including the hippocampus, cortex, VTA and amygdala (P. E. Sawchenko, 1984). Oxytocinergic fibers are widely distributed throughout the brain and can modulate complex socio-sexual behaviors, anxiolysis and stress attenuation (Stoop, 2014).

Magnocellular neurons contain the neurohypophyseal hormones OT and VP and project to the neurohypophysis. These neurons are primarily located in SON and ventral and lateral subdivisions of the PVN in the rat brain. They respond to various stimuli and demonstrate distinct firing pattern (Armstrong, 2007; Freund-Mercier et al., 1983; Wang and Hatton, 2007). For example, in response to suckling, OT-containing neurons exhibit synchronous high frequency burst firing to induce milk ejection. OT neurons also increase their firing frequency in response to dehydration and an increase in plasma tonicity to regulate sodium excretion.
Somato-dendritic Release of Oxytocin

Independent of the classical release from the axon terminals, OT can be released from the dendrites and soma of magnocellular neurons, a process known as volume diffusion. Volume diffusion within the hypothalamus and to extra hypothalamic sites influences a wide network of central OT receptors (OTRs) and has a different temporal profile than peripheral release. Both physiologic as well as psychogenic stressors can elicit dendritic release of OT and can modulate stress-related behaviors (Ebner, 2004). Peptide-evoked dendritic release can prime the secretory vesicles and make them more receptive to subsequent electrical stimuli (Leng et al., 2008; Leng and Ludwig, 2006; Ludwig and Leng, 2006; Ludwig et al., 2002), and can allow for long-lasting behavioral effects (N. Sabatier, 2007; Sabatier, 2006). Dendritic release of OT can lead to very high local concentrations with a prolonged half-life due to slow degradation processes. In rodents, OT has a basal concentration of ~1-10pM in plasma with a half-life of ~2-3 minutes, while in cerebrospinal fluid it has a concentration between 10-50 nM with a longer half-life of ~ 20 minutes. Seminal studies conducted by Ludwig et al. demonstrated the role of voltage gated calcium channels underlying the transmitter release from dendrites. In addition, activation of NMDA receptors on dendrites or release of calcium from intracellular stores can evoke dendritic release (Bourque, 2008; Brown and Bourque, 2006). OT itself can auto-regulate the excitability of its own neuron by directly triggering dendritic release or indirectly by inhibiting GABAergic inputs to the neuron (Oliet et al., 2007). This volume transmission may play a critical role in brain regions associated with behaviors modulated by OT.
Oxytocin Receptors: Distribution and Function

The OT receptor (OTR) is a typical member of the rhodopsin-type (class I) G protein-coupled receptor (GPCR) family. OTRs are considered to be ‘promiscuous’ GPCRs as they can functionally couple to different classes of GTP binding proteins and initiate a cascade of intracellular events. (Gimpl and Fahrenholz, 2001). They can couple to Gq/11 and stimulate the activity of phospholipase C, leading to release of intracellular calcium. They can control cellular excitability, modulate firing patterns, and lead to transmitter release. Within the CNS, OTRs are abundantly expressed in the SON, PVN, and various limbic regions including BNST, amygdala, the nucleus accumbens, ventral tegmental area and the hippocampus. Therefore, OT in the brain is described as a neuromodulator and appears to have broad permissive actions (Breton and Zingg, 1997; Gould and Zingg, 2003; Grazzini et al., 1998; Zingg and Laporte, 2003).

OT and Fluid Homeostasis

The magnocellular neurons receive afferent inputs from circumventricular organs: the organum vasculosum of the lamina terminalis and the subfornical organ (Smith and Ferguson, 2010). These regions lack a blood-brain barrier and can directly sense changes in plasma osmolality and send projections to magnocellular neurons to stimulate OT and vasopressin release in response to hyperosmotic stress (Bourque, 2008). The systemic release of vasopressin results in antidiuresis and conservation of water whereas the secretion of OT promotes natriuresis. Thus, OT secreting magnocellular neurons can coordinate the physiological and behavioral responses to changes in fluid homeostasis.
Crosstalk between Dendritic Oxytocin and Parvocellular Neurons within the PVN

Stress can be defined biologically as actual alterations of homeostasis or anticipated threat to well-being. Stress results in activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. The physiological response to stress involves an efficient recruitment of neural and neuroendocrine systems to maintain the physiological integrity. The PVN of the hypothalamus orchestrates neuroendocrine, autonomic and behavioral responses to regulate energy and fluid homeostasis (Ulrich-Lai and Herman, 2009; Ziegler and Herman, 2002). The PVN can be broadly divided into three major divisions: 1) magnocellular neurons that project directly to the posterior pituitary; 2) parvocellular CRF synthesizing neurosecretory neurons that project to the median eminence and control anterior pituitary hormone secretion; and 3) preautonomic neurons that have descending projections and control the central autonomic system and modulate heart rate and blood pressure.

CRF Synthesizing Neurosecretory Neurons in the PVN and the HPA Axis

CRF serves as an important integrator of stress responses by regulating the HPA axis. The main source of CRF is within the PVN but the peptide is also present at several extra-hypothalamic sites, including hippocampal formation, the lateral septum, the CeA, the BNST and the nucleus accumbens (Aguilera, 1998; Aguilera and Liu, 2012). Within the PVN, the dorsomedial parvocellular CRF neurons are neurosecretory and project to the median eminence serving as the final common pathway of glucocorticoid secretion. Glucocorticoids have catabolic effects on metabolic, inflammatory and immune functions as well as behavioral effects. GCs exert a negative-
feedback control to inhibit further release of CRF, thereby attenuating the stress response (Fig. 1-2).

CRF-synthesizing parvocellular neurosecretory neurons in the PVN constitute a relatively homogenous group located primarily in the dorsomedial region of the PVN.

**Neural Regulation of the HPA Axis**

The PVN receives extensive inputs from local circuits as well as the limbic system. In general, glutamatergic and noradrenergic inputs activate, whereas GABAergic inputs inhibit CRF neurons of the PVN (Miklós and Kovács, 2002; Sawchenko and Swanson, 1982). A major source of the excitatory inputs is from the nucleus of the solitary tract (Cunningham and Sawchenko, 1988). Local inhibitory inputs originate in the peri-PVN regions (Boudaba et al., 2003a; Boudaba et al., 1996; Herman and Cullinan, 1997; Herman et al., 2003), the medial preoptic area, and the dorsomedial nucleus of the hypothalamus. Most regions of the limbic system such as the hippocampus (Sapolsky et al., 1984), the medial prefrontal cortex, the lateral septum (Buijs and Van Eden, 2000) and the posterior division of the BNST (Crane et al., 2003) regulate negative feedback by sending direct or indirect inhibitory signals to the PVN.

**Oxytocin and Stress Response**

Previously published work shows that both physiologic as well as psychogenic stressors can elicit OT release and can modulate stress-related behaviors. Neumann et al. infused OTR antagonist in the PVN of both male and female rats and measured plasma ACTH under control conditions and following exposure to various psychogenic stressors. They observed a significant increase in the ACTH levels showing that endogenous OT can act independent of gender to inhibit basal and stress-induced activation of HPA axis (Neumann et al., 2000a; Neumann et al., 2000b). Another group
used female ovariectomized, estradiol- treated rats, and showed that 30 min restraint fails to increase c-fos mRNA in the PVN only when pre-treated with OT and not with VP (Windle et al., 2004). Also, chronic salt loading can lead to a time-dependent increase in magnocellular CRF mRNA, but decreases parvocellular CRF mRNA, indicating a complex and coordinated response to chronic osmotic stress (Lightman and Young, 1987). More recent work includes the use of OT knockout mice (male and female) that supports the stress attenuating role of OT (Amico et al., 2008a; Amico et al., 2004; Mantella et al., 2005; Nomura et al., 2003).

Recent investigation into the effects of hydromineral imbalance on stress responsiveness in rodents indicated a prominent role for OT signaling. 2.0 M saline injection coupled with 60 minutes of water deprivation results in decreased ACTH, and CORT during a 60 min restraint period that is associated with increased plasma OT levels (Krause et al., 2011). Acute salt loading decreases anxiety-like behavior in elevated plus maze and induces Fos expression in the BNST, ventral lateral septum, and central nucleus of the amygdala (Frazier et al., 2013). Although there is a significant amount of work supporting an interaction between OT and CRF neurons to suppress the HPA axis, the mechanisms underlying this central role are not well understood.

**Preautonomic Neurons and the Sympathetic Tone**

PVN is a convergence point for circuits involved in maintaining body homeostasis ranging from fluid regulation, energy metabolism and immune responses. The cardiovascular portion of the sympathetic tone is regulated through parvocellular PVN neurons that innervate brainstem regions (NTS, RVLM) and spinal cord (Coote, 2005). These neurons are termed as preautonomic neurons and express a combination of
glutamate and peptides (such as VP, OT and CRF) to control sympathetic efferents (Pyner, 2009). The activity of preautonomic neurons is regulated by osmolality (Antunes-Rodrigues et al., 2004) and short-term administration of 2.0M saline can activate preautonomic neurons secondary to the activation of central osmoreceptors located in the circumventricular organs (Brooks et al., 2005; Osborn et al., 2000).

The exaggerated sympathoexcitatory effects of preautonomic neurons due to elevated brain sodium could be relevant for the etiology of salt-induced hypertension and other cardiovascular disease such as chronic heart failure. Various models of hypertension have demonstrated the importance of PVN preautonomic neurons in the generation of neurogenic hypertension either due to impaired inhibition or increased excitation (Allen, 2002; Chen et al., 2010; Toney et al., 2003). Antagonizing either the angiotensin receptors or the glutamate receptors in the PVN or the RVLM reduces blood pressure in certain models of hypertension (Sved et al., 2003). The effect of angiotensin II on RVLM projecting preautonomic neurons relies on several mechanisms as pointed out by various electrophysiological studies (Cato and Toney, 2005; Li and Pan, 2005; Li and Ferguson, 1993). Angiotensin II can act on presynaptic AT1 receptors to increase glutamate release and promote excitability or can attenuate synaptic GABA release to cause disinhibition. Recently, Son et al. revealed a novel crosstalk between dendritic VP and RVLM-preautonomic neurons wherein activation of VP neurons released VP locally to act on V1a receptor expressed on preautonomic neurons to increase their firing rate. This VP-mediated effect also persisted following acute injection of hypertonic saline.
These studies implicate that neuropeptides with a prominent role in fluid homeostasis can act as a neuromodulator to fine-tune the excitation/inhibition balance on preautonomic neurons to affect their activity. Although there is a wide range of information available on how OT can impact stress and anxiety circuitry and promote social affiliation not much is known about whether there is a crosstalk between OT and preautonomic neurons within the PVN. Some studies have pointed to a cardio-protective role of OTRs (Gutkowska and Jankowski, 2012) but the cellular signaling mechanisms still elude us.

**Oxytocin Mediated Modulation of Extrahypothalamic Circuits: OT and the CeA**

**CeA: Integrator of Autonomic and Behavioral Responses to Fear and Anxiety**

The circuits mediating fear and anxiety are related and have been studied for decades. Amygdala has been the focus of many of these studies for its crucial role in the processing of fear and anxiety (Paré, 2003; Phelps and LeDoux, 2005). Amygdala has many subnuclei which are functionally distinct (Fig. 1-3). The lateral amygdala is the convergence point for sensory inputs (LeDoux et al., 1990) which then send projections to the CeA and activate output neurons to trigger expression of fear or anxiety (Pape and Pare, 2010; Phelps and LeDoux, 2005).

The CeA is further subdivided into the lateral division (CeL) and the medial division (CeM) and is mostly comprised of GABAergic medium spiny neurons (Ehrlich et al.). Emerging data is consistent with the idea that CeM is the main output of the amygdala while CeL is critical for acquisition of fear-related memory (Ciocchi et al., 2010). Output neurons from CeM are GABAergic which can affect downstream structures to coordinate motor and autonomic responses to fear.
Recently, (Ciocchi et al., 2010) also discovered two distinct microcircuits that acquired opposite responses to conditional stimulus and are also marked by the expression of protein kinase C-delta (PKCδ) (Haubensak et al., 2010). Haubensak et al. also provided anatomical evidence that PKCδ- neurons project to PKCδ+ neurons which directly inhibit CeM neurons and also project back to PKCδ- neurons to establish a complex, reciprocal feedback circuitry within the CeL.

**CeA and CRF Microcircuitry**

GABA neurons within the CeA also co-express various peptides (Cassell and Gray, 1989) including CRF. CeA is an important extrahypothalamic source of CRF. Unlike the CRF neurons in the PVN which are relatively homogenous population, CRF neurons in the CeA represent a heterogeneous population that has been implicated in various conditions ranging from addiction to memory consolidation, stress responsiveness and alertness (Beckerman et al., 2013; Gilpin et al.; Gray et al., 2015; Herman et al., 2013; Reyes et al., 2011; Silberman and Winder, 2013). CRF receptors are abundantly expressed in CeA (De Souza, 1984) and hyperactivity of CRF system in the amygdala is associated with anxiety-like behavior (Gray et al., 2015; Rainnie et al., 2004). CRF in CeA can also regulate the HPA-axis indirectly through dense projections to the BNST (Sakanaka et al., 1986). Site specific manipulation of CeA CRF expression pointed to a role of CRF in stress-induced anxiety-like behavior with sometimes contradictory results ((Flandreau et al., 2012; Regev et al., 2011; Regev et al., 2012). Specific deletion of GABA-A receptors from CRF neurons enhanced anxiety and disrupted the fear extinction (Gafford et al., 2012). Recently, work from the Bruchas group demonstrated endogenous CRF inputs from CeA to the locus coeruleus that can increase tonic firing of the neurons in the region and induce anxiety-like behaviors.
(McCall et al., 2015). Though the contribution of CRF neurons in the amygdala has been extensively characterized, the role that other neuropeptidergic systems play in modulating the activity of CRF neurons is not well known.

**Source of OT within the CeA: Synaptic Release or Volume Transmission?**

A widely debated question in the field has been the availability of endogenous OT to far-off regions given that the neuropeptide is synthesized within the magnocellular neurons of the PVN and SON. The gross mismatch between the source of OT and the expression of OTRs resulted in the initial hypothesis that dendritic OT can traverse through extracellular space and act on OTRs through volume transmission. This is supported by the evidence that OT can be released in large quantities from dendrites and seem to persist longer centrally compared to its availability in the plasma (Ludwig and Leng, 2006). But, volume transmission alone cannot account for spatial and temporal control that is required to elicit a specific behavioral response. With the observation that there exists a group of distinct parvocellular OT-positive neurons that can project centrally (Sawchenko and Swanson, 1982) resulted in the dogmatic view that magnocellular OT neurons release OT in the periphery where it acts as a hormone while parvocellular OT-positive neurons project centrally to release OT in distinct brain regions where it acts as a neuromodulator. This view was challenged by the work from Stoop lab where they convincingly demonstrated that action potential mediated synaptic release of OT arising from magnocellular neurons in the PVN and SON can modulate fear response in the CeA by directly depolarizing GABAergic neurons in the CeL (Knobloch et al., 2012). Thus, apart from paracrine signaling within the PVN synaptic signaling can also elicit peptide-related behavior relevant to stress and anxiety.
CeA and OT-mediated Anxiolytic Effects

The amygdala and CeA in particular is heavily influenced by peptidergic inputs (Ehrlich et al.; Gray and Magnuson, 1992). OT is one such neuropeptide which has been shown to modulate stress and anxiety responses by activating OTRs in the amygdala (Daniele Viviani, 2011; Huber et al., 2005; Neumann and Landgraf, 2012; Stoop, 2012; Terenzi and Ingram, 2005; Viviani et al., 2010). OT and VP activate different subpopulation of CeL neurons to exert opposing effects (Daniele Viviani, 2011; Huber et al., 2005). Recently, Knobloch et al. demonstrated that optogenetically stimulating hypothalamic oxytocinergic fibers in CeL decreased freezing by increasing GABA signaling in the CeM (Knobloch et al., 2012). These data demonstrate the importance of OT signaling in the CeA in the regulation of the fear and anxiety circuits but how OT can manipulate CRF neurons in the CeA and the neural mechanisms underlying such interaction and its functional relevance has not been probed in-depth.

Experimental Goals

The overall objective of this dissertation was to determine the neural mechanisms by which endogenous OT can modulate stress and anxiety responses. Previous researchers have utilized exogenous application of OT agonist to study the central effects of OT. Although such studies have provided critical information regarding the role of OT, there are limitations to these studies such as different pharmacokinetic profile and high variability. Therefore, acute salt loading paradigm is a unique model to drive central release of OT (Leng and Ludwig, 2008; Ludwig and Leng, 2006) which avoids the limitations of exogenous OT application and elucidate the role of endogenous OT in stress and anxiety-related behaviors (Krause et al., 2011; Smith et al., 2014a). The specific experimental goals of this dissertation are:
1. **To evaluate the crosstalk between OT and CRF neurons in the PVN following 2.0 M NaCl injection:** Exposing male rodents to a psychogenic stressor like restraint following 2.0 M NaCl injection resulted in blunted corticosterone response and reduced neuronal activation of parvocellular neurons in the PVN. In rats, this was dependent on OT-mediated tonic inhibition of parvocellular neurosecretory neurons (Frazier et al., 2013). Taking advantage of a CRF reporter strain, the goal of Chapter 3 was to determine the synaptic mechanisms through which OT-mediated paracrine signaling can modulate the HPA-axis activity by inhibiting the neuronal activity of CRF neurons. This was achieved by combining whole-cell electrophysiology in transgenic mice with cre/lox based selective deletion of OTRs.

2. **To evaluate the role of somatodendritic OT in regulating the excitability of preautonomic parvocellular neurons in the PVN:** An adequate response to a stressful stimulus involves recruitment of both hormonal and autonomic systems to restore the homeostasis. Preautonomic neurons in the PVN represents heterogeneous population of parvocellular neurons that regulate the sympathetic tone and are themselves modulated by different synaptic inputs. A growing body of literature implicates the local GABA-glutamate system in the regulation of neural activity of preautonomic neurons but whether local OT can impact the neuronal discharge of preautonomic neurons is not clearly known. Chapter 4 sought to ask the question whether OT-mediated paracrine signaling in the PVN can modulate the activity of non-
neurosecretory parvocellular neurons and to determine the synaptic mechanisms involved.

3. **To determine if oxytocinergic projections to CeA contribute in the modulation of the amygdala CRF neurons**: OT plays a significant role in modulating anxiety-related behavior by targeting GABA signaling in various stress-related limbic brain areas, especially CeA (Huber et al., 2005; Knobloch et al., 2012; Viviani et al., 2011; Viviani et al., 2010). A subset of GABAergic neurons in the CeA also co-express CRF and is associated with promoting stress-related behavior. The objective of Chapter 5, was to determine whether endogenous OT signaling contributes in the modulation of CRF neurons in the CeA. The effects of OT were observed both in isotonic and hypertonic conditions and relied on the CRF reporter strain to selectively target CRF neurons in CeA.

Together these aims will emphasize the significance of activation of central OTRs by a physiologically relevant stimulus and the role it plays in regulating mood and anxiety-related circuitry. This might provide new insights that could promote the development of novel therapeutic interventions and better understandings of the physiological role of endogenous OT.
Figure 1-1. OT neuropeptide
Figure 1-2. Schematic representation of the HPA axis activity. Adapted from Smith SM, Vale WW (2006). Green: Excitatory; Red: Inhibitory

CRF synthesizing parvocellular neurosecretory neurons.
Figure 1-3. CeA as the hub of fear and anxiety response. Adapted from Tye KM (2013). Green: Glutamatergic projections; Red: GABAergic Projections from BLA impinge on lateral CeA where PKCδ- and PKCδ+ neurons form a reciprocal inhibitory loop. PKCδ+ neurons inhibit output neurons in CeM which regulate downstream structures such as PAG and LC. Furthermore, OT projections from PVN can modulate CeA and projections from CeA can inhibit BNST.
CHAPTER 2
MATERIALS AND METHODS

Generation of CRF Reporter Mice

All studies in chapters 3 and 5 were performed on CRF reporter mice that expressed red fluorescent protein (tdTomato) in neurons that synthesized CRF (Chen et al., 2015; Smith et al., 2014a; Wamsteeker Cusulin et al., 2013) (Fig. 2-1). Briefly, these reporter animals were generated by breeding mice with a mutation of the Gt(ROSA)26Sor locus with a \textit{loxP}-flanked STOP cassette preventing transcription of a CAG promoter-driven sequence coding for tdTomato (Jackson Laboratory Stock # 007914) to mice that expressed Cre-recombinase specifically in neurons with CRF gene (Jackson Laboratory Stock # 012704). CRF-IRES-Cre mice were generated using bacterial artificial chromosome (BAC) recombineering techniques (Taniguchi et al.).

All animals were housed on a 12:12 h light/dark cycle in clear plastic ventilated cages with plumbed water supply. Standard mouse chow (Harlan) was suspended in a wire rack that also supported an accessory water bottle allowing \textit{ad libitum} access to both food and water except where otherwise noted. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida.

Generation of CRF-OTR Knockout Mice

To selectively delete OTRs on CRF neurons, we used cre-\textit{lox} tissue specific knockout (KO) system (Fig. 2-2). To create such a KO strain, we utilized a conditional KO OTR mouse line (Lee et al., 2008a). Briefly, mice with a floxed OTR allele (OTR\textsuperscript{+/flox} or OTR\textsuperscript{flox/flox}) were crossed with CRF-cre line. The offspring had the following genotypes: 1) OTR\textsuperscript{flox/flox}, 2) OTR\textsuperscript{+/-flox}, which were considered wild type and 3) OTR\textsuperscript{+/flox, cre} and OTR\textsuperscript{flox/flox, cre}, which were the CRF-specific OTR KO. The OTR KO mice
appeared to be healthy and displayed no behavioral phenotype distinct from control mice.

**Drugs and Chemicals**

All chemicals used in these experiments were obtained from either Tocris Cookson or Sigma-Aldrich except \([d(CH2)51,Tyr(Me)2,Orn8]\)-oxytocin (OTR-A), which was obtained from Bachem.

**Electrophysiology**

**Slice Preparation**

Male mice (2-5 month old) were divided into two groups: isotonic and hypertonic. The isotonic mice received subcutaneous injections of 0.1 mL of 0.15 M NaCl and had free access to water while hypertonic mice received 0.1 mL of 2.0 M NaCl injections s.c. and subsequently, water was made unavailable (Fig. 2-3). To minimize pain and irritation, each injection was preceded by lidocaine. 1 hour later, mice were administered ketamine (80–100 mg/kg, ip) and were rapidly decapitated using a rodent guillotine. The brain was quickly removed, and coronal sections (300 μm thick) through the PVN were made using a Leica VT 100s vibratome. Slices were incubated for 30 minutes in a dissecting solution maintained at 30-35°C that contained in mM: 124 NaCl, 2.5 KCl, 1.23 NaH2PO4, 2.5 MgSO4, 10 D-glucose, 1 CaCl2, and 25.9 NaHCO3, saturated with 95% O2-5% CO2. After equilibrating at room temperature for at least 30 minutes, slices were transferred to a slice chamber for experimental use.

**Identification of Neurons**

We used CRF-reporter mice to record from tdTomato-expressing neurons in the medial parvocellular division of the PVN. Slices were visualized with infrared differential interference contrast microscopy (IR DIC) using an Olympus BX51W1 microscope.
CRF-expressing neurons were identified under a 40x immersed objective by a 565-nm excitation with rhodamine filter. They can be electrophysiologically identified and distinguished from both magnocellular neurons and preautonomic neurons based on the lack of the expression of both transient outward rectification and a low threshold spike (LTS) respectively (Luther and Tasker, 2000; Tasker and Dudek, 1991) (Fig. 2-4).

Preautonomic non-neurosecretory neurons in the PVN constitute a heterogeneous group located mostly in the ventral parvocellular and posterior parvocellular sub regions of PVN (Stern, 2001). Preautonomic neurons are generally identified using a combination of retrograde tract tracing and electrophysiological parameters. Retrograde tract tracing is used to target projection-specific preautonomic neurons and distinguish between RVLM-projecting and IML projecting neurons. They can be electrophysiologically identified and distinguished from both magnocellular neurons and CRF neurons based on the presence of low threshold spiking (LTS) and high input resistance (Dudek, 1991; Lee et al., 2008b; Luther et al., 2002; Luther et al., 2000; Luther and Tasker, 2000; Stern, 2001) (Fig. 2-5). Electrophysiologically, magnocellular neurons are known to display an A-type potassium current that results in a delayed onset to spike firing in response to depolarizing current steps (Fig. 2-6) (Luther et al., 2002; Luther and Tasker, 2000; Tasker and Dudek, 1991).

**Whole-cell Recording**

For whole-cell recording, slices were continuously perfused at a rate of 1.2-1.5 mL/min with aCSF that contained (in mM): 126 NaCl, 3 KCl, 1.2 NaH₂PO₄, 1.5 MgSO₄, 11 D-glucose, 2.4 CaCl₂, and 25.9 NaHCO₃. This solution was saturated with 95% O₂ and 5% CO₂, and bath temperature was maintained at 28±2°C. The patch electrode was filled with a K-based internal solution that contained (in mM): 130 K-gluconate, 10
KCl, 10 NaCl, 2 MgCl₂, 1 EGTA, 2 Na₂ATP, 0.3 NaGTP, and 10 HEPES, pH adjusted to 7.3 using KOH and volume adjusted to 285–300 mOsm. For experiments that involved the cell impermeant G-protein inhibitor GDP-β-S, a new K-glu internal was prepared that contained 300µM of GDP-β-S instead of GTP. For experiments that involved using a high-chloride K-glu internal, the concentrations used were (in mM): 100 K-gluconate, 40 KCl, 10 NaCl, 2 MgCl₂, 1 EGTA, 2 Na₂ATP, 0.3 NaGTP, and 10 HEPES, pH adjusted to 7.3 using KOH and volume adjusted to 285–300 mOsm.

Whole-cell voltage-clamp recordings were performed using micropipettes pulled from a borosilicate glass using a Flaming/Brown electrode puller (Sutter P-97; Sutter Instruments, Novato, California). Electrode tip resistance was between 4 and 6 MΩ. Voltage-clamp experiments were performed using an Axon Multiclamp 700A or 700B amplifier (Molecular Devices, Sunnyvale, CA). Data were sampled at 20 kHz, filtered at 2 kHz, and recorded on a computer by a Digidata 1400 A/D converter using Clampex version 10 (Molecular Devices, Sunnyvale, CA).

Data Analysis

The analysis of all electrophysiological data was performed using custom software written in OriginC (OriginLab, Northampton, MA) by C.J.F.

Spontaneous Synaptic Events

Spontaneous postsynaptic currents were detected using parameter based event detection software. For event analysis, the data was low pass filtered offline using a Gaussian convolution (kernel time constant = 2 msec). Event parameters were chosen to minimize RMS noise while ensuring accurate detection of vast majority of clear events. Typical threshold amplitude was 6-8 pA and typical threshold area was 20-40 pA*ms. In all cases an algorithm for detecting complex peaks (peaks that occur during
the decay period of a previous event) was employed. This algorithm calculates event amplitude for the second (and subsequent) peaks within the decay period based on extrapolated monoexponential decay from the peak of the initial event.

**Tonic Current Measurement**

Tonic currents were acquired by monitoring the holding current required to voltage-clamp the cells at -70mV throughout the experiments. Total tonic current was calculated from the difference in mean baseline and OTR antagonist currents. To control for variability due to differences in cell size/capacitance, current densities [current density = current (pA)/ capacitance (pF)] were calculated for each cell.

**Statistics**

A two-tailed, one-sample t test was used to assess the significance of the shift following the antagonism of oxytocin receptors [null hypothesis, Δ (pa/pF) = 0] whereas either one-way ANOVA or 2-sample Student’s t-test was used to compare values between groups. For assessing change in membrane resistance (Rm) following application of drug, the values were normalized to baseline and a two-tailed, one-sample t test was used to determine significance. For all cells, baseline was considered as 2 -5 mins and drug effect was measured for a 2-min period at least after the drug was on for 5 mins. All data are expressed as mean ± SEM. P<0.05 was considered significant. Rejection criteria included: unstable baseline or cells that did not last the whole course of the experiment, outliers (Grubb’s test for outliers).
Figure 2-1. Schematics of Cre-lox dependent generation of CRF Reporter mice.
Figure 2-2. Schematics of Cre-lox dependent selective deletion of OTRs from CRF neurons.
Figure 2-3. Schematics of acute salt loading paradigm and slice preparation.
Figure 2-4. Example trace of a parvocellular neurosecretory neuron following depolarizing current.
Figure 2-5. Example trace of a parvocellular preautonomic neuron following a depolarizing current. Notice the presence of a low threshold spike.
Figure 2-6. Example trace of a magnocellular neuron. There’s a characteristic IA current represented by delay in spike firing following a depolarizing current.
CHAPTER 3
OXYTOCIN MEDIATED TONIC INHIBITION OF CRF-SYNTHESIZING NEURONS IN THE MOUSE PVN FOLLOWING PERIPHERAL SALT LOADING

Introduction

The neuropeptide oxytocin is being extensively investigated for its prosocial and anxiolytic effects and an aberrant central oxytocin signaling has been implicated in neurodevelopmental disorders like Autism Spectrum Disorders (ASD) (Guastella et al., 2010; Guastella et al., 2015a; Hollander et al., 2007; Hollander et al., 2002; Modahl et al., 1998; Young and Barrett, 2015). In human subjects, intranasal oxytocin has been shown to promote trust (Kosfeld, 2005) (Kirsch et al., 2005) while suppressing reactivity in response to stress and fear (Daniele Viviani, 2011; Grinevich et al.; Huber et al., 2005; Kirsch et al., 2005; Knobloch et al., 2012; Neumann and Slattery; Stoop, 2012; Viviani et al., 2010). However, the neural mechanisms underlying the central effects of OT are still not clearly understood.

The paraventricular and the supraoptic nuclei of hypothalamus (PVN and SON respectively) are the two main sources of OT. OT-expressing magnocellular neurons within these two nuclei project to posterior pituitary and are responsible for the traditional neuro-hormonal roles of OT (Armstrong et al., 2010; Bourque et al., 2007; Moos et al., 1984; Russell et al., 2003). Recent work exploring the neuromodulatory roles of OT support the idea that oxytocinergic neurons in the PVN send projections to various brain regions, including the amygdala and BNST where they release OT to act mainly on oxytocin receptors (OTRs) to modulate fear and anxiety-related behaviors (Bale et al., 2001; Gimpl and Fahrenholz, 2001; Gould and Zingg, 2003; Hazell et al., 2012; Loup et al., 1991; Sladek and Song, 2012; Sofroniew, 1980; Tribollet et al., 1988;
van den Burg and Neumann, 2011). OTRs are typical class I GPCRs functionally coupled to different Gα proteins (Gq/11, Gi) to induce varied downstream effectors (Gimpl and Fahrenholz, 2001; Gravati et al., 2010).

There’s a growing body of literature describing the prevalence of tonic inhibition in diverse brain regions such as hippocampus, thalamus and cortex (Ferando and Mody, 2012; Glykys and Mody, 2007a, b; Lee and Maguire, 2014; Mody and Pearce, 2004; Semyanov et al., 2004). Despite the physiological relevance of tonic inhibition there is a lack of information regarding the effects of tonic release of neuropeptides such as OT. Interestingly, previous in vitro studies reported the release of OT from the dendrites of magnocellular neurons within the PVN and SON following a systemic injection of 2.0 M NaCl (F. Bergquist, 2008; Ferri and Flanagan-Cato, 2012; Ludwig, 1998b; Ludwig and Leng, 2006; Ludwig and Pittman, 2003; Ludwig et al., 2002; Neumann et al., 2006; Sabatier, 2006; Tobin et al., 2011). Centrally released OT has a much longer half-life in cerebrospinal fluid compared to plasma and can persist for a prolonged period of time independent of axonal release (Ludwig and Leng, 2006). This paracrine release of oxytocin may serve as a tonic signal that integrates fluid homeostasis with stress responsiveness.

Indeed, prior work from our group revealed a tight coupling between hydration state and mood and anxiety-related behaviors. Specifically, we showed that a mild physiological challenge such as acute hypernatremia can alter hypothalamic-pituitary-adrenal (HPA) axis output and plasma CORT responses to restraint, a common psychogenic stressor (Frazier et al., 2013; Krause et al., 2011; Smith et al., 2014b). The responsivity of HPA axis is mediated by corticotropin-releasing factor (CRF) expressing
neurons in the parvocellular region of the PVN which orchestrate the HPA axis by releasing CRF in the anterior pituitary which binds to CRF1 receptors to ultimately release glucocorticoids in the plasma (Aguilera, 1998; Aguilera and Liu, 2012; Hauger et al., 2009; Kasckow et al., 2003; Miklós and Kovács, 2002; Tasker and Dudek, 1993). Therefore, the activity of CRF neurosecretory neurons is predictive of an animal’s stress responsiveness. Previously, we utilized the Cre-lox system to generate male mice that express the red fluorescent protein, tdTomato specifically in CRF synthesizing neurons to demonstrate that relative to controls, mice rendered mildly hypertonic has decreased activation of CRF neurons in response to restraint (Smith et al., 2014a).

An understanding of the modulation of CRF neurons by paracrine release of oxytocin may reveal a novel mechanism by which oxytocin can integrate fluid homeostasis with decreased anxiety and stress to facilitate prosocial behaviors. In the present study, we peripherally injected male CRF-reporter mice with 2.0 M NaCl to drive the central levels of oxytocin and subsequently used patch-clamp electrophysiological methods in acute PVN slices to study the impact of oxytocin receptor on CRF microcircuitry. Utilizing the CRF-reporter mice to identify CRF neurons we report a tonic modulation of CRF expressing neurons in the PVN by oxytocin that was independent of local synaptic activity. Further, deletion of OTRs selectively from CRF neurons revealed that this inhibitory tone was dependent on OTRs expressed on CRF neurons. These studies advance our understanding of central oxytocin signaling and how it modulates CRF neurons through paracrine signaling.
Methods and Results

Oxytocin Exerts an Inhibitory Tone on CRF Neurons following Peripheral Salt Loading

We used CRF-reporter mice to record from tdTomato-expressing neurons in the medial parvocellular division of the PVN (Fig. 3-1 A-B). The expression of tdTomato protein in these mice is under the control of CRF gene and is relatively specific to CRF neurons (Chen et al., 2015; Smith et al., 2014a; Wamsteeker Cusulin et al., 2013). CRF positive neurons within the PVN (n=2) that expressed a transient outwardly rectifying potassium conductance (I_A) which is a hallmark of magnocellular neurons were excluded from further investigation (Fig. 3-1 C).

To study whether acute salt loading can influence the activity of CRF neurons through an OT-mediated paracrine signaling, we voltage-clamped the CRF neurons at -70 mV and bath applied the oxytocin receptor antagonist (OTR-A, 1µM).

Complementary to our previous results in rats (Frazier et al., 2013), CRF neurons from hypertonic mice (n=8) were found to be under the influence of a tonic oxytocinergic inhibition as evident from a downward shift in the current density (CD) in the presence of OTR-A (ΔCD = -1.63±0.66 pA/pF; one-sample t-test, P=0.005 vs. baseline; Fig 3-2 A-B). This downward shift in the current density was not apparent in neurons (n=8) from mice that received 0.15 M NaCl (ΔCD = -0.15±0.09 pA/pF; one-sample t-test, P=0.13 vs. baseline). The average shift in the current density was significantly different between the two groups (F=4.95416, P=0.04, one-way ANOVA). There was no evident change in membrane resistance (Rm) in the 2.0 M NaCl group following application of OTR-A when normalized to baseline (% change=87.46±6.3%; one-sample t-test, P=0.11 vs baseline).
In our recording conditions, the intrinsic membrane properties (membrane resistance and whole cell capacitance) and the frequency and amplitude of spontaneous postsynaptic currents (sPSC) did not differ between hypertonic and isotonic animals (Table 3-1).

**Oxytocinergic Inhibition of CRF Neurons is Independent of Local Synaptic Activity**

Accumulating evidence suggest that oxytocin can increase GABAergic signaling in different brain regions such as hippocampus and central amygdala by activating OTRs. Therefore, the tonic inhibitory current observed in CRF neurons could be a result of oxytocinergic modulation of GABAergic interneurons to increase ambient GABA levels in the PVN. CRF neurons are known to express high affinity extrasynaptic GABA-A receptors which can be tonically active and detect changes in basal GABA levels.

To test this hypothesis, we incubated the acute brain slices from hypertonic mice in a solution that contained antagonists for ionotrophic glutamate receptors (DNQX, 20 μM, APV, 40 μM) and GABA-A receptors (PTX, 100 μM). Additionally, the solution contained TTX, a voltage-gated sodium channel blocker (1 μM) to prevent action potential mediated release of neurotransmitters. After an incubation time of 1 h, CRF neurons (n=7) were patched using a K-gluconate based internal and voltage-clamped at -70 mV. All the above mentioned antagonists were present in the bath solution throughout the experiment. Under these conditions, bath application of OTR-A (1 μM) still caused a downward shift in current density (ΔCD = -0.77±0.24 pA/pF; one-sample t-test, P=0.02 vs. baseline; Fig 3-3 A-B). The average tonic inhibition observed in presence of all the synaptic blockers did not vary significantly from the one seen in Fig. 2A (F=1.34145, P=0.27, one-way ANOVA).
Direct Activation of GPCRs Expressed on CRF Neurons is Responsible for the Inhibitory Tone

An alternative explanation for the oxytocin-mediated tonic inhibition of CRF neurons is the direct activation of G-protein-coupled OTRs expressed postsynaptically on the CRF neurons. To test this hypothesis, we first substituted GTP for GDP-β-S (300µM) in the patch pipette. GDP-β-S is a non-hydrolysable analog of GDP that competitively inhibits G-protein activation by GTP and thus would effectively inactivate G-protein signaling in the recorded neuron. A potent and more selective OTR antagonist L-368,899 hydrochloride (1µM) was used instead of OTR-A ((Busnelli et al., 2013)). When CRF neurons from hypertonic mice (n=6) were patched with a GDP-β-S containing internal solution, the OT-induced inhibitory tone was eliminated across all the neurons recorded (ΔCD = -0.37±0.17 pA/pF; one-sample t-test, P=0.08 vs. baseline; Fig 3-3 C-D) even though we observed a robust inhibitory tone in CRF neurons (n=5) that were patched with a regular K-gluconate internal solution (ΔCD = -1.80±0.64 pA/pF; one-sample t-test, P=0.04 vs. baseline; Fig 3C-D). Comparing the two groups demonstrated a significant difference between the control and the GDP-β-S group (F=5.49994, P=0.04, one-way ANOVA) indicating the direct involvement of GPCRs expressed on the CRF neurons.

Selective Deletion of OTRs from CRF Neurons Eliminated the OT Mediated Tonic Inhibition following Peripheral Salt Loading

To further implicate the role of activation of OTRs expressed on CRF neurons, we generated animals that had OTRs deleted selectively from CRF neurons using the cre-lox system (see Chapter 2). Both wild type littermate controls and CRF-OTR KO mice were rendered hypertonic following 2.0 M NaCl injection and putative CRF neurosecretory neurons were identified (Fig. 3-4A) based on known electrophysiological
characteristics such as high membrane resistance and a lack of I\textsubscript{A} current following current injection in current clamp mode. Putative CRF neurons from control mice (n=8) exhibited an OTR-dependent tonic inhibition in the presence of L-368 (ΔCD = -0.91±0.37 pA/pF; one-sample t-test, P=0.04 vs. baseline; Fig 3-4B-C). This downward shift was abolished in neurons (n=6) from CRF-OTR KO mice (ΔCD = -0.23±0.14 pA/pF; one-sample t-test, P=0.16 vs. baseline). These results suggest that activation of OTRs expressed on the CRF neurons is crucial for the tonic inhibitory current observed following peripheral salt loading.

**Discussion**

Overall, our findings suggest that peripheral salt loading suppresses stress-responsiveness by engaging oxytocinergic signaling to create a tonic current that inhibits CRF neurons in the PVN. According to our model, 2.0 M NaCl injection possibly triggers release of central OT, which acts through paracrine signaling on CRF neurons in the PVN to blunt the HPA axis. These observations reveal a novel mechanism by which oxytocin may impact the CRF microcircuitry and curb stress-responsiveness of an animal.

Apart from OT, administration of hypertonic saline results in dynamic increase in both central and circulating levels of VP (Stricker and Sved, 2000; Stricker and Verbalis, 1986). However, the greatest rise in concentrations of VP is observed within 30 minutes following injection of 2.0 M NaCl (Stricker and Verbalis, 1986) while we waited 60 minutes following injection to prepare brain slices for patch clamp recordings. As such, I believe we were able to minimize the potential influence of dendritic VP on stress responsiveness. This is further supported by the fact that following salt loading...
the animals displayed an anxiolytic phenotype while VP is anxiogenic and plays an opposing role when compared to OT (Neumann and Landgraf, 2012).

Excitability of CRF-expressing parvocellular neurons in the PVN is predictor of an animal’s stress responsiveness, as such, it is not surprising that CRF neurons in the PVN receive a variety of heterogeneous afferent inputs from local hypothalamic and adjacent forebrain regions that help integrate stressful and homeostatic stimuli to modulate the sensitivity of the HPA axis. Therefore, inhibition of CRF-expressing neurons provides an effective way to reduce stress-related behavior. Previously, we utilized the Cre-lox system to generate male mice that express the red fluorescent protein, tdTomato, in cells that have the CRF gene to demonstrate that relative to controls, mice rendered mildly hypertonic had decreased HPA activation and a blunted rise in plasma CORT in response to restraint. Subsequently, hypertonic mice showed more c-Fos induction, which is a marker for neuronal activation, in oxytocin containing neurons with concomitant reduction in c-Fos expression in CRF expressing neurons; suggestive of a putative interaction between the two populations within the PVN (Smith et al., 2014a).

Knowing that decrease in CRF neuronal activation was associated with increase in OT activity in hypertonic male mice, we hypothesized an extrasynaptic, OT-mediated tonic inhibitory current resulting in a decreased neuronal gain. We did not find any changes in intrinsic properties following 2.0 M NaCl injection when compared to control mice. However, we found a significant tonic inhibitory current that was unmasked following addition of OTR antagonist only in hypertonic group. Therefore, we believe
that the reduction in plasma CORT levels following salt loading is due to increased inhibition of CRF neurons by dendritically released OT.

Given the importance of tonic inhibition of CRF neurons by extrasynaptic GABA-A receptors (Bali and Kovács, 2003; Cullinan et al., 2008; Ferguson and Latchford, 2000; Herman and Cullinan, 1997; Herman et al., 2003; Herman et al., 2004; Herman et al., 2002) and the known excitatory effects of OT on GABA signaling (Huber et al., 2005; Knobloch et al., 2012; Viviani et al., 2010) showing that the OT mediated tonic inhibition persisted following blockade of GABA-A receptors and fast ionotropic glutamate receptors along with action potential dependent synaptic activity was a significant finding. Further, the tone was abolished in the presence of GDP-β-s indicating the involvement of a GPCR signaling cascade in the CRF neurons. Our result is contrary to studies showing the inhibitory effect on CRF expression following icv administration of OT is through recruitment of GABAergic interneurons (Bülbül et al., 2011; Smith et al., 2016). This may be due to the involvement of both direct and indirect mechanisms through which OT can modulate the stress response and their activation might depend on the nature of the stressor. The data presented in this chapter support the hypothesis that OT exerts a direct effect on CRF neurons via OTRs since the OT mediated tonic current was lost in CRF selective OTR knockout mice.

In sum, data presented in this chapter is of paramount significance because it provides a mechanistic insight into how OT mediated paracrine signaling can lead to tonic suppression of CRF neurons in the PVN and might provide a better understanding of exaggerated stress responses in the future.
Figure 3-1. Identification and whole-cell patch-clamp recordings from CRF neurons in the PVN. A) For both the groups of mice, acute brain slices were prepared 1 h after receiving 0.1 ml of either 0.15 M NaCl or 2.0 M NaCl injections. A representative coronal section of PVN slice showing robust expression of CRF neurons. B) Using a combination of DIC and epifluorescence microscopy we can readily record from these neurons. Scale bars=10 µM. C) Representative current-clamp traces of CRF positive neurons in response to hyperpolarizing and depolarizing current injections (10 pA current step for 500 msec). CRF positive neurons that had a prominent IA current were excluded from the study.
Table 3-1. Peripheral salt loading does not affect basic intrinsic properties of CRF neurons.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Size (n)</th>
<th>Group</th>
<th>Mean ± SEM</th>
<th>F-value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rm (MΩ)</td>
<td>7</td>
<td>0.15 M</td>
<td>741.90±136.33</td>
<td>0.2403</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.0 M</td>
<td>665.92±87.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cm (pF)</td>
<td>8</td>
<td>0.15 M</td>
<td>10.29±2.29</td>
<td>0.6771</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.0 M</td>
<td>8.15±1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sPSC freq (Hz)</td>
<td>8</td>
<td>0.15 M</td>
<td>4.81±2.21</td>
<td>1.066</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.0 M</td>
<td>7.92±1.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sPSC amp (pA)</td>
<td>8</td>
<td>0.15 M</td>
<td>15.17±1.69</td>
<td>0.010</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.0 M</td>
<td>15.39±1.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as means ± SEM. Values were recorded from CRF positive neurons in the PVN using a K-gluconate based internal solution. Membrane resistance (Rm) and whole cell capacitance (Cm) were calculated from whole-cell capacitive transients observed in response to a voltage step (-10 mV in amplitude and 50 msec in duration) delivered to neurons voltage clamped at -70 mV. For spontaneous frequency and amplitude data, the data was filtered and event parameters were chosen to minimize RMS noise (see Methods). n represents the number of cells in each group.
Figure 3-2. Oxytocin receptor-mediated tonic inhibition of CRF neurons in the PVN following 2.0 M NaCl injection. A) Bath application of 1 µM OTR antagonist (OTR-A) caused a downward shift in the current density (pA/pF) in CRF neurons only in hypertonic group. B) Summary data of the mean current density showing a scatter plot for each recorded cell (see results). Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. * (P<0.05).
Figure 3-3. Oxytocinergic inhibition of CRF neurons is independent of synaptic activity. A) Bath application of 1 µM OTR-A at 5 min caused a downward shift in current density (pA/pF) in CRF neurons from hypertonic mice in the presence of ionotropic glutamate blockers (DNQX 25µM, APV 5µM), GABA-A (PTX, 100µM) antagonists and voltage-gated sodium channel blocker (TTX, 1µM). B) Summary data of the mean current density showing a scatter plot for each recorded cell (see results). Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. * (P<0.05).
Figure 3-4. Oxytocinergic inhibition of CRF neurons is dependent on direct activation of GPCRs on CRF neurons. A) Bath application of 1 µM of a different OTR antagonist (L-368) at 5 min significantly reduced the shift in current density (pA/pF) in CRF neurons from hypertonic mice with GDP-β-s (300 µM) in the recording pipette compared to neurons recorded with regular K-glu internal. B) Summary data of the mean current density showing a scatter plot for each recorded cell (see results). * (P<0.05).
Figure 3-5. Cre-dependent deletion of OTRs on CRF neurons abolish the tonic oxytocinergic inhibition following peripheral salt loading. A) Representative current-clamp traces of putative CRF neurosecretory neuron in response to hyperpolarizing and depolarizing current injections (10 pA current step for 500 msec, square pulse 500ms). B) Bath application of 1 µM of L-368 at 5 min caused a downward shift in current density (pA/pF) in CRF neurons from hypertonic mice in the littermate control group that was abolished in neurons from CRF-OTR KO mice. C) Summary data of the mean current density showing a scatter plot for each recorded cell (see results). Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. * (P<0.05).
Figure 3-6. Proposed model of OT mediated tonic inhibition of CRF neurons following peripheral salt loading.
CHAPTER 4
TONIC INHIBITION OF PREADUONOMIC NEURONS IN THE MOUSE PVN: CROSSTALK BETWEEN OXYTOCIN AND GABA-A RECEPTORS

Introduction

Acute fluctuations in the fluid/electrolyte balance are detected by osmosensitive regions of the forebrain which then recruit specific neurons in the paraventricular nucleus of the hypothalamus (PVN) to restore homeostasis (Antunes-Rodrigues et al., 2004; Fry and Ferguson, 2007; Shi et al., 2008; Smith and Ferguson, 2010; Toney et al., 2003; Toney and Stocker, 2010). Both acute and chronic hyperosmotic stimuli have been associated with an exaggerated sympathetic tone that can contribute to salt-sensitive cardiovascular diseases (Biancardi et al., 2010; Chen et al., 2010; Guyenet, 2006; Kc and Dick, 2010; Stern, 2015; Toney and Stocker, 2010). The preautonomic neurons of the PVN send long projections to distinct regions of the brainstem and the spinal cord to regulate the sympathetic output and the autonomic responses to stress (Hjemdahl, 2011; Pyner, 2009; Steptoe, 2012; Strazzullo et al., 2009). Interestingly, OT, an anti-stress neuropeptide is also synthesized in the PVN and activation of OTRs can be cardio-protective (Gutkowska and Jankowski, 2012; Michelini et al., 2003; Morris et al., 1995), however, whether there is a possible crosstalk between OT and preautonomic neurons within the PVN, has not yet been explored.

Recent studies by various groups have raised the possibility of somatodendritically released neuropeptides such as vasopressin and Neuropeptide Y in regulating the excitability of preautonomic neurons resulting in altered sympathetic tone (Cassaglia et al., 2014; Son et al., 2013). Following increase in hypertonicity due to an injection of 2.0 M NaCl, there is a sustained release of both VP and OT (F. Bergquist, 2008; Leng and Ludwig, 2008; Ludwig, 1998a; Ludwig and Pittman, 2003; Ludwig et al.,
2002; Tobin et al., 2011) leading to the possibility of OT impacting the neuronal activity of preautonomic neurons in the PVN through volume transmission.

Previous studies from our group have shown that subcutaneous injection of 2.0 M NaCl can generate a tonic inhibitory current on parvocellular CRF synthesizing neurosecretory neurons via activation of OTRs (Frazier et al., 2013; Krause et al., 2011; Smith et al., 2014b), thus in the present study, we utilized a similar acute salt loading paradigm to evaluate whether central release of OT following hypernatremia can also exert a tonic influence on preautonomic neurons.

Putative preautonomic neurons were identified based on the presence of low threshold spiking (LTS) and high input resistance (Dudek, 1991; Lee et al., 2008b; Luther et al., 2002; Luther et al., 2000; Luther and Tasker, 2000; Stern, 2001) and whole-cell patch clamp recordings were performed to determine the effect of OT-mediated signaling on these neurons. Bath application of a selective OTR-antagonist (L-368, 1µM) revealed a prominent OTR-mediated inhibitory tone on preautonomic neurons in the isotonic animals that was lost in the hypertonic group. Intriguingly, this tonic current was dependent on both GABA-A receptor and adenosine 1 (A1) receptor mediated signaling. Thus, we have compelling data suggesting the involvement of basal levels of OT in tonic control of putative preautonomic neurons in the PVN through a complex polysynaptic and/or glial-neuronal connectivity which is lost after peripheral salt loading.

**Methods and Results**

All the experiments in this chapter were conducted on male C57/BL wild type mice and animals received either 0.15 M NaCl or 2.0 M NaCl injection subcutaneously
following which the 2.0 M group were water deprived for 1 h. Slices were prepared and whole cell patch clamp recordings were conducted as described in Chapter 2.

**Oxytocin Exerted an Inhibitory Tone on Preautonomic Neurons that was Lost following Peripheral Salt Loading**

Preautonomic neurons represent a heterogeneous neuronal population located mostly in the ventral parvocellular and posterior parvocellular sub regions of PVN (Stern, 2001). To study whether acute salt loading can influence the activity of preautonomic neurons, putative preautonomic neurons were identified (Fig 4-1 A) based on their location and known electrophysiological parameters: high input resistance and expression of low threshold spike (LTS). When voltage clamped at -70 mV, neurons from the isotonic group had an average input resistance of $1083.53 \pm 215.60 \, \text{M}\Omega$ (n=8) that did not differ from the hypertonic group (n=5; $1506.13 \pm 290.66 \, \text{M}\Omega$; $F=1.408$; $P=0.26$). The oxytocin receptor antagonist (L-368, 1µM) was bath applied following a baseline period of 5 min. Preautonomic neurons recorded from the isotonic mice (n=8) were found to be under the influence of a tonic oxytocinergic inhibition as evident from a downward shift in the current density (CD) in the presence of L-368 ($\Delta\text{CD} = -1.21\pm0.34 \, \text{pA/pF}$; one-sample t-test, $P=0.009$ vs. baseline; Fig 4-1 B-C). This downward shift in the current density was not apparent in neurons (n=5) from mice that received 2.0 M NaCl ($\Delta\text{CD} = -0.05\pm0.19 \, \text{pA/pF}$; one-sample t-test, $P=0.81$ vs. baseline). The average shift in the current density was significantly different between the two groups ($F=6.2230$, $P=0.03$, one-way ANOVA).

**Oxytocinergic Inhibition of Preautonomic Neurons is Dependent on GABAergic Signaling**

RVLM projecting preautonomic neurons are known to be under tonic GABAergic inhibition and dysregulation of GABA signaling has been implicated in the increased
discharges from preautonomic neurons resulting in hypertension and myocardial
infarction (Biancardi et al., 2010; Chen and Toney, 2009; Park et al., 2009; Park et al.,
2007; Potapenko et al., 2013; Sonner and Stern, 2007). Therefore, the tonic inhibitory
current observed in putative preautonomic neurons could be a result of oxytocinergic
modulation of GABAergic interneurons to increase ambient GABA levels in the PVN.

To test this hypothesis, we incubated the acute brain slices from isotonic mice in
a solution that contained antagonists for ionotropic glutamate receptors (DNQX, 20 µM,
APV, 40 µM) and GABA-A receptors (PTX, 100 µM) and GABA-B receptors (CGP, 10
µM). After an incubation time of 1 h, preautonomic neurons (n=6) were patched using a
K-gluconate based internal and voltage-clamped at -70 mV. All the above mentioned
antagonists were present in the bath solution throughout the experiment. Under these
conditions, bath application of L-368 (1 µM) was elicited a very small inhibitory current
as evident by a downward shift in current density (\(\Delta CD = -0.16\pm0.05\) pA/pF; one-sample
t-test, \(P=0.02\) vs. baseline; Fig 4-2 A). This was significantly smaller when compared to
the tonic current observed without any blockers (Fig. 4-2 B \(F=6.7159, P=0.02\), one-way
ANOVA).

**Direct Activation of Extrasynaptic GABA-A Receptors on Preautonomic Neurons
is not Required for the Inhibitory Tone**

The occlusion of the oxytocinergic inhibition of preautonomic neurons by blockers
of GABAergic signaling may be due to an OT-dependent enhancement of GABAergic
inputs to these neurons. Both extrasynaptic GABA-A receptors and GABA-B receptors
are expressed by preautonomic neurons in the PVN and can be involved in the
generation of a tonic inhibitory current (Chen and Pan, 2006; Han et al., 2010; Li et al.,
2008; Page et al., 2011; Pandit et al., 2015). To test the hypothesis that GABA-A
receptor mediated tonic current is responsible for the observed oxytocinergic effect on preautonomic neurons, we substituted the regular K-glu internal with a high chloride internal solution in the patch pipette. This manipulation would effectively increase the intracellular chloride concentration resulting in extrusion of chloride following the opening of GABA-A receptor coupled chloride channel making GABA excitatory instead of inhibitory. We incubated the acute brain slices from isotonic mice in a solution that contained antagonists for ionotropic glutamate receptors (DNQX, 20 µM, APV, 40 µM) and GABA-B receptors (CGP, 10 µM). When preautonomic neurons (n=5) were patched with a high chloride containing internal solution, we still observed an OT-induced robust inhibitory tone as indicated by a downward deflection of the holding current (ΔCD = -1.07±0.35 pA/pF; one-sample t-test, P=0.04 vs. baseline; Fig 4-3 A-B). The inability of the antagonist to reverse the polarity of the chloride gradient suggests an involvement of a polysynaptic network independent of the extrasynaptic GABA-A receptor. Additionally, since we observed the tonic current in the presence of a GABA-B blocker, it is indicative of the tonic current being independent of GABA-B receptors.

Blockade of Adenosine Type 1 Receptors Abolished the OT Mediated Tonic Inhibition of Preautonomic Neurons

To further investigate the plausible mechanism of OT mediated inhibition of preautonomic neurons, we pre-incubated the acute slices in a selective adenosine receptor subtype 1 (A1-R) antagonist (DPCPX, 1 µM). Adenosine, which is a known gliotransmitter and a degradation product of ATP can modulate the sympathetic tone in the PVN via activation of A1-Rs on both pre and postsynaptic terminals on preautonomic neurons (Han et al., 2011; Li et al., 2010). In the presence of DPCPX, the OT-mediated inhibition was abolished (ΔCD = 0.09±0.18 pA/pF; one-sample t-test,
P=0.65 vs. baseline) implicating a possible crosstalk between OTR and A1-R (Fig. 4-4 A-B).

**Discussion**

Collectively, our findings suggest that under isotonic conditions, basal levels of OT suppress sympathetic tone by engaging oxytocinergic signaling to create a tonic current that inhibits preautonomic-like neurons in the PVN. Intriguingly, 2.0 M NaCl injection which is known to trigger release of central OT, fails to maintain this OT-mediated tonic inhibition.

Cardiovascular homeostasis involves the coordinated regulation of sympathetic tone to maintain blood pressure and blood volume (Guyenet, 2006). Along with PVN, RVLM represent the two dominant regions essential for maintaining tonic regulation of blood pressure (Coote, 2005; Toney et al., 2003). The preautonomic neurons project to autonomic nuclei in brainstem and spinal cord and are responsible for the activation of sympathetic nerve tone (Sawchenko and Swanson, 1982). A subset of descending PVN neurons project to RVLM and spinal cord for control of blood pressure (Coote, 2005). Thus, modulation of the excitability of preautonomic parvocellular neurons in the PVN can alter the sympathetic tone. Challenges to central osmotic balance either following dehydration or excess dietary salt intake can alter the excitability of central sympathetic networks (Bardgett, 2014; Holbein and Toney, 2015; Stocker et al., 2010; Toney et al., 2003; Toney and Stocker, 2010). A dysregulated sympathetic tone is also associated with chronic pathophysiological states like salt-sensitive hypertension and myocardial infarction (Chen et al., 2010; Li and Pan, 2007; Li et al., 2014). Although there is a wide range of information available on how neuropeptides such as Angiotensin-II (Braga et al., 2011; Cato and Toney, 2005; Chen et al., 2010; Claflin and Grobe, 2015; De
Wardener, 2001; Ferguson, 2009) or VP (Son et al., 2013) modify the firing discharge of preautonomic neurons not much is known about the crosstalk between OT and preautonomic neurons within the PVN.

Our study provides evidence that basal levels of OT can tonically inhibit preautonomic neurons. PVN in general (Herman et al., 2004; Herman et al., 2002; Ulrich-Lai and Herman, 2009; Ziegler and Herman, 2002) and preautonomic neurons in particular are both under a strong tonic GABAergic influence (Biancardi et al., 2010; Park et al., 2009; Park et al., 2007; Stern et al., 2003). Therefore, OT can indirectly modulate preautonomic neurons by targeting the GABA neurons in the peri-PVN region. The data presented here show that the tonic inhibition of preautonomic neurons indeed require the activation of GABA-A receptors.

**Neuronal-glial interaction: A possible mechanism for oxytocinergic inhibition of preautonomic neurons.** Surprisingly, even though OT recruits the GABA-A receptor, we show the lack of direct involvement of extrasynaptic GABA-A receptors. Using a high chloride internal in the recording pipette it is possible to alter the chloride homeostasis inside a neuron in such a way that activation of GABA-A receptor would lead to depolarization of the cell. Since, alteration of chloride gradient did not alter the polarity of the OTR-mediated tonic current we think this alludes to a complex, polysynaptic network with plausible involvement of neuronal-glia interactions.

Prior studies have implicated both astrocytes and adenosine, a known gliotransmitter in modulation of neuronal activity and GABAergic-astrocyte signaling as a lesser known form of cell communication (Ferreira-Neto et al., 2015; Gordon et al., 2005; Haam et al., 2014; Halassa et al., 2009; Hines and Haydon, 2014; Li et al., 2010;
Losi et al., 2014; Zhang et al., 2003). The tripartite system has extended the functions of astrocytes to short term and long term modulation of synaptic transmission. Previously, electrophysiological experiments performed on hippocampal slices revealed that astrocytes expressed GABA-A receptors similar to neurons but activation of astrocytic GABA-A receptors leads to depolarization due to larger intracellular chloride concentration in astrocytes (Fraser et al., 1994; MacVicar et al., 1989). One of the outcomes of GABAergic activation of astrocytes is increase in an astrocytic calcium to cause release of gliotransmitters.

Adenosine is a well-known gliotransmitter that can act both presynaptically and postsynaptically to modulate synaptic transmission (Hines and Haydon, 2014). A1-R is a GPCR that has been shown to inhibit the RVLM-projecting preautonomic neurons in the PVN (Han et al., 2011; Li et al., 2010). Figure 4-4 supports the dependence of OT-mediated inhibition of preautonomic neurons on A1-receptor mediated signaling since in the presence of an A1-R antagonist, OT failed to tonically inhibit preautonomic neurons. Further work is required to distinguish between the pre- and postsynaptic effects of A1-R. Also, utilizing calcium imaging technique, it is possible to quantify the responsiveness of astrocytes to GABA. To further validate the electrophysiological findings presented in this chapter, a combination of in situ hybridization and immunohistochemistry techniques can be used to determine localization of GABA-A receptors on astrocytes surrounding the PVN region and also to look at astrogliosis in response to peripheral salt loading. Figure 4-5 outlines a plausible mechanism that ties all the pieces of evidence to provide an insight into a novel mechanism by which oxytocin may impact the sympathetic tone.
Loss of OT-mediated inhibition of preautonomic neurons following hyperosmotic stress: To cope with dehydration, there is a compensatory increase in the sympathoexcitation of preautonomic neurons (Toney and Stocker, 2010). Increase in sympathoexcitatory response can either result from increased excitatory drive on to preautonomic neurons or via disinhibition of tonic GABAergic inputs. Interestingly, the same hyperosmotic stimulus can cause dendritic release of both VP and OT with the ability to impact the activity of neighboring preautonomic neurons. Work from Stern lab elegantly demonstrated an interpopulation crosstalk between dendritic VP and RVLM-projecting presympathetic neurons mediated through V1a receptors (Son et al., 2013). Combining 2-photon calcium imaging with uncaging of glutamate, Stern et al. demonstrated that activation of V1a-mediated calcium dependent non-selective cation channel causes an increase in firing activity of RVLM-projecting neurons. In contrast, our experimental data suggest that following 2.0 M NaCl injection, the OT-mediated tonic inhibition of preautonomic neurons seemed to be lost. It is an interesting observation as salt loading is known to cause somatodendritic release of OT in the PVN (Ludwig and Leng, 2006) which should increase the ambient OT concentrations in the PVN when compared to isotonic group. One plausible explanation could be astrogliosis following 2.0 M NaCl that causes retraction of the astrocytic processes surrounding the preautonomic neurons resulting in reduced accessibility of gliotransmitters such as adenosine to bind to A1-Rs expressed on preautonomic neurons.

Compared to the OT-mediated tonic inhibition of CRF neurons following salt loading as demonstrated in Chapter 2 the loss of tonic inhibition in preautonomic neurons allude to the complexities of paracrine signaling within the same nucleus.
Preliminary results from our laboratory suggest that OTRs are not expressed on RVLM-projecting preautonomic neurons unlike CRF neurons where they are responsible for the tonic inhibitory current. It is possible that under isotonic conditions synaptic levels of OT modulate the preautonomic neurons while following increase in hypertonicity there is an inhibition of synaptic OT release but increase in somatodendritic OT. This correlates with decreased tonic inhibition of preautonomic neurons while increased inhibition of CRF neurons. While moderately complex, this hypothesis is bolstered by findings in the literature pointing to the existence of OT-positive parvocellular neurons in the PVN (Atasoy et al., 2012) capable of synaptic release of OT outside of the PVN. Also, alpha melanocyte-stimulating hormone (a MC4 agonist) has been shown to differentially regulate synaptic versus dendritic release of OT from magnocellular neurons (Sabatier et al., 2003) resulting in inhibition of synaptic release but potentiation of dendritic release of OT.

In conclusion, the data presented in this chapter demonstrate a hitherto unknown modulation of preautonomic neurons by endogenous levels of OT within the PVN that might have implications in control of sympathetic activity and could possibly be a side-effect of OT therapy. Future experiments are warranted to test this hypothesis and parse out the exact mechanism of oxytocinergic inhibition of preautonomic neurons.
Figure 4-1. Oxytocin receptor-mediated tonic inhibition of preautonomic neurons in the PVN under basal conditions. A) Representative trace of a preautonomic-like neuron following application of a depolarizing current step in the current clamp mode. B) Bath application of 1 µM OTR antagonist (L-368) caused a downward shift in the current density (pA/pF) in preautonomic neurons. This OT-mediated tonic inhibition is absent in neurons from hypertonic mice. C) Summary data of the mean current density showing a scatter plot for each recorded cell (see results). Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. * (P<0.05).
Figure 4-2. OT tonically inhibits preautonomic neurons via GABAergic signaling. 
A) Bath application of 1 μM OTR antagonist (L-368) at 5 min reduced the downward shift in current density (pA/pF) from isotonic mice in the presence of ionotropic glutamate blockers (DNQX 25μM, APV 5μM), GABA-A (PTX, 100μM) and GABA-B (CGP, 10 μM) antagonists. 
B) Summary data of the mean current density showing a scatter plot for each recorded cell (see results). Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. * (P<0.05).
Figure 4-3. Tonic inhibition of preautonomic neurons is independent of direct activation of GABA-A receptors. A) Bath application of 1 µM L-368 at 5 min elicited a downward shift in current density (pA/pF) from isotonic mice in the presence of ionotropic glutamate blockers (DNQX 25µM, APV 5µM) and GABA-B (CGP, 10 µM) antagonists when recorded from preautonomic neurons using a high chloride internal solution. B) Summary data of the mean current density showing a scatter plot for each recorded cell (see results). Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. * (P<0.05).
Figure 4-4. Oxytocinergic inhibition of preautonomic neurons is dependent on A1-receptor activity. A) Bath application of 1 µM L-368 at 5 min abolished the downward shift in current density (pA/pF) from isotonic mice in the presence of A1-R antagonist (DPCPX, 1µM). B) Summary data of the mean current density showing a scatter plot for each recorded cell (see results). Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. * (P<0.05).
Figure 4-5. Proposed model of OT mediated tonic inhibition of preautonomic-like neurons in the PVN involving neuron-glia crosstalk.
CHAPTER 5
OTR-DEPENDENT TONIC MODULATION OF CRF NEURONS IN THE MOUSE CeA

Introduction

The amygdaloidal complex is a functionally and anatomically heterogeneous structure with a critical role in regulating fear-related behaviors (Allsop et al., 2014; Duvarci and Pare, 2014; Ehrlich et al., 2009; Gafford and Ressler; Pare and Duvarci, 2012). The basolateral amygdala is a site of convergence for sensory inputs related to danger that is relayed to the central amygdala (CeA). Neurons in the CeA integrate an array of inputs to coordinate autonomic and behavioral components of fear responses. CeA is also the major output site of amygdala and sends out projections to distinct brain structures including hypothalamus, brainstem, BNST and periaqueductal gray (Cai et al., 2014; Ciocchi et al., 2010; Haubensak et al., 2010; Phelps and LeDoux, 2005).

CeA in itself is composed of several distinct subnuclei (McDonald, 1982) with extensive local connectivity (Gafford and Ressler; Lammel et al., 2014). CeA is comprised mostly of GABAergic neurons (Paré and Smith, 1993; Sun and Cassell, 1993) that can also co-express various peptides ranging from somatostatin, neurotensin and CRF (Cassell and Gray, 1989). In fact, CeA is a major site of extrahypothalamic expression of CRF. CRF has an anxiogenic effect and is a well-known modulator of the stress response both via activation of the HPA axis in the PVN and via modulation of synaptic transmission in limbic regions associated with stress and anxiety such as BNST, hippocampus and hindbrain structures (for review, see (Gray, 1993). Stress increases CRF mRNA in the CeA and alterations in the CRF system in the CeA have been associated with anxiety-like behavior (Bale, 2005). Therefore, a better
understanding of neuromodulators that affect the activity of CRF neurons may provide a better understanding of the pathophysiology of stress and anxiety related disorders.

Several studies have shown that increases in central OT signaling in the amygdala is associated with anxiolytic phenotypes. Indeed, recently Knobloch et al., showed using in vitro and in vivo optogenetics that endogenous OT release activated a local GABAergic circuit within the central amygdala and decreased freezing responses in female rats (Knobloch et al., 2012). Also, in the lateral central amygdala, local application of OT selectively activates a subpopulation of GABAergic interneurons thereby attenuating behavioral responses to fear (Huber et al., 2005; Terenzi and Ingram, 2005; Viviani et al., 2010).

There is an impressive amount of evidence suggesting the role of OT in suppressing stress response via modulation of CRF system within the PVN however, the exact mechanisms underlying the interaction between the two opposing systems is not clearly known. OT released within the PVN or SON can reach CeA either through volume transmission or synaptic release. The present study investigated the mechanisms by which endogenous levels of OT affect CRF neuron activity in the CeA using whole cell electrophysiology. We utilized an acute salt loading paradigm to activate OT producing neurons in the PVN and SON of hypothalamus and using a CRF-reporter animal line, recorded from CRF neurons in the CeA. Here, we report that basal levels of OT generate a tonic inhibitory conductance on CRF neurons from the CeA. Bath application of L-368, a selective OTR antagonist revealed this tonic current that is independent of both GABA signaling and direct activation of a GPCR expressed on CRF neurons. Following 2.0 M NaCl injection we observed a tonic excitation of a subset
of CRF neurons. The results demonstrate that OT can influence the activity of CRF neurons in the CeA and may have implications in physiological and pathophysiological states.

**Methods and Results**

**Identification and Characterization of CRF Neurons in the CeA**

To study whether endogenous OT can influence the activity of CRF neurons in the CeA, we injected male CRF-reporter mice with 0.15 M NaCl and 1 h later 300 μm coronal sections of CeA were prepared. CRF neurons were identified as the neurons that expressed the tdTomato protein in the CeA (Fig. 5-1 A-B). The expression of tdTomato protein in these mice is under the control of CRF gene and is relatively specific to CRF neurons (Chen et al., 2015). As reported in literature, CRF neurons were confined mostly to the lateral division of CeA (Asan et al., 2005).

CRF positive neurons within the CeA exhibited a regular spiking phenotype (Schiess et al., 1999) (Fig. 5-1 C) and displayed varying degrees of Ih current when voltage clamped at -100mV.

**Basal Levels of OT Exert an Inhibitory Tone on CRF Neurons**

CRF positive neurons in the CeA were voltage-clamped at -70 mV and holding current was monitored throughout the experiment. Following a baseline period of 5 mins the oxytocin receptor antagonist (L-368, 1µM) was bath applied. CRF neurons (n=7) were found to be under the influence of a tonic oxytocinergic inhibition as evident from a downward shift in the current density (CD) in the presence of L-368 ($\Delta$CD = -0.17±0.06 pA/pF; one-sample t-test, P=0.03 vs. baseline; Fig 5-2 A-B) with one outlier cell showing OT-mediated tonic excitation. The change in tonic current was also associated with a decrease in membrane resistance (Rm; n=8) following application of L-368 when
normalized to baseline (% change=72.66±3.0%; one-sample t-test, P=0.00001, baseline=1).

**Oxytocinergic Modulation of CRF Neurons is Independent of GABA and Glutamate Activity**

Accumulating evidence suggest that oxytocin can increase GABAergic signaling in CeA by activating OTRs. CRF neurons are known to express high affinity extrasynaptic GABA-A receptors which can be tonically active and detect changes in basal GABA levels. Therefore, the tonic inhibitory current observed in CRF neurons could be a result of oxytocinergic modulation of GABAergic interneurons to increase ambient GABA levels in the CeA.

To test this hypothesis, we incubated the acute brain slices in a solution that contained antagonists for ionotropic glutamate receptors (DNQX, 20 µM, APV, 40 µM) and GABA-A and GABA-B receptors (PTX, 100 µM, CGP, 10 µM). After an incubation time of 1 h, CRF neurons (n=7) were patched using a K-glucurate based internal solution and voltage-clamped at -70 mV. All the above mentioned antagonists were present in the bath solution throughout the experiment. Under these conditions, bath application of L-368 (1 µM) still elicited tonic current in 6 out of 7 cells (ΔCD = -0.21±0.06 pA/pF; one-sample t-test, P=0.02 vs. baseline; Fig 5-3 A-B) with one outlier showing the opposite shift in current density. The average tonic modulation observed in presence of all the synaptic blockers did not vary significantly when compared to the control (F=0.28104, P=0.6, one-way ANOVA).

**Direct Activation of GPCRs Expressed on CRF Neurons is not Required for the Inhibitory Tone**

An alternative explanation for the oxytocin-mediated tonic modulation of CRF neurons is a direct activation of a G-protein-coupled receptor expressed on the CRF
neurons. To test this hypothesis, we first substituted GTP for GDP-β-S (300µM) in the patch pipette. GDP-β-S is a non-hydrolysable analog of GDP that competitively inhibits G-protein activation by GTP and thus would effectively inactivate G-protein signaling in the recorded neuron. When CRF neurons from isotonic mice (n=5) were patched with a GDP-β-S containing internal solution, the OT-induced tonic current not only persisted (ΔCD = -0.57±0.18 pA/pF; one-sample t-test, P=0.03 vs. baseline; Fig 5-4 A-B), it was significantly bigger in magnitude when compared to the tonic inhibitory current observed in the control group (F=5.53, P=0.04, One-way ANOVA).

**A Subset of CRF Neurons are Tonically Excited by OT Following Peripheral Salt Loading**

Next we asked whether 2.0 M NaCl injection, a stimulus known to drive central release of OT can alter the OT-mediated tonic inhibition of CRF neurons observed in basal conditions. Fig. 5-5 shows no change in the neuronal gain function following salt loading when neurons were held at -70 mV by and 50 pA incremental steps were applied in both negative and positive directions of -70 mV.

Even though peripheral salt loading did not affect the passive properties of CRF neurons, it resulted in a bi-directional modulation of CRF neurons by OT with a subset of neurons (n=4 out of 8) exhibiting an OT-induced robust excitatory tone as indicated by an upward deflection of the holding current (ΔCD =0.34±0.04 pA/pF; one-sample t-test, P=0.004 vs. baseline; Fig 5-6 A-B). The other 4 cells demonstrated OT-mediated tonic inhibition (ΔCD =-0.17±0.07 pA/pF; one-sample t-test, P=0.08 vs. baseline; Fig 5-6 A-B). This bimodal modulation of CRF neurons by OT persisted even in the presence of fast ionotropic glutamate receptor blockers (DNQX, 20 µM, APV, 40 µM), GABA-A and GABA-B receptor blockers (PTX, 100 µM, CGP, 10 µM) as shown in Fig. 5-7 (A-B) with
n=5 cells showing tonic inhibition (ΔCD = -0.22±0.07 pA/pF; one-sample t-test, P=0.03 vs. baseline) and n=4 cells displayed an OT-mediated tonic excitation (ΔCD = 0.25±0.08 pA/pF; one-sample t-test, P=0.05 vs. baseline).

**Discussion**

CeA is an important part of the limbic circuitry and is deeply involved with modulation of the fear response (Allsop et al., 2014; Anthony et al., 2014; Botta et al., 2015; Ciocchi et al., 2010; Duvarci and Pare, 2014; Ehrlich et al., 2009; Gafford and Ressler; Paré, 2003; Pare and Duvarci, 2012; Sah et al., 2003). CeA consists of ~90% GABAergic neurons which can co-release other neuropeptides such as CRF. The CRF system in the CeA has been implicated in various conditions from addiction to memory consolidation, stress responsiveness and alertness (Beckerman et al., 2013; Gilpin et al.; Gray et al., 2015; Herman et al., 2013; Reyes et al., 2011; Silberman and Winder, 2013). In the recent years, the anxiolytic effects of oxytocin and its role in modulating the activity of amygdala has garnered lots of attention (Ciocchi et al., 2010; Daniele Viviani, 2011; Huber et al., 2005; Knobloch et al., 2012; Silberman and Winder, 2013; Viviani et al., 2010). Here, we provide evidence that centrally released OT under basal conditions and in response to peripheral salt loading can tonically modulate the CRF neurons in the CeA. Our findings suggest that basal levels of oxytocin suppress CRF neurons in a GABA- and GPCR-independent manner whereas following salt loading a higher subset of CRF neurons seemed to be excited by OT. Further work is needed to figure out the exact mechanism responsible for the bimodal effects of OT on CRF neurons. There is a trend supporting the idea that salt loading might influence the effect of OT on CRF neurons by increasing the probability of tonic excitatory current when compared to the small number of CRF neurons that respond in an excitative manner in
the isotonic group. Implementing a Fisher’s exact test (2X2 contingency table) to evaluate the probability of getting higher number of cells that were excited by OT following salt loading when compared to isotonic conditions we observed a trend indicating that we need to increase our sample size to be conclusive.

Published work from other groups indicate synaptic projections from PVN OT neurons to the CeA that can release OT when stimulated (Knobloch et al., 2012). We hypothesize the effects of OT seen in our experimental condition also relies on synaptic release of OT but cannot conclusively rule out volume diffusion of OT from either PVN or SON as a plausible source of OT in the CeA. Further investigation is warranted to determine whether the tonic modulation of CRF neurons relies on synaptic OT. We can test this by incubating the slices in TTX to block action potential mediated release.

The variable effect of OT on CRF neurons can be accounted for by the fact that CRF neurons represent a heterogeneous population. CRF neurons are mostly confined to lateral division of CeA. Within lateral division of CeA, PKC-δ neurons play key roles in coding responses to fear conditioning. A high proportion of PKC-δ+ neurons express OTRs (~64%) while a small proportion of CRF neurons (~16.9%) co-express PKC-δ (Haubensak et al., 2010). There’s a possibility of overlap between the groups such that CRF neurons expressing OTRs are the ones getting excited by OT whereas CRF neurons without OTRs might be inhibited by OT. Also, CRF neurons within CeA comprise of both projection neurons and interneurons as such a combination of optogenetics and circuit tracing may be a way forward for selectively targeting different population of CRF neurons to study the interaction between OT and CRF system.
The observation that tonic inhibition of CRF neurons by OT not only persisted in the presence of GDP-β-s but got bigger in magnitude may allude to the possibility of a complex mechanism wherein both the excitatory and the inhibitory factors contribute simultaneously to elicit the effects of OT on CRF neurons. Under basal condition OT-mediated inhibition might be predominant but following acute salt loading the balance might shift in the favor of excitation. More work is needed to critically assess the underlying mechanism for the dual response of OT.

Generally, OT system is considered anxiolytic whereas CRF system is considered anxiogenic and they seem to counter-balance each other. The functional relevance of tonic modulation of CRF neurons in the CeA by OT is still an unanswered question. Previously, we have shown that 2.0 M NaCl injection is associated with animals spending more time in the open arm in the elevated plus maze paradigm indicative of an anxiolytic phenotype (Frazier et al., 2013; Smith et al., 2014b). Whether tonic inhibition of CRF neurons by OT plays any role in this effect is something that is yet to be determined. Overall, the data presented in Chapter 5 show the involvement of OT signaling in the modulation of CRF neurons in the CeA and imply a complex role for OT signaling in amygdala.
Figure 5-1. Identification and whole-cell patch-clamp recordings from CRF positive neurons in the CeA. A) A representative coronal section of CeA slice showing robust expression of CRF neurons. B) Using a combination of DIC and epifluorescence microscopy we can readily record from these neurons. Scale bars=10 µM. (C) Representative current-clamp traces of CRF positive neurons in response to hyperpolarizing and depolarizing current injections (50 pA current step for 500 msec).
Figure 5-2. Oxytocin receptor-mediated tonic modulation of CRF neurons in the CeA following 0.15 M NaCl injection. (A) Bath application of 1 µM OTR antagonist (L-368) caused a downward shift in the current density (pA/pF) in CRF neurons with the exception of one cell that showed an upward shift in current density. (B) Summary data showing a scatter plot for each recorded cell. The mean delta current density between 10 and 12 mins into the experiment was compared to the baseline. Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. Dash box represents the outlier cell.
Oxytocinergic modulation of CRF neurons is independent of GABAergic and glutamatergic activity. A) Bath application of 1 µM OTR antagonist (L-368) caused a downward shift in the current density (pA/pF) in CRF neurons in the presence of all the blockers with the exception of one cell that showed an upward shift in current density. B) Summary data showing a scatter plot for each recorded cell. The mean delta current density between 10 and 12 mins into the experiment was compared to the baseline. Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM. Dash box represents the outlier cell.
Figure 5-4. Oxytocinergic inhibition of CRF neurons is independent of direct activation of GPCRs. A) Bath application of 1 µM OTR antagonist (L-368) caused a downward shift in the current density (pA/pF) in CRF neurons recorded with 300 µM GDP-β-S that was significantly different from the control cells. B) Summary data showing a scatter plot for each recorded cell from both the control and GDP-β-S group. The mean delta current density between 10 and 12 mins into the experiment was compared to the baseline and to the mean of each group (see results). Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM.
Figure 5-5. No change in neuronal gain function following peripheral salt loading. A) In current clamp mode, the neurons were held around -70 mV and 50 pA current step was injected ranging from -100 pA to +400 pA. The average action potential frequency in each group were plotted against the current injected to generate an I/O curve (input/output curve). B) The mean of the last three data points in each group were calculated and plotted as the mean maximal AP frequency.
Figure 5-6. A subset of CRF neurons in the CeA shows OTR-mediated tonic excitation CeA following 2.0 M NaCl injection. (A) Bath application of 1 µM OTR antagonist (L-368) caused both a downward and an upward shift in the current density (pA/pF) in CRF neurons. (B) Summary data showing a scatter plot for each recorded cell. The mean delta current density between 10 and 12 mins into the experiment was compared to the baseline. Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM.
Figure 5-7. The bidirectional modulation of CRF neurons persisted in the presence of blockers of glutamate and GABAergic transmission. (A) Bath application of 1 µM OTR antagonist (L-368) caused both a downward and an upward shift in the current density (pA/pF) in CRF neurons. (B) Summary data showing a scatter plot for each recorded cell. The mean delta current density between 10 and 12 mins into the experiment was compared to the baseline. Dotted line with in the box plots illustrate the mean and the whiskers represent the SEM.
Figure 5-8. Proposed model of OT mediated tonic modulation of CRF neurons in the CeA.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Summary of the Key Findings

The role of OT in reproduction and sexual behavior has been well-documented but its role as an anxiolytic agent that promotes social attachment and attenuates stress is currently under investigation. The breadth of its role in modulating the limbic circuitry to influence social salience is not fully understood. A thorough understanding of oxytocinergic actions in the normal physiology has important implications for the therapeutic applications of OT in aberrant pathophysiological conditions like ASD and schizophrenia. The goal of this dissertation was to present experimental evidence that provides insight into the role of endogenous OT in modulating stress and anxiety. Using an acute salt loading paradigm to drive central release of OT, I demonstrated the importance of OT mediated tonic inhibition of CRF neurons as a mode of stress reduction in animals.

Many studies have suggested that OT is responsible for inhibition of the HPA axis in response to psychogenic stressors (Blume et al., 2008; Ring et al., 2006; Windle et al., 2004). OT deficient mice display enhanced anxiety-related phenotype ((Amico, 2008; Amico et al., 2008a; Amico et al., 2008b; Mantella et al., 2005; Mantella et al., 2004; Nomura et al., 2003) that can also be mimicked pharmacologically by using an antagonist to block OTRs. At the end of Chapter 3, I showed that in male mice OT can tonically inhibit CRF neurons in the PVN of hypothalamus that results in reduced plasma CORT levels. To identify and selectively target CRF neurons we utilized the Crf-IRES-Ai4 reporter strain that co-expressed tdTomato in neurons with CRF mRNA. This approach removes ambiguity and the reliance on electrophysiological and
morphological parameters to identify putative parvocellular neurosecretory neurons. The
tonic inhibition of CRF neurons was independent of synaptic activity but required the
activation of OTRs expressed on CRF neurons. The selective deletion of OTRs from
CRF neurons attenuated the effects of OT on CRF neurons elucidating a direct
relationship between central release of OT in the PVN and reduction in CRF activity.
Thus, OT-mediated paracrine signaling in the PVN may contribute to reduction in stress
responsiveness.

PVN is crucial for neural regulation of both the endocrine and autonomic
responses to stress (Herman, 2010; Herman and Cullinan, 1997; Herman et al., 2003;
Herman et al., 2004; Herman et al., 2002; Ulrich-Lai and Herman, 2009). The
sympathetic tone of an animal is maintained by the preautonomic neurons in the PVN
that project to the RVLM or IML to regulate sympathetic outflow (Guyenet, 2006;
Kenney et al., 2003; Pyner, 2009). Prior studies have shown that microinjection of OT in
the RVLM can induce a pressor response (Coote, 2007; Sermasi and Coote, 1994;
Sofroniew, 1980; Yang et al., 2009) but to date there is no direct evidence that
somatodendritic release of OT within the PVN can affect PVN-preautonomic neurons.
Recent evidence suggests that somatodendritic release of vasopressin can excite
RVLM projecting preautonomic neurons in the PVN (Son et al., 2013; Stern, 2015). To
the best of my knowledge, the data presented in Chapter 4 are the first evidence of
oxytocinergic inhibition of PVN-preautonomic neurons under basal conditions.

Preliminary data suggest the involvement of GABA-A receptors in the tonic inhibition of
preautonomic-like neurons by OT. Interestingly, the lack of involvement of GABA-A
receptors expressed on these neurons but the dependence on A1-receptor mediated
signaling alludes to a complex, polysynaptic network that might involve neuronal-glial interactions.

In Chapter 4, Figure 4-5 outlines a plausible mechanism that hints at a novel mechanism by which OT may impact the sympathetic tone. Prior studies have implicated both astrocytes and adenosine, a known gliotransmitter in modulation of neuronal activity (Hatton, 2004). Rapid glial plasticity and retraction of glial processes has been reported in both PVN and SON (Theodosis and Poulain, 1993; Theodosis et al., 2006; Theodosis, 2008). GABAergic-astrocyte signaling is a lesser known form of cell communication that is known to increase astroglial calcium levels in turn leading to the release of gliotransmitters such as adenosine or ATP (Fraser et al., 1994; Halassa et al., 2009; Losi et al., 2014; MacVicar et al., 1989). It is also important to note that this tonic inhibition of preautonomic neurons by OT is lost following peripheral salt loading which may contribute to the increase in sympathoexcitation drive.

OTRs are expressed abundantly throughout the limbic system including amygdala, BNST, lateral septum and nucleus accumbens (Gimpl and Fahrenholz, 2001). There is evidence for axonal release of OT in the CeA from PVN magnocellular neurons (Knobloch et al., 2012) but no known report of whether OT is released into the CeA after peripheral salt loading and if it can interact with CRF neurons in the CeA. Taking advantage of the CRF reporter strain, I provided evidence for a tonic modulation of CRF neurons by OT in the CeA. Basal levels of OT predominantly inhibited CRF neurons independent of glutamatergic, GABAergic and direct GPCR-mediated signaling. Following peripheral salt loading, a subset of CRF neurons were tonically excited by OT. The physiological relevance of tonic modulation of CRF neurons in the
CeA by oxytocin is still an unanswered question. This is compounded by the fact that CRF neurons in the CeA represent a heterogeneous population including both interneurons and projection neurons. Further work is needed to establish a definitive relevance of the data presented.

**Future Directions**

**Acute salt loading as a model to study pathophysiology:** Challenges to fluid homeostasis such as changes in plasma sodium concentration can impact hypothalamic regulation of stress responsiveness and mood (Krause et al., 2011; Smith et al., 2015). PVN and select limbic brain regions represent the convergence center for regulating both real and perceived threats to homeostasis. One of the compensatory mechanisms to alleviate fluid imbalance is somatodendritic release of OT (Balment et al., 1980; Ludwig, 1998b; Ludwig and Leng, 2006). As such, acute salt loading paradigm represents a promising model to study the role of endogenous OT with respect to mood and anxiety and circumvents the need to exogenously apply an agonist to drive the central oxytocinergic system. Thus, a peripheral salt injection paradigm can be an interesting tool to study central oxytocin system in both physiological state and in pathophysiological conditions with known dysregulation of OT signaling such as ASD (Modi and Young, 2012; Young and Barrett, 2015).

**Stress response of CRF-OTR knockout mice:** A future line of study could also utilize the CRF-OTR knockout mice and examine the stress response when exposed to a repertoire of stressors as a valuable tool for further exploration of the interaction of OT signaling and CRF neurons. It would be interesting to more fully explore the crosstalk between OT and CRF systems to determine if OT elicits similar response on CRF activity in different stress paradigms and compare the basal stress levels of the
knockouts to the controls. Does knockout of OTRs selectively from CRF neurons affect anxiety like phenotype which is more governed by the amygdaloidal circuitry? These are some of the questions that can help shape our understanding of the role that OT plays in modulating the stress signals.

**Open questions and challenges:** The growing interest in oxytocin signaling and the plethora of tools available for micro dissecting different circuits in the brain with both temporal and spatial precision open up a whole new era of research. The preliminary success with intranasal delivery of oxytocin in ameliorating symptoms of neuropsychiatric disorders such as schizophrenia (Guastella et al., 2015b), generalized anxiety (Kirsch et al., 2005; Parker et al., 2005) and neurodevelopmental conditions like ASD (Hollander et al., 2007; Hollander et al., 2002) is promising on one hand but also warrants more thorough investigation of oxytocinergic circuits and the role they play in normal physiology. Accumulating evidence support the notion that OT enhances affiliative prosocial behavior and attenuates stress (Blume et al., 2008; Neumann et al., 2000a; Neumann, 2008; Neumann et al., 2000b; Neumann and Landgraf, 2012; Neumann and Slattery); however, the effects of OT are not always positive and may be context-dependent. There are studies demonstrating that OT can induce aggressive and anti-social behavior and promote envy (Campbell, 2008; Shamay-Tsoory et al., 2009). Hence, we one should be more cautious and rigorous when interpreting the results of OT administration. Also, according to the NIMH, anxiety-related disorders affect twice as many adult women as men (Kessler et al., 2005a; Kessler et al., 2005b) while most of the studies in rodents utilize male animals. Thus, there is a need for more
anxiety-related studies in female rodents especially given the importance of OT in female reproductive and maternal behavior.

A problematic issue within the field of research using neuropeptides as putative therapeutics is that of variable pharmacokinetics and difficulty in delivery to the brain. An outstanding question in the field is how a peripheral injection of OT can elicit a central response without crossing blood brain barrier. A comparison of acute versus chronic oxytocin treatment is needed to ensure the long term safety of oxytocin therapy (Huang et al., 2014; Peters et al., 2014).

In conclusion, the work conducted in this dissertation signify the complexities of central OT signaling. Within the PVN, OT-mediated paracrine signaling inversely affected two surrounding neuronal populations, CRF and preautonomic neurons respectively following peripheral salt loading. In the CeA possibly through a synaptic mechanism OT can also have a bimodal effect on CRF neurons. Taken together, these data suggest that OT acting either through paracrine signaling or synaptically, may be an important factor contributing to inter-neuronal communication resulting in integration of neuroendocrine and autonomic responses to stress and anxiety. Thus, the findings contained within this dissertation are a small step toward the ultimate goal of using basic science to study the neurobiological underpinnings of the OT system that can be then used to design better therapeutics.
APPENDIX
DECREASE IN BASAL GLUTAMATERGIC TRANSMISSION TO CRF NEURONS IN THE MOUSE PVN FOLLOWING CHRONIC SALT LOADING

Introduction: Chronic salt loading induces synaptic and neuropeptide plasticity in the PVN to serve as a compensatory mechanism to cope with increased sympathetic outflow and attenuated HPA axis activity (Dahl and Heine, 1961). Chronic dehydration caused by substituting water with 2% saline for a 5-day period alters the CRF mRNA expression in the PVN and the SON (Amaya et al., 2001; Dohanics et al., 1990; Lightman and Young, 1987). In the PVN, the CRF expression pattern shifts from parvocellular to magnocellular neurons and concomitantly there is an increase in VP expression in the parvocellular region (Amaya et al., 1999).

Chronic salt loading also results in dramatic structural changes in the PVN including retraction of astrocytic processes and formation of new GABA and glutamate synapses (Hatton, 2004; Miyata et al., 1994; Theodosis and Poulain, 1993). There is evidence for increased frequencies of spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs) in SON magnocellular neurons after chronic dehydration (Di and Tasker, 2004). Thus, the goal of this study was to elucidate the changes in glutamate and GABAergic transmission to CRF neurons in the PVN following 5-day of chronic dehydration.

Methods: Male CRF reporter mice were used for the study. The control group had ad libitum access to water while the experimental group had water replaced with a 2% NaCl solution for five days (Chronic). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida. At the end of five days, the animals were sacrificed and whole-cell electrophysiology was performed on identified CRF neurons.
As mentioned in Chapter 2, a high chloride K-glu internal solution was used in the recording pipette in the presence of the ionotropic glutamate receptor antagonists 6,7-dinitroquinoxaline-2,3-Dione (DNQX; 20 μM) and DL-2-amino-5-phosphonovaleric acid (APV; 40 μM) to isolate sIPSCs. For recording sEPSCs, a regular K-glu internal was used in the presence of GABA-A receptor antagonist picrotoxin (PTX, 100 μM). Slices were continuously perfused at a rate of 1.2-1.5 mL/min with modified aCSF to increase overall excitability of the slice and contained (in mM): 124 NaCl, 5 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 10 D-glucose, 2.5 CaCl₂, and 25.9 NaHCO₃. This solution was saturated with 95% O₂ and 5% CO₂, and bath temperature was maintained at 28±2°C.

**Results:** To test whether chronic salt loading altered GABAergic and glutamatergic transmission to CRF neurons in the PVN, we performed whole-cell patch clamp recordings in an acute PVN slice preparation. CRF neurons were voltage-clamped at -70 mV and the basal frequencies of spontaneous excitatory postsynaptic sEPSCs and sIPSCs were observed for 5 min.

**Decrease in sEPSCs frequency in CRF neurons of PVN following chronic salt loading:** Preliminary results suggest a significant reduction in the frequency of sEPSCs recorded in CRF neurons from chronic animals relative to controls (Fig. A1), yet there was no significant difference in sEPSC amplitude. The sEPSC frequency changed from 4.95 ± 1.38 Hz (n= 6, control) to 1.28 ± 1.09 Hz, chronic, F= 7.378, P=0.02) while the amplitude stayed the same (11.84 ± 1.29 pA to 12.45 ± 0.75 pA, respectively; F= 0.1805, P=0.68).
No change in sIPSCs frequency in CRF neurons of PVN following chronic salt loading:

Contrary to the changes in glutamate transmission to CRF neurons, there seemed to be no changes in GABAergic transmission. The sIPSC frequency for control animals (n=6) were 5.08 ± 0.99 Hz as compared to salt-loaded animals (n=6; 3.95 ± 1.32 Hz; F= 0.4714; P=0.50), the amplitude of sEPSCs were not different between the two groups (26.39 ± 5.78 pA in control vs 29.61 ± 4.53 pA in salt loaded mice; F=0.1926; P=0.67).

**Interpretation and future experiments:** The presynaptic changes in the average frequencies of sEPSCs in PVN CRF neurons after chronic salt loading is mediated by changes in glutamate levels in the PVN. An overall reduction in excitatory inputs to CRF neurons with no change in inhibitory inputs can shift the excitation/inhibition balance and result in reduced activity of the CRF neurons. As the activity of parvocellular CRF neurons is reflected in the activity of HPA-axis, this could be one of the compensatory mechanisms that can cause the known attenuation of HPA-axis with chronic dehydration ((Amaya et al., 2001). Interestingly, this observation differs from the plasticity changes associated with magnocellular neurons following chronic dehydration where there is an increase in both sEPSCs and sIPSCs frequencies (Di and Tasker, 2004) and also from neurotransmitter plasticity in the PVN induced by chronic stress (Flak et al., 2009).

One plausible explanation could be the dynamic plasticity of astrocytic processes that can alter the ambient glutamate levels in the PVN. Changes in ambient levels of glutamate following chronic salt loading has been shown to tonically activate
presynaptic metabotropic glutamate receptors in the SON (Boudaba et al., 2003b) which would lead to decreased release probability of glutamate from synaptic terminals. Further work is needed to parse out the mechanistic details of this reduced glutamate transmission and its functional relevance.
Figure A-1. Reduction in sEPSCs frequency in CRF neurons of PVN following 5-day chronic salt loading. A) Representative traces of sEPSCs from control and chronic salt-loaded mice. B-C) Bar graph showing average sEPSC frequency and amplitude in both the groups. Mean ± SEM.
Figure A-2. No change in sIPSCs frequency in CRF neurons of PVN following 5-day chronic salt loading. A) Representative traces of sEPSCs from control and chronic salt-loaded mice. B-C) Bar graph showing average sIPSC frequency and amplitude in both the groups. Mean ± SEM.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Dipa was born in the eastern part of India but spent most of her teenage years in a small sleepy town in northern India dreaming about becoming a mad scientist. Undergraduate studies took her to Manipal University in southern India where she received her bachelors in pharmaceutical sciences and developed an interest in pharmacology and decided to pursue her Masters in Pharmaceutical Sciences from BITS, Pilani. At BITS, under the able guidance of Dr. Radhakrishnan she fell in love with neuroscience and moved to US for her graduate studies in the Dept. of Pharmacodynamics at University of Florida in the fall of 2010. In April 2011, she joined the laboratory of Dr. Charles J. Frazier and started studying endocannabinoid signaling in the hippocampus. In spring of 2012, she made a foray into the world of neuroendocrine as a part of an exciting collaboration with Dr. Eric G. Krause. Over the course of her graduate career she has published three peer-reviewed research papers and is in the process of submitting four more papers. Her graduate work was supported partially by a Graduate Alumni Fellowship and a Predoctoral Fellowship from the American Heart Association.

Following graduation, Dipa plans to move to Chapel Hill, North Carolina to begin working as a postdoctoral fellow in the laboratory of Dr. Thomas L. Kash at University of North Carolina.