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The Impact of Environmental and Psychological Stressors on Markers of Stress Axis Activation and the Beneficial Effects of Manual Therapy

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Doctor of Philosophy

in

Neuroscience

by

Kurt Andrew Spurgin

December 2014

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ABSTRACT OF THE DISSERTATION

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by

Kurt Andrew Spurgin

Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, December 2014
Dr. Margarita Curras-Collazo, Chairperson

Centrally-acting Pituitary adenylate cyclase-activating polypeptide (PACAP) is involved in hypothalamo–pituitary–adrenocortical (HPA) and sympatho-adrenal (SA) responses to stress. Evidence for an intra-adrenal PACAPergic system is mounting although information is lacking about its responsiveness to different stressors, especially as it compares to responses seen in other adrenal stress systems involving glucocorticoids and catecholamines. My work examines the impact of various physiological, psychological, and environmental stressors on markers of HPA and HSA activity in the adrenal gland and hypothalamus. Markers evaluated in these studies include mRNA expression of adrenal PACAP, steroidogenic acute regulatory protein (StAR), a chaperone protein crucial for making cholesterol available for the initiation of steroid biosynthesis and

melanocortin receptor accessory protein (MRAP), essential for trafficking of the adrenocorticotropic hormone receptor, melanocortin 2 (MC2R), CA biosynthetic enzymes tyrosine hydroxylase (TH) , and phenylethanolamine N-methyltransferase (PNMT), which methylates norepinephrine to epinephrine. In addition, I attempted to place markers for altered gene expression within the context of current perceptions on the etiology of hypertension. Lastly, I developed a reproducible procedure for studying a possible non-invasive treatment for hypertension using the application of variable pressures during massage-like stroking in rats.

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Introduction

Throughout fifteen years of clinical practice, I have seen a significant number of patients presenting with fibromyalgia, complex regional pain syndrome (CRPS), and other forms of chronic debilitating pain. The manual therapy and rehabilitation techniques employed in my clinic are generally quite effective at providing these patients with some temporary relief. However, I was frequently frustrated by the chronic nature of these patients' conditions. As I spent time with my most challenging patients, I began to notice an interesting pattern emerging in their medical histories. In addition to widespread diffuse pain, many of these patients suffered with symptoms such as irritable bowel syndrome, sleep disorders, sound sensitivity, urinary frequency, anxiety, and depression. Furthermore, these patients often reported high levels of perceived stress in their lives prior to developing chronic pain. I began to believe that chronic stress may be profoundly affecting the physiology of some individuals and making them much more likely to develop chronic debilitating pain syndromes. Further, I was intrigued by the possibility that the widespread symptoms in these patients were somehow caused by a global dysfunction of the stress-response systems. It seemed likely that some common mechanism must be involved in creating a broad-spectrum of dysfunction. This was the genesis of my interest in chronic stress related research.

Chronic stress has been shown to have a profoundly negative effect on a wide range of biological processes and has been implicated in disorders such as hypertension, heart

disease, stroke, anxiety, depression, fibromyalgia, chronic regional pain syndrome, gulf war illness, chronic fatigue syndrome, truncal obesity, and schizophrenia (Buskila 2001; Lohmeier 2001; Nicholls, Martin et al. 2001; Bosworth, Bartash et al. 2003; Rosengren, Hawken et al. 2004; Charmandari, Tsigos et al. 2005; Charmandari, Tsigos et al. 2005; Martinez-Lavin 2007; Albazaz, Wong et al. 2008; Sparrenberger, Cichelero et al. 2008; Wyller, Saul et al. 2008; de Mos, Sturkenboom et al. 2009; Spruill 2011). The widespread prevalence and societal costs of stress-related disorders is astounding. Estimates suggest that fibromyalgia alone affects approximately 1 in 20 women and nearly 5 million adults in the United States (Lawrence, Felson et al. 2008). Major depression is the leading cause of disability worldwide (Lopez and Murray 1998). Nearly 1 in 5 soldiers returning from Iraq and Afghanistan suffer from post-traumatic stress disorder (Adamson, Burnam et al. 2008). And, nearly 31% of U.S. adults have high blood pressure (CDC 2008) resulting in an annual cost of more than $76.6 billion in health care services/missed work (Lloyd-Jones, Adams et al. 2009). All told, the estimated societal cost of stress-related disorders reaches well into the tens of billions of dollars annually (Lloyd-Jones, Adams et al. 2009).

The impacts of chronic stress are often most profoundly felt by socioeconomically challenged populations. An illustration of this can be seen if we examine the impact of hypertension on Mexican immigrants to the United States. Mexico has lagged far behind the United States in the treatment of hypertension (Rodriguez, Malvezzi et al. 2006).

Cardiovascular disease-related mortality remains at very high levels in Mexico and essential hypertension has been identified as a major risk factor in Mexican populations (Rosas Peralta, Lara Esqueda et al. 2005; Rodriguez, Malvezzi et al. 2006). A recent study reported that increased incidence of hypertension in Mexico has resulted in annual costs of $485-$622 USD per patient for the management of hypertension and its consequences (Arredondo, Zuniga et al. 2005). This trend is expected to result in resources being diverted from the management of other health problems and infectious disease toward the treatment of hypertension. Furthermore, there is evidence to suggest that the Hispanic population in the US is particularly predisposed toward cardiovascular disease. Hispanic Americans have higher rates of uncontrolled and untreated hypertension than non-Hispanic whites (Vaeth and Willett 2005) and cardiovascular disease is the leading cause of disability and death for Hispanic Americans (NIH 2002). Even more alarming are recent finding suggesting that for immigrants from Mexico who settle in US border states, the degree of integration with the resident population and culture is positively linked to the incidence of hypertension (Vaeth and Willett 2005). Clearly, understanding maladaptive autonomic, neuroendocrine, and behavioral responses to chronic stress would have a broad impact on public health.

In recent years, a number of researchers have been examining the concept that early life stressors can make some individuals more susceptible to certain disease states in adulthood (Barker, Eriksson et al. 2002; Barker, Bagby et al. 2006). Some of the most

intriguing studies have demonstrated that adverse conditions during fetal development can increase cardiovascular reactivity to stressors in childhood. Importantly, increased cardiovascular reactivity in children is predictive of adult cardiovascular disease (Falkner, Kushner et al. 1981; Parker, Croft et al. 1987; Barker 2000; Sanders, Fazzi et al. 2004). For instance, the offspring of obese rat dams have aberrant autonomic control of cardiovascular function and increased cardiovascular reactivity to stressors (Samuelsson, Morris et al. 2010; Rudyk, Makra et al. 2011). These and other findings have lead to the development of a "reactivity hypothesis" (Light 2001) that mirrors my early intuition that exposure to chronic stress may predispose some individuals to developing disease states later in life.

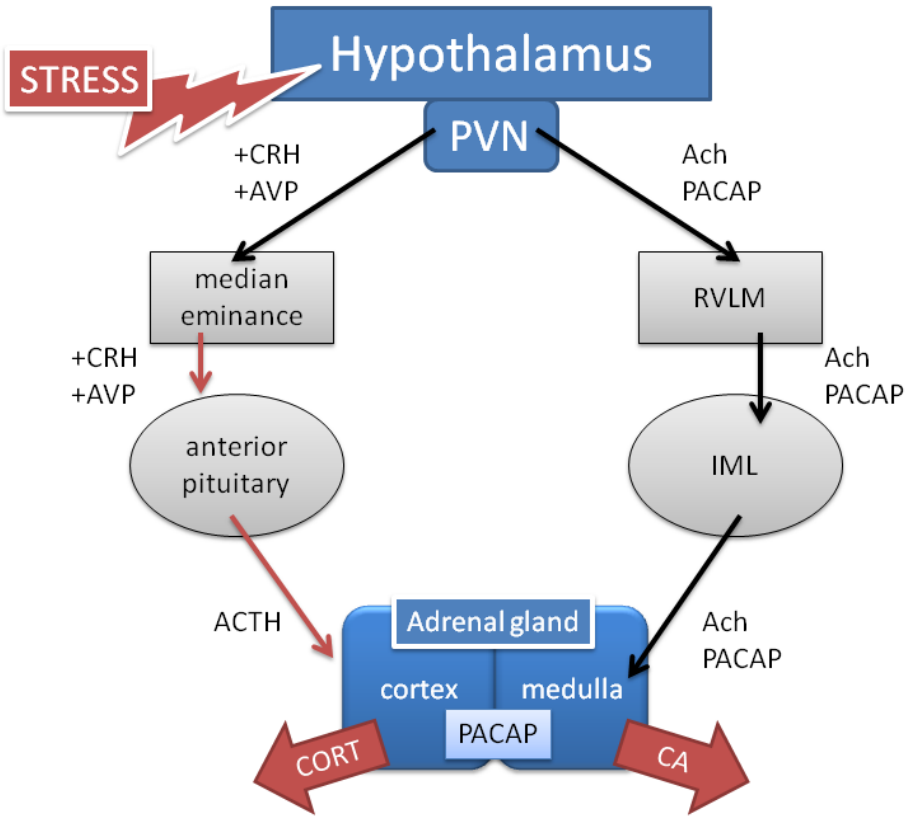
The effects of chronic stress are manifest in part through increased cortisol secretion via activation of the HPA axis (Stein, Filho et al. ; García-Bueno, Madrigal et al. 2005; Zafir and Banu 2008). Physiological responses to stress also include activation of the hypothamo-sympathoadrenal (HSA) axis (Kvetnanský, Pacák et al. 1995; Seematter, Binnert et al. 2004; Carter, Durocher et al. 2008). And, stressors such as immobilization and swim stress have been show to impact renin-angiotinsin system (RAS) activity (Kosunen, Pakarinen et al. 1976; Kosunen 1977; Armando, Tjurmina et al. 2003; Saavedra and Benicky 2007; Saavedra, Sánchez-Lemus et al. 2010). Because stress responses often vary depending on the type of stressor applied, comparing different types

of stressors should offer key insights into how these stress response systems are integrated.

The adrenal gland serves as an important location for integration of output from these major stress systems. Catecholamines, glucocorticoids, and mineracorticoids produced in the adrenal gland by activation of these stress axes play an important role in regulating stress responses, blood pressure, and energy homeostasis. At the level of the adrenal glands, pituitary adenylate cyclase-activating polypeptide (PACAP) appears to be of critical importance for homeostatic communication within these stress-activated systems (Arimura 1998; Davis-Taber, Baker et al. 2008; Ghzili, Grumolato et al. 2008). Importantly, recent findings have confirmed an association between PACAP signaling and both post-traumatic stress disorder (Ressler, Mercer et al. 2011) and major depression (Hashimoto, Hashimoto et al. 2011). And, PACAP signaling has also been shown to participate in the maintenance of the resting HR of spontaneously hypertensive rats (Farnham, Inglott et al. 2011).

A central hypothesis to be explored in this dissertation is that various environmental, psychological, and physiological stressors can disrupt HPA, HSA, and RAS functioning. Further, I examine the role that PACAP and its receptors play in maladaptive autonomic, neuroendocrine, and behavioral responses to chronic stress. Lastly, I provide evidence that a calibrated manual therapy method may prove useful in examining a promising therapeutic modality for mediating the deleterious effects of chronic stress.

Fig 1. The major stress axes. Stress activates the hypothamo-pituitary-adrenal (HPA) and hypothamo-sympatho-adrenal (HSA) axis. Parvocellular neurons project to the median eminence where they release corticotropin releasing-hormone (CRH) and arginine vasopressin (AVP) into the pituitary portal system. CRH and AVP cause the release of adrenocotropic hormone (ACTH) into the peripheral circulation. ACTH binds to melanocortin 2 receptors (MC2R) in the adrenal cortex to initiate corticosterone (CORT) synthesis and release. In the HSA axis, pre-autonomic neurons from the PVE project to the rostral ventral lateral medulla (RVLM) where pre-ganglionic sympathetic neurons arise and traverse via the interomedial lateral cell column in the spinal cord. Acetylcholine (Ach) and pituitary adenylate cyclase activating peptide (PACAP) work synergistically in this pathway. Preganglionic neurons project to the adrenal medulla where they stimulate catecholamine (CA) synthesis and release from adrenal chromaffin cells. PACAP plays an important role in coordinating stress axis responses in the adrenal gland.


STRESS
Hypothalamus
PVN
+CRH
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Ach
PACAP
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RVLM
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PACAP
anterior
pituitary
IML
ACTH
Adrenal gland
Ach
PACAP
cortex
medulla
PACAP
CORT
CA

Chapter 1

Intra-adrenal PACAP mRNA is regulated by various stressors in association with markers of glucocorticoid hormone production and catecholamine biosynthesis

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Abstract

Centrally acting pituitary adenylate cyclase-activating polypeptide (PACAP) is involved in hypothalamo–pituitary–adrenocortical (HPA) and sympathoadrenal (SA) responses to stress. Adrenal gland gene expression of PACAP was compared to that of HPA and SA markers using quantitative PCR in C57Bl/6 (WT) under different stress conditions: acute restraint stress, chronic restraint stress and social isolation stress. Acute restraint stress for 1 hour increased mRNA expression of adrenal PACAP, steroidogenic acute regulatory protein (StAR), and melanocortin receptor accessory protein (MRAP), essential for trafficking of the adrenocorticotropic hormone (ACTH) receptor, melanocortin 2 (MC2R). Changes in the latter likely increase the responsiveness of the adrenal steroid systems to endocrine triggers such as ACTH. In contrast, adrenal PACAP gene expression decreased significantly during chronic restraint stress (1 hr daily for 2 weeks) concomitant with elevated StAR and CYP11B1. Adrenal MRAP and MC2R expression was similar to controls after chronic stress. Social isolation stress had no impact on mRNA levels of all HPA and SA relevant genes. However, socially isolated PACAP KO mice had significantly increased adrenal mRNA levels of MRAP and MC2R as compared to isolated WT mice. The changes in adrenal PACAP expression occurred in association with CA release during acute stress and with induction of tyrosine hydroxylase (TH) in isolated PACAP KO mice only, suggesting a complex association between intra-adrenal PACAP and catecholamines. Taken together, our present findings suggest that acute and chronic restraint stress as well as isolation stress differentially impacts the intra-adrenal PACAPergic system. Changes in local PACAP signaling in the adrenal gland may

influence steroid endocrine function by association with steroid biosynthesis and/or ACTH receptor responsiveness. Additional study is necessary to further elucidate the exact physiological role that adrenal PACAP plays in HPA and HSA responses to stress.

Introduction:

The adrenal gland serves as an important organ for the integration of output from the two major stress systems, the hypothalamo–pituitary–adrenocortical (HPA) and the hypothalamo–sympathoadrenal (HSA) axis. Catecholamines (CA), glucocorticoid and steroid hormones produced in adrenal medulla and cortex, respectively, by activation of these stress axes play an important role in the maintenance of carbohydrate metabolism, stress management, and energy homeostasis. Pituitary adenylate cyclase-activating polypeptide (PACAP) has emerged as a potential important master regulator of both the HPA and HSA, and of anxiogenic behavior critical for stress management (Arimura 1998; Ghzili, Grumolato et al. 2008; Hammack, Roman et al. 2011; Stroth, Liu et al. 2011; Dore, Iemolo et al. 2013). Further, PACAP has recently been linked to stress-related disorders such as post-traumatic stress disorder (Ressler, Mercer et al. 2011) and major depression (Hashimoto, Hashimoto et al. 2011).

PACAP is abundant in the adrenal medulla (Shioda, Shimoda et al. 2000), and PACAP immunoreactivity and PACAP mRNA have both been identified in

norepinephrine-immunoreactive adrenal chromaffin cells. PACAP is synthesized in adrenomedullary chromaffin cells and PACAP specific mRNA has been identified in the adrenal medulla (Kantor, Heinzlmann et al. 2002; Mazzocchi, Malendowicz et al. 2002; Conconi, Spinazzi et al. 2006) suggesting a local source of PACAP. Indeed, in addition to PACAPergic fibers innervating the adrenal gland via splanchnic nerves there are also intrinsic PACAPergic fibers (Frodin, Hannibal et al. 1995; Dun, Miyazaki et al. 1996; Holgert, Holmberg et al. 1996; Moller and Sundler 1996; Nielsen, Hannibal et al. 1998). PACAP acts locally by binding to PAC1 receptors in the adrenal medulla (Watanabe, Masuo et al. 1992; Shiotani, Kimura et al. 1995) and VPAC receptors in the adrenal cortex (Mazzocchi, Malendowicz et al. 2002; Conconi, Spinazzi et al. 2006). PACAP receptors are coupled to Gs proteins leading to activation of second messengers which can influence gene expression, CA synthesis and release (Ohtaki, Watanabe et al. 1990; Ohtaki, Masuda et al. 1993).

Because of its robust expression in the adrenal gland PACAP has received significant attention as a potent regulator of catecholamine secretion. Both acetylcholine (ACh) and PACAP work synergistically to stimulate catecholamine release by the adrenal during stimulation of the splanchnic nerve (Malhotra and Wakade 1987; Guo and Wakade 1994; Lamouche, Martineau et al. 1999; Lamouche and Yamaguchi 2003). Importantly, PACAP-induced catecholamine release lasts much longer than release mediated by mixed muscarinic/nicotinic ACh signaling (Wakade and Wakade 1983; Boksa and Livett 1984;

Watanabe, Masuo et al. 1992; Guo and Wakade 1994). PACAP is also critical for induction of adrenal mRNA levels of the catecholamine biosynthetic enzymes, TH and PNMT, under some conditions of restraint stress (Stroth and Eiden 2010).

PACAP produced by chromaffin cells in the mammalian adrenal gland may act on adrenomedullary and adrenocortical endocrine processes indirectly through the local release of catecholamines (Nussdorfer 1996; Nussdorfer and Malendowicz 1998; Vaudry, Gonzalez et al. 2000). Local PACAP may also influence adrenal processes involving the major stress hormone, corticosterone which is under the regulation of the HPA. For example, PACAP-38 elicits glucocorticoid secretion in rats by stimulating an intramedullary CRH-ACTH system and by activation of ACTH receptors or by way of adrenomedullary CAs (Nussdorfer 1996; Nussdorfer and Malendowicz 1998).

At the level of the hypothalamus, PACAP is a potent secretagogue of central peptides involved in regulation of the HPA, i.e. corticotropin-releasing hormone (CRH) (Kageyama, Hanada et al. 2007; Stroth, Holighaus et al. 2011; Stroth, Liu et al. 2011) and arginine-vasopressin (Shioda, Shuto et al. 1997; Shioda, Toshihiko et al. 1998) and HPA responses appear to be sustained by PACAP-dependent mechanisms (Stroth, Holighaus et al. 2011). For example, PACAP-deficient mice exhibit impaired corticosterone response to restraint stress (Stroth and Eiden 2010) and deletion of the PACAP gene blunts stress-induced expression of c-Fos, an immediate early gene used as a marker of cell

activation, in CRH-producing neurons of the PVN (Tsukiyama, Saida et al. 2011). Interestingly, PACAP stimulation of CORT cannot, in all cases, be attributed to central CRH receptors, suggesting that intra-adrenal mechanisms may contribute significantly (Dore, Iemolo et al. 2013). At the level of the adrenal gland, a few studies have demonstrated that PACAP can elicit secretion of glucocorticoid as well as the mineralocorticoid aldosterone (Edwards and Jones 1994; Andreis, Malendowicz et al. 1995; Mazzocchi, Malendowicz et al. 2002); however, the regulation and significance of the intra-adrenal PACAP system during stress is not well understood.

In the present study we examined the impact of different stressors on the mRNA expression of PACAP and key factors regulating glucocorticoid and catecholamine (CA) biosynthesis. We used archival tissue from PACAP gene deleted mice to explore possible changes in adrenal function under prolonged mild stress conditions. Our results show that acute and chronic stress differentially affect PACAP gene expression, which is intricately linked to stress-induced changes in the expression of StAR, MRAP, CYP11β1 but not TH. Moreover, PACAP gene deletion upregulates MC2R and TH mRNA expression during chronic isolation stress further suggesting a potential participation of PACAP in adrenomedullary and adrenocortical stress responses. Taken together, these findings point to an intra-adrenal PACAP system that may intrinsically regulate adrenal steroid endocrine responses to stress.

Methods:

Animals: PACAP knockout (PACAP$^{-/-}$ (Colwell 2004) animals (backcrossed to C57BL/6 for greater than ten generations) were used (Colwell 2004). At one month of age, PCR was conducted on tail tissue to verify they were homozygous PACAP knockouts (PACAP KO). Confirmed male PACAP KO mice and C57BL/6 wild-type (WT) controls from the same colony were maintained in accordance with the guidelines in National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were group-housed 3 per cage in standard polycarbonate plastic cages with heat-treated pine shavings as bedding unless otherwise noted. Food pellets (Purina Lab Diet) and water were provided *ad libitum* except during experimental period. Temperature was maintained at 21 ± 2 °C and relative humidity at 50 ± 10% under a 12/12 h light/dark cycle (lights on from 7:00–19:00h). All experiments were performed between 13:00-18:00h). Experiments were approved by the IACUC on animal care and use at the University of California.

Social isolation stress: Adult male C57Bl6 mice or PACAP$^{-/-}$ mice at 3-5 months of age were individually housed for 8 weeks without enrichment toys placed in their cages. Prior research has demonstrated that chronic individual housing of mice can impact behavioral phenotypes (Võikar, Polus et al. 2005) and it has been recommended that researchers avoid single housing (Van Loo, Van Zutphen et al. 2003). However, recent work has demonstrated that individual housing does not appear to have any significant impact on

basal plasma CORT levels or adrenal TH activity (Arndt, Laarakker et al. 2009) or basal immune-endocrine levels, although single housing increases stress reactivity (Bartolomucci, Palanza et al. 2003). Age-matched control mice were group housed, three mice per cage. Mice were sacrificed by decapitation and adrenals collected and stored at -80^{o}C until used for qPCR analysis and CA assay.

Restraint stress: Restraint devices were made from 50 ml clear polystyrene centrifuge tubes which were modified with numerous air holes for ventilation (Castilla-Ortega, Hoyo-Becerra et al. 2011). Restraint devices were placed on a wire cage top to allow for adequate ventilation and cooling of the animals. For acute stress, C57Bl6 mice were placed in the restraint devices for a single 1-hour session and then sacrificed 1 hour after removal from the restraint device. This paradigm was chosen because it is too brief to trigger induction of genes participating in CA biosynthesis (Nankova, Tank et al. 1999; Kubovcakova, Tybitanclova et al. 2004; Xu, Chen et al. 2007; Kvetňanský, Krizanova et al. 2008; Stroth and Eiden 2010). This approach enables examination of early changes in the expression of PACAP and associated gene partners. For chronic stress, C57Bl6 mice were placed in the restraint devices for a 1 hour period daily for two weeks and then sacrificed one hour after the final stress session. Mice were sacrificed by decapitation and adrenals collected. All mice used were adult males between 4-6 months of age.

Quantitative Polymerase Chain Reaction (qPCR): RNA was isolated from the left adrenal gland using phenol-chloroform extraction. qPCR was employed to examine genes of interest that are involved in regulating glucocorticoid and mineralocorticoid synthesis in and release from the adrenal gland. We examined the HPA-relevant genes encoding steroidogenic acute regulatory protein (StAR), cytochrome P450 11 β 1 (CYP11 β 1), melanocortin 2 receptor (MC2R), and melanocortin receptor accessory protein (MRAP). **Figure 1.1** provides a brief overview of the role these genes play in steroid synthesis. qPCR was also used to examine adrenomedullary markers, tyrosine hydroxylase (TH), the first synthetic and rate-limiting enzyme in the biosynthesis of catecholamines, and phenylethanolamine N-methyltransferase (PNMT) which methylates norepinephrine to epinephrine (Wong and Tank 2007).

qPCR was performed using the Bioline SensiFAST™ SYBR No-ROX One-Step Kit and the Bio-Rad CFX 96 Real-Time PCR Detection System. Total RNA was prepared from frozen adrenal glands using phenol-chloroform extraction. A temperature gradient from 52-59^{o} C was used on the Bio-Rad CFX 96 station to capture all ideal annealing temperatures. The reaction was run for 40 cycles. Genes of interest were normalized to the housekeeping gene β-actin. The nucleotide sequences of qPCR primers are listed in Table 1. Results are presented as relative expression which was determined via the equation described in Pfaffl (Pfaffl 2001). In other experiments we determined that β-actin gene expression was constant under several stress conditions.

CA assay: Catecholamine content in adrenal glands was determined via fluorometric detection using a modification of published protocols (Wakade 1981; Wakade, Blank et al. 1991; Guo and Wakade 1994). The assay used is a non-specific catecholamine assay that detects dopamine, epinephrine and norepinephrine. In our lab this assay has a sensitivity of 0.125 mM, sufficient to detect changes at the adrenal level. To obtain a standard curve, norepinephrine was dissolved in 0.05 N perchloric acid (PCA) and serial dilutions were made. For samples, the right adrenal gland was dissected at sacrifice, snap frozen on dry ice and later homogenized in 400 ul PCA. Eighty-five μL of each blank, standard, or sample was added to each well of a sterile 96 well plate. Using a multi-well pipette 3.7 μL of $K_3Fe(CN)_6$ solution was added to each well and the plate was shaken gently. The reaction was stopped after 3 minutes by adding 34 μL of ascorbic acid solution to each well. Absorbance was measured at 450nm and 560nm. Absorbances obtained at 560 and those obtained for blanks at 450 nm were subtracted from sample absorbances. Absorbance was converted to catecholamine concentration using the best-fit four-power polynomial equation. All curves used had a correlation coefficient greater than 0.99. Data are expressed in mM units.

Statistical analysis. General linear model ANOVA was used where data met normal distribution/equal variance assumptions. Two-group comparisons were determined using Mann Whitney test or Student's t-test. Multiple group comparisons were made using

Student–Newman–Keuls test applied following general linear model ANOVA. Statistical significance was acknowledged at an alpha level of 0.05 or lower.

Results:

Acute restraint stress upregulates adrenal PACAP and gene markers associated with MC2R trafficking. In the present study one hour restraint did not significantly alter TH (0.81 ± 0.20 vs 1.02 ± 0.12 fold expression; n=8) or PNMT (1.35 ± 0.26 vs 1.03 ± 0.12 fold expression; n=12) mRNA expression levels in the adrenal gland (stressed vs controls) (**Fig. 1.2A**). This finding is consistent with reports that TH and PNMT are not significantly elevated in mice after 1 hour restraint stress (Stroth and Eiden 2010). In spite of the lack of gene induction, adrenals from stressed mice showed a marked loss of CA content as compared to that of controls (3.10 ± 0.24 vs 4.71 ± 0.46 mM; $p<0.05$, n=9), suggesting rapid bulk release of CA (**Fig. 1.2B**). **Fig. 1.3** shows the effects of this treatment on gene expression of PACAP and markers for steroid synthesis and MC2R trafficking in the adrenal gland. Specifically, mRNA gene expression was significantly elevated in stressed animals vs controls for PACAP (1.75 ± 0.25 vs 1.01 ± 0.07 fold expression; n=13, $p<0.05$), StAR (6.56 ± 1.33 vs. 1.18 ± 0.51 fold expression; n=7, $p< 0.05$) and MRAP (4.35 ± 0.44 vs. 1.01 ± 0.08 fold expression; n=8, $p<0.001$).

Chronic stress upregulates adrenal genes associated with the synthesis of glucocorticoids and concomitant with reduced PACAP gene expression. Adrenal expression of adrenomedullary axis markers, TH and PNMT, was not significantly elevated when compared to unstressed controls in tandem with a significant reduction in PACAP mRNA expression (**Fig. 1.4B**). Mean values for adrenal PACAP mRNA expression in chronically stressed mice vs. unstressed controls were 0.40 ± 0.03 vs. 1.14 ± 0.28 fold expression respectively (n=14; $p>0.01$). In contrast, **Fig. 1.4A** shows that mean values for adrenal StAR and CYP11B1 were elevated after chronic restraint stress. Mean mRNA values for StAR in stressed and unstressed-controls were 1.30 ± 0.06 and 0.95 ± 0.17 fold expression, respectively (n=14; $p<0.05$). Corresponding mean mRNA values for CYP11β1 mRNA were 1.14 ± 0.05 and 0.92 ± 0.10 fold expression, respectively (n=13; $p<0.05$). Other adrenal genes associated with ACTH signaling (MC2R and MRAP) remained at control levels.

Chronic social isolation upregulates MC2R and MRAP, concomitant with upregulation of TH mRNA in PACAP deficient mice. Adrenal CA content is unaffected by PACAP KO. Individual housing did not significantly change any of the genes of interest in wild type mice (**Figs. 1.5, 1.6**). We tested the effect of single housing on adrenal markers of HPA axis activity and examined the compounded effects of PACAP gene deletion. An ANOVA that compared mRNA expression levels in group housed WT mice, single housed WT mice and single-housed PACAP KO mice yielded a statistically significant

difference amongst the groups for MRAP ($F_{2,20}$=5.608, n=29, p<0.05 **Fig 1.6C**). Similar results were found for MC2R ($F_{2,26}$=4.317, n=28, p<0.05, **Fig 1.6D**) and TH ($F_{2,28}$=5.568, n=31, p<0.05, **Fig 1.5A**) in separate ANOVAs. Post-hoc comparisons revealed no statistically significant difference in expression of any of the genes of interest between single housed WT and group housed WT. Other labs have shown that adrenals taken from group housed PACAP KO animals did not show elevated basal expression of HPA or HSA markers relative to WT mice (Stroth, Holighaus et al. 2011; Stroth, Kuri et al. 2013). Adrenal CA content was unaltered by PACAP gene deletion (2.53 ± 0.32 vs 2.59 ± 0.43 mM; n=9, **Fig 1.5**.). Genes associated with CORT production in the adrenal gland, StAR and CYP11β1, were unaffected by social isolation stress as previously shown (Serra, Sanna et al. 2007) or by the combination of social isolation and PACAP gene deletion.

Discussion:

In this study we have shown that acute and chronic stress regulates PACAP gene expression in the adrenal gland. However, the adrenal is a source of substantial PACAP and PACAP mRNA measured in the adrenal gland is likely being produced and released by adrenomedullary chromaffin cells and acting in an autocrine or paracrine manner within the adrenal gland as shown in previous reports (Shiotani, Kimura et al. 1995; Kantor, Heinzlmann et al. 2002; Mazzocchi, Malendowicz et al. 2002; Conconi, Spinazzi et al. 2006). PACAP mRNA is expressed in cultured adrenal medullary cells lacking

nerve fibers (Mazzocchi, Malendowicz et al. 2002). PACAP appears to be released in the adrenal medulla and cortex by PACAPergic nerve fibers (Hannibal, Mikkelsen et al. 1995; Dun, Miyazaki et al. 1996; Holgert, Holmberg et al. 1996; Moller and Sundler 1996; Nielsen, Hannibal et al. 1998). Other sources of PACAP in adrenal gland may include sympathetic fibers in splanchnic nerve innervating the adrenal are not likely to contain PACAP mRNA (Tabarin, Chen et al. 1994; Shiotani, Kimura et al. 1995) (for review see (Giuditta, Tai Chun et al. 2008). Indeed, PACAP mRNA expression has been demonstrated in cultured adrenal medullary cells that are lacking innervation from splanchnic nerve fibers (Mazzocchi, Malendowicz et al. 2002). Therefore, our present findings that PACAP mRNA in the adrenal gland is rapidly induced in mice following a single 1-hour restraint is consistent with reports that intra-adrenal PACAP plays an important role in adrenal responsiveness to acute restraint stress (Stroth, Liu et al. 2011).

Intra-adrenal PACAP may play a significant role in the regulation or signaling associated with adrenal glucocorticoid and/or mineralocorticoid hormones. We found that, concomitant with increased PACAP expression, adrenal StAR and MRAP gene expression was also induced rapidly following acute stress. These findings are consistent with reports that adrenal StAR gene transcription is stimulated within minutes of ACTH binding to MC2R (Jo, King et al. 2005). In the HPA axis, PACAP has been shown to stimulate ACTH release (and enhance corticosterone production) (Hart, Gowing et al. 1992; Andreis, Malendowicz et al. 1995; Neri, Andreis et al. 1996) via c-Fos induction

and CREB phosphorylation in CRF neurons in the PVN (Agarwal, Halvorson et al. 2005; Norrholm, Das et al. 2005). Similarly, PACAP-dependent corticosterone synthesis and secretion from the adrenal gland of mice (Stroth and Eiden 2010; Stroth, Holighaus et al. 2011) may involve an intra-adrenal CRH-ACTH system (Shiotani, Kimura et al. 1995; Nussdorfer 1996; Conconi, Spinazzi et al. 2006). The early elevation in StAR mRNA following acute stress seen in our study is expected for enhanced corticosterone secretion since StAR is critical for transport of cholesterol from cellular stores to mitochondria initiating the first committed step in steroid biosynthesis, the conversion of cholesterol into pregnenolone. The latter is the precursor for progesterone, the required substrate for glucocorticoid and other adrenal steroid hormones production (Peters, Clausmeyer et al. 1998). mRNA levels of MRAP, essential for the cell surface trafficking and signaling of MC2R receptors responding to ACTH, were also elevated concomitant with elevated PACAP gene expression during acute stress. This finding supports a role for intra-adrenal PACAPergic system in facilitating the responsiveness of adrenal steroid endocrine systems to HPA signaling and/or to stress. A picture is emerging, from our work and that of others, that PACAP produced locally can regulate these adrenal steroid systems independent of the HPA since PACAP can trigger corticosterone and aldosterone release from isolated adrenal gland and adrenal cell cultures (Mazzocchi, Malendowicz et al. 2002).

In contrast to upregulated PACAP mRNA expression following our relatively brief application of acute stress (one hour), gene markers for CA biosynthesis (i.e., TH and PNMT mRNA) were not elevated in agreement with previous reports showing significant increases in TH and PNMT mRNA following 6 hours of unrelieved restraint, but not 5 h after a 1 h period of restraint (Stroth and Eiden 2010). Longer periods of acute stress appear to be required for induction of TH and PNMT genes (Kubovcakova, Tybitanclova et al. 2004). Our findings indicate that early PACAP-dependent responses to stress may not be associated with the induction of CA biosynthesis. However, because adrenal CA content was significantly reduced in acutely stressed mice, PACAP responses may be linked to enhanced CA signaling and/or secretion from the adrenal gland. This is consistent with previous reports showing that PACAP treatment can regulate catecholamine release from the isolated adrenal gland and adrenal cell cultures (Wakade and Wakade 1983; Boksa and Livett 1984; Guo and Wakade 1994; Watanabe, Shimamoto et al. 1995; Mazzocchi, Malendowicz et al. 2002).

Prior work has shown that chronic restraint stress produces sustained elevation of plasma CORT levels and that this adrenal response does not habituate to such stress (Pitman, Ottenweller et al. 1988). This is consistent with CORT's critical role in fat metabolism, anti-inflammatory effects, CNS alertness, and stress management. In the present study we demonstrate that adrenal mRNA levels of genes associated with CORT synthesis, StAR and CYP11β1 (which is specific to CORT but not aldosterone), were significantly

elevated while that of PACAP was significantly blunted following chronic restraint. Interestingly, reduced PACAP expression in chronically stressed animals coincides with a return of MRAP expression to control levels. This hints at a possible compensatory role for intra-adrenal PACAP in the expression and trafficking of ACTH receptors. This connection is further supported by our finding that the primary changes noted in PACAP KO animals subjected to chronic social isolation primarily involved changes in MRAP and MC2R expression (see below).

Changes in StAR and CYP11β1 occurred in the absence of concomitant induction of TH and PNMT genes nor changes in adrenal CA content. We found near basal levels of TH and PNMT mRNA after repeated restraint stress treatments of 1 hour consistent with prior reports by Tóth et al (2008) which found no increase TH mRNAs in the brainstem. As with acute stress, TH and PNMT induction may require longer bouts of restraint stress (Xu, Chen et al. 2007). These findings suggest that intra-adrenal PACAP is primarily associated with adrenocortical cells involved in CORT induction and signaling without involvement of adrenal CAs.

Prior research has demonstrated that chronic individual housing of mice can impact behavioral phenotypes (Võikar, Polus et al. 2005) and it has been recommended that researchers avoid single housing (Van Loo, Van Zutphen et al. 2003). However, recent work has demonstrated that individual housing does not appear to have any significant

impact on basal plasma CORT levels or adrenal TH activity (Arndt, Laarakker et al. 2009). Bartolomucci et al. also found that basal immune-endocrine levels were unaltered in individually housed animals, but they did note that single housing increased stress reactivity (Bartolomucci, Palanza et al. 2003). Consistent with these findings we found no significant effect of chronic social isolation on adrenal markers of either CORT or sympathoadrenal markers in wildtype animals. In contrast, isolated PACAP knockout mice displayed elevated TH mRNA expression, an effect that may be due to compensation resulting from changes in cholinergic neural drive to the adrenal via splanchnic nerve-mediated control (Smith and Eiden 2012; Stroth, Kuri et al. 2013). Hamelink and others (2002) demonstrated that PACAP knockout mice under control conditions have normal adrenal TH and PNMT immunoreactivity as well as normal epinephrine production. Only unAder prolonged insulin shock does TH induction and epinephrine secretion become compromised (Hamelink, Lee et al. 2002).

Regulation of adrenal MC2R and MRAP in PACAP KO mice is likely to be complex due to an abrogated central and systemic PACAPergic system and its disregulation of pituitary ACTH release. In light of this, upregulated adrenal MC2R (and MRAP) mRNA levels (without StAR and CYP11β1 mRNA alterations) in isolation-stressed PACAP KO mice could reflect a compensatory response by the adrenal glucocorticoid system to reduced circulating ACTH. Indeed, central CRF mRNA and corticosterone levels are reduced in PACAP deficient mice after restraint stress (Stroth and Eiden 2010).

Alternatively, the lack of changes in StAR and CYP11β1 mRNA expression may also suggest that changes in PACAP gene expression under these conditions do not appear linked to the induction of genes for CORT biosynthesis.

Taken together, our present findings suggest that local PACAP signaling may influence adrenal processes associated with steroid biosynthesis and/or ACTH receptor responsiveness. An intra-adrenal PACAPergic system appears to respond differentially to acute and chronic restraint stress and sometimes in a manner exclusive of sympathoadrenal responses. PACAP gene deletion, which likely alters central and adrenal PACAP systems, results in altered CORT activation during chronic social isolation. Additional study is necessary to further elucidate the exact physiological role that adrenal PACAP plays in adrenal endocrine function and in HPA and HSA responses to stress.

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Table 1.1: Sequences of qPCR primers used to probe for adrenal genes markers involved in regulating glucocorticoid/ mineralocorticoid and catecholamine biosynthesis and induction. Primers listed are for HPA-relevant genes encoding steroidogenic acute regulatory protein (StAR), cytochrome P450 11 β 1 (CYP11 β 1), melanocortin 2 receptor (MC2R), and melanocortin receptor accessory protein (MRAP). qPCR primers for adrenomedullary markers include tyrosine hydroxylase (TH), the first synthetic and rate-limiting enzyme in the biosynthesis of catecholamines, and phenylethanolamine N-methyltransferase (PNMT) which methylates norepinephrine to epinephrine. Also listed are primers for pituitary-adenylate-cyclase activating polypeptide (PACAP) and the primers used for housekeeper β-actin

Gene	Accession #	Primer	Sequence (5'-3')	Annealing Temp (°C)
β actin	NM_007393.3	forward	TAGGCACCAGGGTGTGATGGTGG	59.7
		reverse	GCAGCACAGGGTGCTCCTCAG	
PACAP	NM_009625.2	forward	AGGTGCTGGTGTTGGAATGAATGC	61.1
		reverse	AATGCATGAGGGCAAGGGTAGGAA	
StAR	NM_011485.4	forward	CTGCTTGGTTCTCAACTGGAAG	59.5
		reverse	CACCTCCAAGCGAAACACCT	
MRAP	NM_029844.3	forward	TGAAAGCCAACAAGCATTCCA	60.3
		reverse	GGCACAGAGGGAGGTTGAAG	
MC2R	NM_001271716.1	forward	TGTATATGTTCCGGCCTTTCCTG	60.4
		reverse	TTGCTGGTTGAGGGTAGAGGAT	
CYP11β1	NM_001033229.3	forward	AGAGGGTCGTCCACAGTCC	59.5
		reverse	ACCAACAGGATAGAGCCTCAAG	
TH	NM_009377.1	forward	TCACTGTGGAGTTTGGGCTGTGTA	60.1
		reverse	ACACCGGCTGGTAGGTTTGATCTT	
PNMT	NM_008890.1	forward	TGCCCACTTTGAGGACATCACCAT	59.8
		reverse	TGAGGCAGGCATGCTGACTATACA	

Fig 1.1. The adrenal steroid biosynthesis pathway. Biosynthesis of adrenal steroids is intitiated in the mitochondria after cholesterol gets transferred there from cellular stores, a process that critically depends on steroidogenic acute regulatory protein (StAR) a chaperone protein. Increased activity of StAR, as occurs during ACTH-mediated stimulation and hypoxia stress (Stocco and Clark 1996; Raff, Hong et al. 2003), facilitates the first committed step in steroid biosynthesis, the conversion of cholesterol into pregnenolone, the precursor for progesterone, the required substrate for glucocorticoid and mineralocorticoid production (Peters, Clausmeyer et al. 1998). The mitochondrial enzyme, steroid 11β-hydroxylase(encoded by cytochrome P450 11 β 1 (CYP11β1), converts 11-deoxycorticosterone to corticosterone in the adrenal cortex. Secretion of both aldosterone and corticosterone are stimulated by activation of adrenal melanocortin 2 receptor (MC2R) when it is bound by adrenocorticotropic hormone (ACTH) (Chida, Nakagawa et al. 2007). Melanocortin receptor accessory protein (MRAP) is essential for the cell surface trafficking and signaling of MC2R receptors.

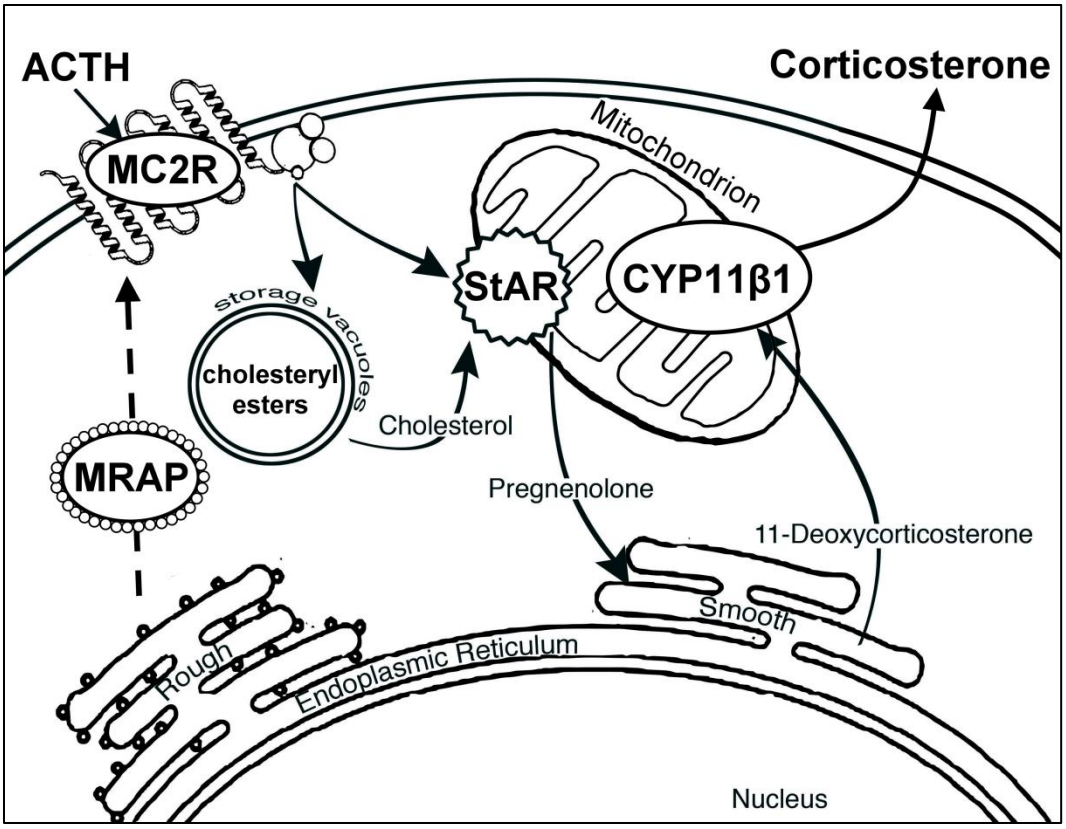

ACTH
Corticosterone
MC2R
Mitochondrion
StAR
CYP11β1
storage vacuoles
cholesteryl esters
Cholesterol
MRAP
Pregnenolone
11-Deoxycorticosterone
Smooth
Rough
Endoplasmic Reticulum
Nucleus

Fig 1.2. Acute restraint stress increases PACAP mRNA in the adrenal gland but not other markers associated with sympathoadrenal activity. A. qPCR analysis was performed on the adrenal glands of male C57Bl6 mice after a single restraint stress. mRNA gene expression of PACAP was significantly elevated in stressed animals vs controls. Expression of TH and PNMT were unaffected by acute restraint stress. Transcript expression levels were normalized to the housekeeping gene β-actin. **B.** Adrenal CA content was reduced following acute restraint stress. PACAP= pituitary adenylate cyclase activating polypeptide, TH= tyrosine hydroxylase, PNMT = phenylethanolamine N-methyltransferase, CA=catecholamine. Asterisk indicates statistical significance in comparison to unstressed controls at $p<0.05$.

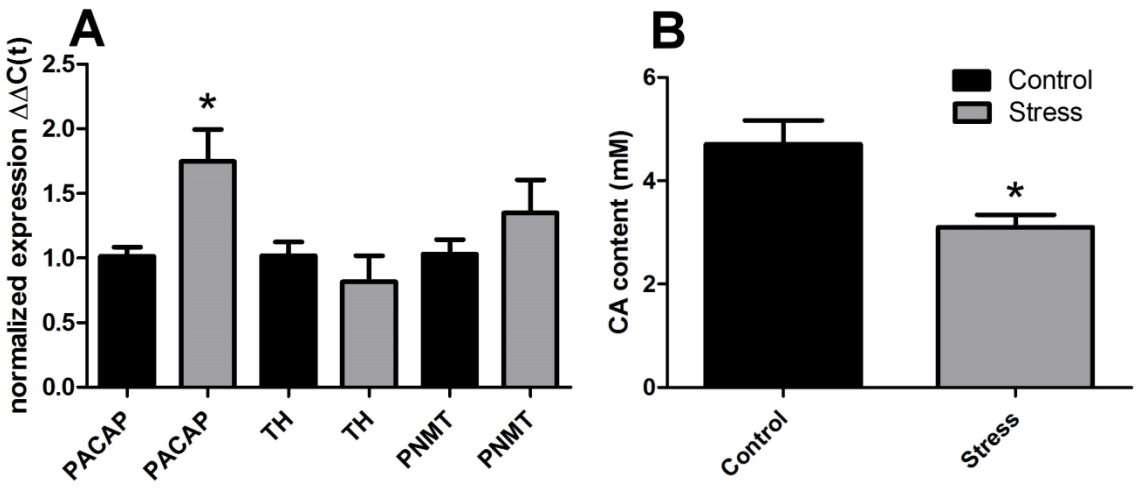

A
normalized expression ΔΔC(t)
2.5
2.0
1.5
1.0
0.5
0.0
*
PACAP
PACAP
TH
TH
PNMT
PNMT
B
CA content (mM)
6
4
2
0
Control
Stress
*
Control
Stress

Fig 1.3. Acute restraint stress increases gene markers for corticosterone production and ACTH receptor in the adrenal gland. qPCR analysis was performed on the adrenal glands of male C57Bl6 mice after a single restraint stress. mRNA gene expression of StAR, and MRAP were elevated in stressed animals vs controls. Transcript expression levels were normalized to the housekeeping gene β-actin. PACAP= pituitary adenylate cyclase activating polypeptide, StAR = steroidogenic acute regulatory protein, MC2R = adrenal melanocortin 2 receptor, MRAP = melanocortin receptor accessory protein, CYP = cytochrome P450 11β 1. Asterisks indicate statistical significance in comparison to unstressed controls at $p<0.05$ (*) and $p<0.001$ (***).

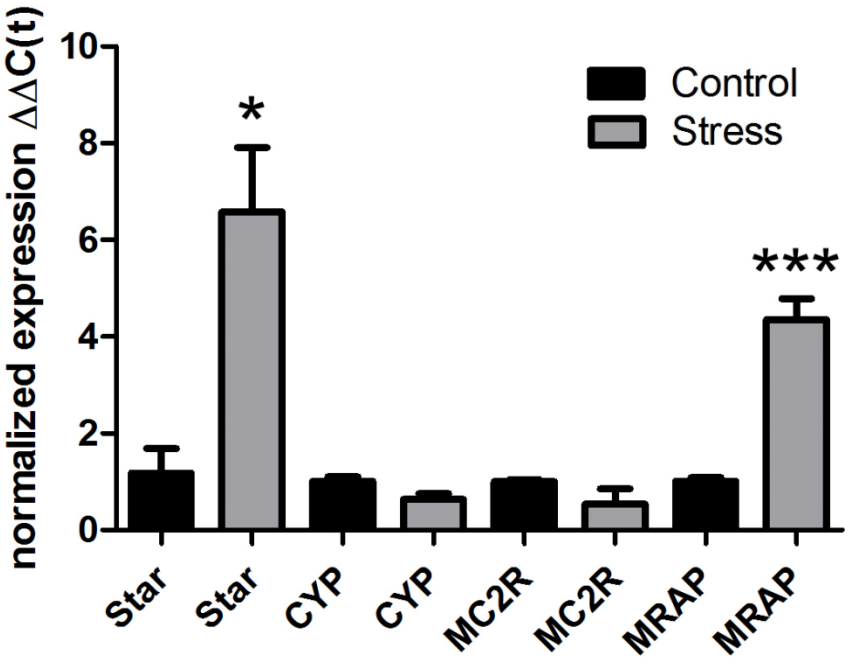

normalized expression ΔΔC(t)
10
8
6
4
2
0
*
***
Control
Stress
Star
Star
CYP
CYP
MC2R
MC2R
MRAP
MRAP

Fig 1.4 Chronic restraint stress significantly elevates the expression of adrenal HPA genes associated with CORT production and blunts PACAP expression. A. qPCR analysis was performed on the adrenal glands of male C57Bl6 mice after two weeks of daily restraint stress. Chronic restraint stress in WT mice significantly increased adrenal StAR and CYP11β1 mRNA expression vs. unstressed controls. Adrenal expression of additional HPA axis markers (MRAP and MC2R), and sympathoadrenal markers (TH and PNMT) were no different after repeated stress as compared to no stress (control). **B.** Chronic stress in WT mice significantly decreased adrenal PACAP mRNA expression vs. unstressed controls. Transcript expression levels were normalized to the housekeeping gene β-actin. PACAP KO mice and WT controls. PACAP= pituitary adenylate cyclase activating polypeptide, StAR = steroidogenic acute regulatory protein, MC2R = adrenal melanocortin 2 receptor, MRAP = melanocortin receptor accessory protein, CYP = cytochrome P450 11β 1, TH= tyrosine hydroxylase, PNMT = phenylethanolamine N-methyltransferase. Asterisks indicate statistical significance in comparison to unstressed controls at $p<0.05$ (*) and $p<0.01$ (**).

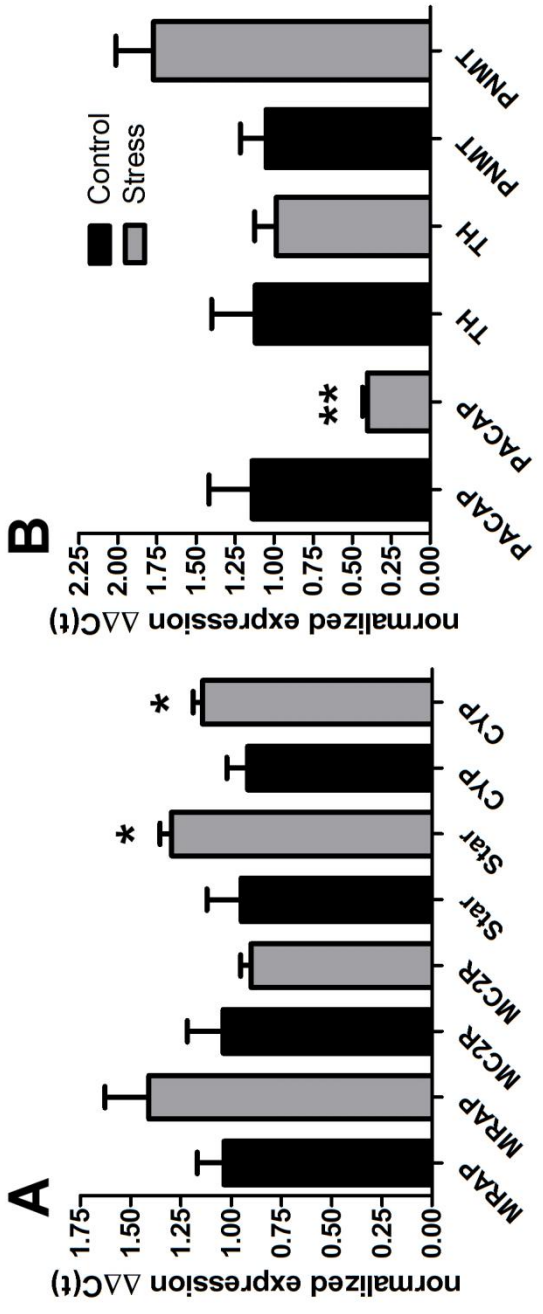
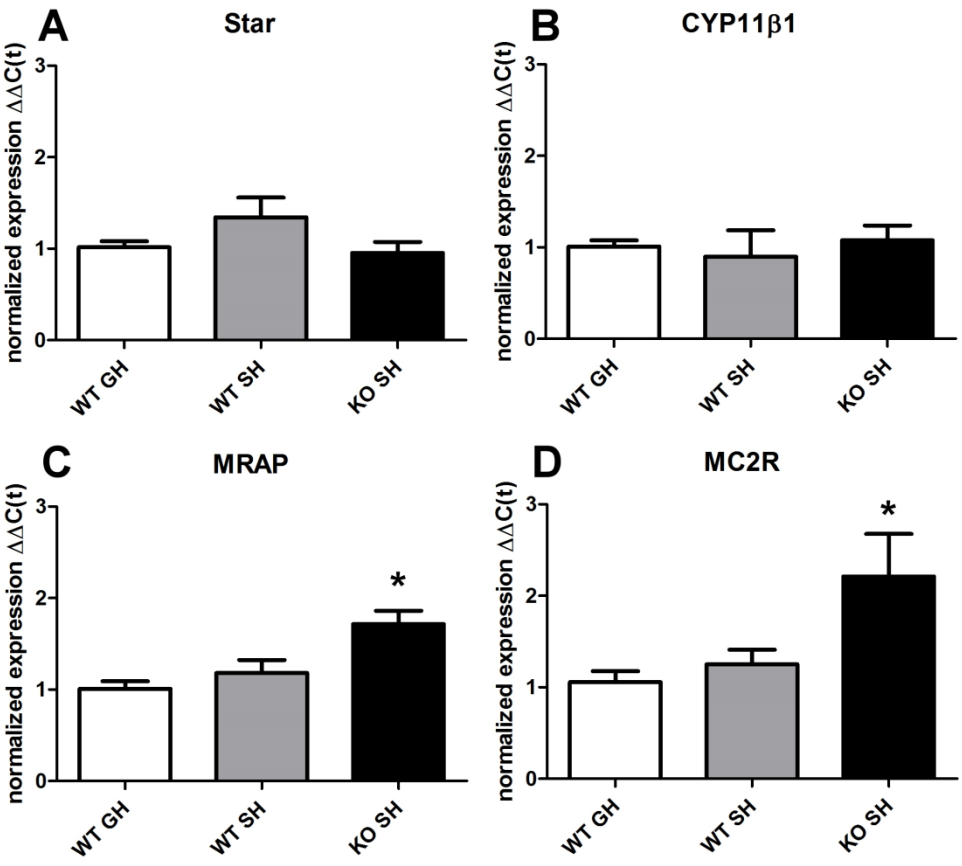

A
normalized expression ΔΔC(t)
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
MRAP
MRAP
MC2R
MC2R
Star
Star
CYP
CYP
*
*
B
normalized expression ΔΔC(t)
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25
Control
Stress
PACAP
PACAP
**
TH
TH
PNMT
PNMT

Fig 1.5. Chronically isolated PACAP knockout mice show upregulated TH mRNA in adrenal gland. Adrenals collected from PACAP knockout and adult WT male mice subjected to isolation stress for 8 weeks were analyzed using qPCR. **A**. Single housing (SH) did not significantly impact TH or PNMT gene expression in WT mice. In comparison to single-housed WT mice (SH), PACAP KO mice showed increased adrenal TH mRNA expression. **B.** Neither isolation stress nor PACAP gene deleted mice showed changes in PNMT mRNA. **C**. Adrenal CA content is similar in all groups examined. Transcript expression levels were normalized to the housekeeping gene β-actin. GH = group housed, SH = single housed, KO = PACAP KO; WT = wild type C56Bl/6, TH= tyrosine hydroxylase, PNMT = phenylethanolamine N-methyltransferase, CA=catecholamines. Asterisk indicate statistical significance when compared to single-housed WT mice at $p<0.05$.

A
normalized expression ΔΔC(t)
TH
*
WT GH
WT SH
KO SH
B
normalized expression ΔΔC(t)
PNMT
WT GH
WT SH
KO SH
C
CA concentration (mM)
WT
KO

Fig 1.6. Chronically isolated PACAP knockout mice display upregulated MC2R and MRAP mRNA levels in adrenal gland. qPCR analysis was performed on the adrenal glands harvested from male C57Bl6 and PACAP KO mice after 8 weeks of social isolation (single housing- SH). **A,B**. Mean StAR and CYP11β1 mRNA levels were not significantly different in PACAP KO mice as compared to WT mice subjected to single housing. **C.D.** In comparison to isolated WT mice, PACAP KO mice showed increased mean mRNA levels of MC2R and MRAP. Isolation stress by itself did not impact gene expression in WT mice Transcript expression levels were normalized to the housekeeping gene β-actin. GH = group-housed, SH = single-housed, KO = PACAP -/-; WT = wild type C56Bl/6, StAR = steroidogenic acute regulatory protein, MC2R = adrenal melanocortin 2 receptor, MRAP = melanocortin receptor accessory protein, CYP = cytochrome P450 11β 1. Asterisk indicates statistical significance when compared to unstressed controls at $p<0.05$.


A
Star
normalized expression ΔΔC(t)
0
1
2
3
WT GH
WT SH
KO SH
B
CYP11β1
normalized expression ΔΔC(t)
0
1
2
3
WT GH
WT SH
KO SH
C
MRAP
normalized expression ΔΔC(t)
0
1
2
3
*
WT GH
WT SH
KO SH
D
MC2R
normalized expression ΔΔC(t)
0
1
2
3
*
WT GH
WT SH
KO SH

Chapter 2

Developmental PBDE exposure produces hypertensive responses associated with altered sympathoadrenal and hypothalamic-pituitary-adrenal endocrine activity

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Abstract:

Organohalogens such as polychlorinated biphenyls (PCBs) have been linked to hypertension and metabolic syndrome. We have previously shown that polybrominated diphenyl ethers (PBDEs) produce exaggerated pressor responses to physiological stress, suggesting that life-long exposure may potentially contribute to hypertension. To examine participation of the sympathetic nervous system (SNS) in this effect we examined the sensitivity of pressor responses to ganglionic blockade and measured sympathoadrenal parameters of catecholamine and corticosterone synthesis and release. Male offspring were exposed to the industrial PBDE mixture, DE-71, or corn oil vehicle perinatally (GD6-PND21). In adulthood, offspring received hyperosmotic (3.5 M NaCl) or normosmotic (0.9g%) saline injection (0.6cc/100g, i.p.) with or without ganglionic blockade. Hypersomotic treatment produced a significant increase (23.0 ± 2.55 % of baseline) in systolic blood pressure 3 hours post-treatment in PBDE-dosed but not in oil-dosed controls ($p<0.001$, $n=22$). Ganglionic blockade was effective, reducing the PBDE-induced pressor responses to hyperosmotic stimulation to 12.9 ± 1.3% of baseline ($p<0.01$, $n=12$). PBDE-exposed animals also had significantly lower adrenal catecholamine (CA) content under hyperosmotic conditions ($p<0.05$, $n=23$) but heart rate variability analysis did not indicate increased sympathetic drive ($n=24$, $p<0.05$). Taken together, these findings indicate a significant sympathoadrenal involvement in the PBDE-mediated pressor effect. Adrenal mRNA levels of pituitary adenylate cyclase-activating polypeptide (PACAP) and tyrosine hydroxylase (TH) were elevated

during hyperosmotic challenge but not in PBDE-dosed rats, suggesting impaired compensatory catecholamine biosynthesis (n=17-18, p<0.01-0.001). Hyperosmotic challenge markedly elevated plasma corticosterone in PBDE-dosed rats (CORT; 200.27 ± 51.51, p<0.05, n=16) vs. PBDE-dosed normosmotic controls (27.58 ± 3.53). A similar trend was found with acute GB treatment alone pointing to SNS involvement in PBDE-mediated disruption of HPA activity (p<0.001, n=19). In combination, these results suggest that developmental exposure to PBDEs results in disruption of sympathoadrenal and HPA endocrine activity associated with elevated pressor responses to hyperosmotic stimulation.

Introduction

Polybrominated diphenyl ethers (PBDEs) are commonly used brominated flame-retardants (BFRs) that are added to polyurethane foam and plastics and can be found prominently in indoor environments (Darnerud, Eriksen et al. 2001; (EPA) 2010). PBDEs and the structurally analogous compounds polychlorinated biphenyls (PCBs) act as endocrine disruptors and neurotoxins (Costa and Giordano 2007; Kodavanti and Curras-Collazo 2010). Because of an increased rate of manufacture over 30 years, human exposure to BFRs has increased dramatically (WHO 1994; Kodavanti and Curras-Collazo 2010). Alarmingly, PBDE concentrations in human breast milk doubled every 5 years between 1984 and 1997 due, in part, to stricter flammability standards imposed in the U.S. and Europe (Darnerud et al, 2001; Akutsu, Kitagawa et al. 2003; Law et al, 2014).

Children are particularly at high risk for PBDE accumulation showing 2-7 fold greater body burdens for PBDEs than adults because of dust inhalation, hand-to-mouth, placental and breast milk transfer (Johnson-Restrepo and Kannan 2009). New reports show high PBDE body burdens even in children from underdeveloped countries like Mexico, Nicaragua, Korea and China (Pérez-Maldonado, Ramírez-Jiménez et al. 2009; Moon, Lee et al. 2011; Su, Liu et al. 2011) and a recent study found detectable levels of PBDEs in over 75% of children sampled (Gump, Yun et al. 2014). It is suspected that the effects of childhood exposure to these toxicants may persist well into adulthood (Costa and Giordano 2007; Bloom, Jansing et al. 2014).

PCBs and PBDEs have been linked to hypertension and metabolic syndrome in adults (Kreiss, Matthew et al. 1981; Huang, Lessner et al. 2006; Everett, Mainous et al. 2008; Lim, Lee et al. 2008). In particular, a positive association has been found between serum PCB levels and blood pressure in some human subpopulations after controlling for major confounders including age, sex, body mass index, and socioeconomic class (Stehr-Green, Welty et al. 1986; Goncharov, Bloom et al. 2010). Recently, an association has been found between childhood exposure to PBDEs and cardiovascular responses to stress (Gump, Yun et al. 2014). However, there is a paucity of experimental animal studies focused on cardiovascular function and of supporting causal evidence. The few animal studies that have focused on cardiovascular function reported increased cardiovascular risk factors in PCB-exposed rats (Lind, Örberg et al. 2004) and PBDE-exposed zebrafish (Lema, Schultz et al. 2007). Our laboratory has recently shown that developmental

exposure to PBDEs can produce elevated pressor responses to acute hyperosmotic stimulation, a stimulus which does not normally raise systolic blood pressure (Shah, Coburn et al. 2011). These PBDE-triggered pressor responses approach basal blood pressure levels seen in spontaneously hypertensive rats (SHR). The persistent effects of PBDEs, therefore, may result in exaggerated cardiovascular reactivity, exacerbate autonomic responses to stress, and contribute to the pathogenesis of hypertension.

PBDEs have been studied with respect to stress responses. For instance, increased reactivity to stress and elevated plasma corticosterone levels have been noted following DE-71 exposure in mice (Fowles, Fairbrother et al. 1994). Importantly, PBDEs can target the adrenal gland of mammals which is critical for hypothalamo-sympatho-adrenal (HSA) and hypothalamo-pituitary-adrenal (HPA) endocrine responses to physiological and psychogenic stress (Darnerud, Eriksen et al. 2001; Hakk, Larsen et al. 2002). The adrenal gland is highly vascularized and has a high capacity for uptake and storage of lipophilic toxins (Hinson and Raven 2006). One such toxin, PCB 126, for example, increases steroid biosynthesis by altering the expression of synthetic enzymes (Li and Wang 2005). PBDEs (and PCBs) have been demonstrated to alter catecholamine (CA) levels although few studies have focused on the physiological consequences of this and implications for stress responses (Messeri, Bickmeyer et al. 1997; Westerink and Vijverberg 2002; Dingemans, de Groot et al. 2008). In this study we explore whether exaggerated pressor responses seen in PBDE-exposed rats are associated with altered sympathetic drive, HSA, and HPA parameters.

Our results show that PBDE-induced pressor responses to hyperosmotic stimulation are sensitive to autonomic ganglionic blockade. PBDE-dosed rats also show reduced reserves of adrenal catecholamine during hyperosmotic challenge and altered sympathoadrenal activity. Related to the HPA axis stimulated levels of plasma corticosterone appeared elevated but without corresponding changes in gene expression for adrenal glucocorticoid biosynthesis. Our findings indicate that both HSA and HPA endocrine axis are disrupted by developmental exposure to PBDEs. Our findings may have important implications for potential dysregulation of physiological responses to stress and the development of hypertension following developmental exposure to PBDEs. A preliminary account of our findings has been reported in abstract/poster form at the Center for Neuronal-Glial Interactions (CGNI) conference, UC Riverside in January 2014.

Methods:

Animals: All animals were maintained in accordance with the guidelines in National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Long-Evans rats were group-housed 3-4 per cage after weaning in standard polycarbonate cages with heat-treated pine shavings as bedding. Food (Purina Lab Diet) and water were provided *ad libitum* except during the experimental period. Temperature and relative humidity were maintained at 21 ± 2 °C and 50 ± 10%, respectively, under a 12/12 h light/dark cycle. All experiments were performed between 13:00- 18:00h). Experiments were

designed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the IACUC on animal care and use at the University of California, Riverside.

Perinatal Exposure to PBDEs: Dams (6/dose group) were weighed and dosed with the commercial pentaPBDE mixture DE-71 (30.6 mg/kg/day) from gestational day (GD) 6 through postnatal day (PND) 21, except on the day of birthing (PND 0). Dosing was accomplished by dissolving DE-71 (supplied by the Great Lakes Chemical Corporation, El Dorado, AR, lot# 1550OI18A) in corn oil which was loaded (2ml/kg b.w./day) into cheese puff snacks (Cheetohs, Frito Lays). Control dams received cheese puffs loaded with corn oil vehicle (2ml/kg b.w./day). Rat litters were reduced to 8 pups to optimize lactational transfer from mother to pups. Pup weight and size were monitored to determine non-specific effects of DE-71. No significant differences were found in litter size or pup viability between vehicle and DE-71 groups as described previously (Shah, Coburn et al. 2011). In **Figure 2.3** we show that body weights taken between PND 23-59 (before experimentation) were not significantly different between male offspring of oil- and PBDE-dosed dams.

The commercial pentaPBDE mixture, DE-71 contains the individual congeners PBDE 47, 99, 100, 153 and 154 which are biologically relevant (Kodavanti and Curras-Collazo 2010). The dose of DE-71 (close to the lowest-observed-adverse-effect level (LOAEL) value of 1 mg/kg/day; Darnerud et al, 2001) was selected to match that used in our

previous report showing pressor responses to hyperosmotic stimulation in PBDE- but not oil-dosed hyperosmotic rats (Shah, Coburn et al. 2011). This dosing regimen yields PBDE levels in milk and fat of pregnant dams of ~1000 μg/g lipid but the concentration in neonatal gut and brain are significantly lower (Kodavanti, Coburn et al. 2010). This concentration is one to two orders of magnitude higher than what was reported for human milk from the U.S. (Johnson-Restrepo and Kannan 2009). However, the concentration used is more realistic with regard to highly exposed infants and toddlers since California dust samples from low income California households contained PBDE levels that are 20 times higher than those reported elsewhere in US (Quirós-Alcalá, Bradman et al. 2011).

In vivo osmotic challenge and plasma osmolality measurements. Normosmotic and hyperosmotic groups received 0.15 M or 3.5 M NaCl containing 10 mM HEPES, pH 7.4 (0.6 cc/100g body weight, i.p.), respectively (Shah, Coburn et al. 2011). Water was withheld to prevent normalization of plasma osmolality by drinking (Gillard, Coburn et al. 2007). This method was chosen because it produces elevation of plasma osmolality without appreciably altering blood volume (Dunn, Brennan et al. 1973; Landgraf, Neumann et al. 1988). Aliquots of plasma samples taken via cardiac puncture was brought to room temperature in order to measure plasma osmolality using a vapor-pressure osmometer (Wescor).

Non□Invasive Blood Pressure (NIBP): NIBP was measured by tail-cuff plethysmography using a manual blood pressure monitoring system with manual deflation (IITC) as

described (Shah, Coburn et al. 2011). Systolic blood pressure (highest pressure at which the heart pulses return), mean arterial pressure (highest amplitude pulses) and heart rate was recorded (Rodriguez-Iturbe, Zhan et al. 2003; Kamba, Tam et al. 2006). All measures were obtained under light anesthesia (1.75% isoflurane in O_2; 2L/min) after a brief induction (5% isoflurane in O_2; 1L/min). A heat lamp was used to maintain the animal's temperature near 37^o C. Baseline NIBP recordings were made 5-7 days prior to manipulation and values were recorded as an average of three NIBP recordings. Diastolic blood pressure was determined using MAP = diastolic BP + 1/3 PP, where PP is calculated as systolic BP − diastolic BP. When compared to simultaneous measurements with an invasive catheter and transducer method, NIBP values vary no more than 7%. "Sensitivity" of the NIBP transducers are ± 1 mmHg in the range of 30-300 mmHg. The pulse-based tail-cuff method used here provides values with a strong correlation (0.98) to those generated using radiotelemetry (Whitesall, Hoff et al. 2004).

Ganglionic Blockade (GB): To determine the degree to which pressor responses to hyperosmotic stimulation in PBDE-exposed rats is dependent on sympathetic drive we examined NIBP and other parameters in the absence and presence of ganglionic blockade. Pentolinium tartrate (R278084, Sigma) was used to block systemic nicotinic acetylcholine receptors in autonomic ganglia. Pentolinium was prepared in a 25% polyvinylpyrrolidone (PVP-40T, Sigma) solution and injected subcutaneously (19.2 mg/kg b.w.). This method allows for gradual release during the experimental period (Osmond, Mavrogiannis et al. 1998; Amfilochiadis, Papageorgiou et al. 2004;

Papageorgiou, Simos et al. 2009). In order to determine whether the dose/duration of the pentolinium treatment was fully blocking ganglionic transmission, we tested the effect of acute treatment with a different ganglionic blocker, hexamethonium chloride (Sigma H2138, 10 mg/kg b.w., i.p.) 2.5 hr after pentolinium administration (and 20 minutes prior to NIBP testing).

Heart rate variability: Heart rate variability was recorded via electrocardiogram (ECG) under light anesthesia (1.75% isoflurane in O_2; 1L/min) following a brief induction (5% isoflurane in O_2; 2L/min). ECG recordings were obtained using PowerLab 15T data acquisition hardware controlled by *LabChart8* software (ADInstruments). Data from the ECG recording was exported and smoothed with a Savitzky-Golay filter (Savitzky and Golay 1964) using custom Python software (in collaboration with Dr. Chris Wilson, Loma Linda University, Loma Linda, CA). Data was analyzed via open source gHRV software (Milegroup) using time-domain parameters, non-linear analysis, and frame-based analysis. Band limits for HRV were high frequency 0.150-0.400 (HF) and low frequency 0.050-0.150 (LF). Data was reported as LF/HF ratio.

Plasma corticosterone (CORT): Following NIBP and ECG recording, anesthesia was increased (4% isoflurane in O_2; 2L/min). An incision was made in the chest to expose the heart and blood was removed via cardiac puncture using a 16g needle. Blood was immediately centrifuged at 4^{o}C and plasma was stored at -20^{o}C until analysis. Plasma CORT was measured using a commercially available competitive enzyme-linked

immunosorbent assay (ELISA) kit (Abcam, AB108821). The CORT assay is cross-reactive with mouse, rat, human, and baboon and is sensitive to 0.3 ng/ml in a range of 0.391 ng/ml to 100 ng/ml.

CA assay: At sacrifice adrenals were harvested and snap frozen and stored at -80 oC until later use. Catecholamine content in adrenal glands was determined via fluorometric detection using a modification of published protocols (Wakade 1981; Wakade, Blank et al. 1991; Guo and Wakade 1994). The assay used is a non-specific catecholamine assay that detects dopamine, epinephrine and epinephrine. To obtain a standard curve, norepinephrine was dissolved in 0.05N perchloric acid (PCA) and serial dilutions were made. For samples, the right adrenal gland was dissected at sacrifice, snap frozen on dry ice and later homogenized in 400 ul PCA. Eighty-five μL of each blank, standard, or sample was added to each well of a sterile 96 well plate. Using a multi-well pipette 3.7 μL of $K_3Fe(CN)_6$ solution was added to each well and the plate was shaken gently. The reaction was stopped after 3 minutes by adding 34 μL of ascorbic acid solution to each well. Absorbance was measured at 450nm and 560nm. Absorbances obtained at 560 and those obtained for blanks at 450 nm were subtracted from sample absorbances. Absorbance was converted to catecholamine concentration using the best-fit four-power polynomial equation. All curves used had a correlation coefficient greater than 0.99. Data are expressed in mM units. In our lab this assay has a sensitivity of 0.125 mM, sufficient to detect changes at the adrenal level.

Quantitative Polymerase Chain Reaction (qPCR): Total RNA was prepared from snap-frozen adrenal glands using phenol-chloroform extraction. qPCR was used to examine adrenomedullary markers, tyrosine hydroxylase (TH), the first synthetic and rate-limiting enzyme in the biosynthesis of catecholamines (CA), and phenylethanolamine N-methyltransferase (PNMT) which methylates norepinephrine to epinephrine (Wong and Tank 2007). We also measured gene expression of pituitary adenylate cyclase-activating polypeptide (PACAP) which is a potent regulator of CA release and is emerging as a master regulator of autonomic, endocrine and behavioral parameters of stress responses (Malhotra and Wakade 1987; Guo and Wakade 1994; Lamouche, Martineau et al. 1999; Stroth, Holighaus et al. 2011; Smith and Eiden 2012). qPCR was also employed to examine gene markers of activity and responsiveness of the HPA, including genes involved in regulating glucocorticoid synthesis in and release from the adrenal gland. We examined the HPA-relevant genes encoding 1) steroidogenic acute regulatory protein (StAR) a chaperone protein important for biosynthesis of corticosterone (CORT) from cholesterol (Stocco and Clark 1996; Raff, Hong et al. 2003), 2) melanocortin 2 receptor (MC2R), which responds to ACTH, and 3) melanocortin receptor accessory protein (MRAP) which is essential for the cell surface trafficking and signaling of MC2R receptors (Chida, Nakagawa et al. 2007).

The nucleotide sequences and accession numbers of qPCR primers are listed in **Table 2.1**. PCR primers were tested first via standard RT-PCR and gel electrophoresis to confirm that the primers generated a single band of the expected PCR product size.

Primers were then tested via qPCR with a melt curve to rule out the presence of primer-dimers. qPCR was performed using the Bioline SensiFAST™ SYBR No-ROX One-Step Kit and the Bio-Rad CFX 96 Real-Time PCR Detection System. A temperature gradient from 52-59^{o} C was used on the Bio-Rad CFX 96 station to capture all ideal annealing temperatures. The reaction was run for 40 cycles. Genes of interest were normalized to the housekeeping gene β-actin. Results are presented as relative expression using the equation described in Pfaffl (Pfaffl 2001).

Statistical analysis. Multifactor analysis was carried out using two-way ANOVA where data met normal distribution/equal variance assumptions. Pairwise comparisons were made using a post-hoc Bonferroni test applied following general linear model ANOVA. Two-group comparisons were determined using Student's t-test. Statistical significance was acknowledged at an alpha level of 0.05.

Results

Plasma osmolality is elevated in all groups stimulated with hypertonic saline injection. Ganglionic blockade does not significantly alter plasma osmolality. **Figure 2.2** shows that hyperosmotic stimulation used as described maintains plasma osmolality elevated over the course of the 3 hr experimental period. A two-way ANOVA comparing all experimental groups revealed a significant effect of hyperosmotic stress on plasma osmolality ($F_{1,17}$=53.86, n=21, $p<0.0001$) as expected. Bonferonni post-hoc analysis

showed a significant difference between PBDE norm and PBDE hyper ($t=5.197$, $n=11$, $p<0.001$). Similar effects of hyperosmotic treatment were observed in oil controls ($t=5.796$, $n=11$, $p<0.001$). There was no significant effect of ganglionic blockade on plasma osmolality.

Exaggerated pressor responses to hyperosmotic stress displayed in rats developmentally exposed to DE□71 is significantly reduced by ganglionic blockade. We have previously shown that developmental exposure to DE-71 (30 mg/ml) in *conscious, tamed* rats predisposed these animals to exaggerated blood pressure responses to hyperosmotic stimulation (Shah, Coburn et al. 2011). In this study we first tested whether a similar effect of developmental PBDEs was displayed in *anesthetized* rats. **Table 2.2** shows the absolute NIBP values obtained for systolic and diastolic blood pressure, and heart rate prior to osmotic challenge. There were no statistically significant differences between the groups. NIBP values measured were as expected for rats under isoflurane anesthesia (Albrecht, Henke et al. 2014). In **Fig. 2.3A** we compare the average values (mean ± s.e.m.) for systolic blood pressure, expressed as a percent of baseline, for 4 groups of rats: oil-dosed normosmotic (oil Norm), oil-dosed hyperosmotic (oil Hyper), PBDE-dosed normosmotic (PBDE Norm), and PBDE-dosed hyperosmotic (PBDE Hyper). A two-way ANOVA of systolic blood pressure recorded from oil controls and PBDE animals receiving normosmotic or hyperosmotic treatment showed statistically significant effects of interaction ($F_{1,18}=5.437$, $p<0.05$, $n=22$), PBDE exposure ($F_{1,18} = 27.06$, $p<0.0001$), and hyperosmotic treatment ($F_{1,18} = 13.84$, $p<0.01$). Post-hoc comparisons indicated that

PBDE-dosed hyperosmotic rats but not corresponding controls displayed significantly elevated systolic blood pressure as compared to PBDE-dosed normosmotic controls ($t=5.327$, $n=11$, $p<0.001$).

To determine the degree to which exaggerated pressor responses to hyperosmotic stimulation in PBDE-exposed rats was dependent on sympathetic drive we examined NIBP recordings in the presence of pentolinium tartrate (19.2 mg/kg b.w.) to block systemic nicotinic acetylcholine receptors in autonomic ganglia. **Figure 2.3A** shows that the stimulated pressor responses unique to PBDE hyperosmotic rats was partially but significantly reduced under ganglionic blockade. A two-way ANOVA of systolic blood pressure values in hyperosmotically stimulated PBDE rats in the presence or absence of pentolinium showed significant effects of interaction ($F_{1,20}=6.385$, $p<0.05$, $n=24$), and PBDE exposure ($F_{1,20}=50.90$, $p<0.0001$). Under ganglionic blockade, PBDE-dosed hyperosmotic rats had significantly reduced blood pressure (12.85 ± 1.3% elevation over baseline), as compared to PBDE hyperosmotic group without GB (22.99 ± 2.55 % elevation over baseline; $t=3.824$, $n=12$, $p<0.001$). To rule out incomplete ganglionic blockade we performed separate experiments in which an additional acute treatment with Hex (10mg/kg, i.p.) was given 2.5 hr after administration of pentolinium and 20 min prior to NIBP. Additional treatment with Hex produced no additional decrease in systolic blood pressure in PBDE hyperosmotic rats indicating that the dose and duration of pentolinium used was completely efficacious (**Fig. 2.3B**).

Hyperosmotic stimulation of rats developmentally exposed to DE□71 produced elevated diastolic and mean arterial pressure without sensitivity to ganglionic blockade. **Figure 2.4A** shows the mean (± s.e.m.) values for mean arterial blood pressure (MAP) for all experimental groups tested at 3 hr post-injection. A two-way ANOVA of mean arterial pressure recorded from oil and PBDE-dosed animals under normosmotic and hyperosmotic conditions showed significant effects of interaction ($F_{1,18}$=10.37, p<0.001, n=22), PBDE exposure ($F_{1,18}$=17.77, p<0.001), and hyperosmotic treatment ($F_{1,18}$=28.15, p<0.001). Post-hoc comparisons using Bonferroni test indicated that PBDE-dosed hyperosmotic animals had significantly elevated MAP relative to PBDE-dosed normosmotic rats (t=5.034, n=11, P<0.001). Similar changes were seen in PBDE Hyper + GB group (t=2.999, n=14, p<0.05). Therefore, PBDE-dosed rats receiving GB did not display a significant reduction in mean arterial pressure (9.84 ± 2.67% elevation over baseline), as compared to PBDE hyperosmotic without GB (14.85 ± 1.03 % elevation over baseline; t=1.347, n=14, p=0.20).

Mean values of diastolic blood pressure showed a similar trend as those of MAP. **Figure 2.4B** shows that in PBDE-dosed hyperosmotic rats diastolic blood pressure increased to a significantly greater extent (14.09 ± 1.89%) than in PBDE-dosed normosmotic rats (-1.26 ± 1.48 %). A two-way ANOVA of diastolic blood pressure values recorded from oil- and PBDE-dosed animals under normosmotic and hyperosmotic conditions showed significant effects of interaction ($F_{1,20}$= 9.089, p<0.01, n=24), PBDE exposure ($F_{1,20}$=18.45, p<0.001), and hyperosmotic treatment ($F_{1,20}$=24.53, p<0.0001). Post-hoc

comparisons using Bonferroni test indicated that PBDE-dosed hyperosmotic rats had significantly elevated diastolic blood pressure as compared to PBDE-dosed normosmotic rats ($t=5.133$, $n=13$, $p<0.001$). Similar changes were seen in PBDE Hyper + GB group ($t=3.881$, $n=19$, $p<0.01$). Therefore, PBDE-dosed hyperosmotic rats receiving GB did not display a significant reduction in diastolic blood pressure (13.58 ± 2.7% elevation over baseline), as compared to PBDE hyperosmotic without GB (14.09 ± 1.89 % elevation over baseline; $t=0.058$, $n=12$, $p=0.88$).

Perinatal dosing with PBDE results in reduced heart rate variability as measured by LF/HF ratio. Because GB treatment significantly reduced the exaggerated pressor responses seen in PBDE-dosed hyperosmotic rats, we estimated the relative contribution of parasympathetic and sympathetic tone in PBDE-dosed animals. **Figure 2.5** shows that PBDE hyperosmotic rats failed to show a higher LF/HF ratio indicative of sympathetic overactivity when compared to PBDE-dosed normosmotic rats. In contrast, PBDE-dosed normosmotic rats displayed significantly lower LF/HF ratio compared to oil-dosed normosmotic controls (4.91 ± 0.88 vs 2.74 ± 0.32, $n=15$, $p<0.05$, **Fig. 2.5A**). No changes were found in heart rate between any groups studied (**Fig. 2.5B**).

Developmental exposure to DE□71 markedly reduces adrenal CA content during hyperosmotic stimulation in adulthood. To examine the possibility of altered HSA activity in rats perinatally dosed with DE-71 we examined adrenal CA content in all groups. **Figure 2.6** shows similar adrenal levels of CA amongst all groups except

hyperosmotic PBDE-dosed which had 76% lower adrenal CA content vs normosmotic treated PBDE-dosed animals. Mean adrenal catecholamine (CA) content for PBDE-exposed rats under hyperosmotic vs normosmotic conditions was 2.21 ± 1.29 vs. 1.29 ±0.43 mM. A two-way ANOVA revealed significant effects of hyperosmotic stimulation ($F_{1,19}$=4.669, $p<0.05$, n=23). Post-hoc analysis via Bonferroni test revealed that PBDE-dosed hyperosmotic rats had significantly reduced adrenal CA concentration when compared with PBDE-dosed normosmotic rats (t=3.031, n=14, $p<0.05$).

Upregulated adrenal PACAP and TH expression in response to hyperosmotic stimulation is blunted in stimulated DE□71 exposed animals. To examine markers of catecholamine biosynthesis and signaling we used qPCR analysis of adrenals collected from all experimental group. **Figure 2.7 A-C** show mRNA levels for genes of interest in PBDE-dosed and oil-dosed rats receiving normosmotic or hyperosmotic stimulation. Specifically, we examined PACAP mRNA expression since it is produced by adrenal chromaffin cells and is a potent regulator of catecholamine secretion (Shiotani, Kimura et al. 1995; Nussdorfer 1996; Conconi, Spinazzi et al. 2006). **Figure 2.7A** shows that mRNA levels in PBDE-dosed hyperosmotic rats were reduced vs oil-dosed hyperosmotic controls. Mean (±s.e.m.) values for PACAP were 0.66 ± 0.27 vs 2.07 ± 0.27, respectively. A two-way ANOVA for adrenal PACAP expression revealed significant effects of PBDE dosing ($F_{1,31}$ = 21.87, $p<0.0001$, n=35) and hyperosmotic treatment ($F_{1,31}$=6.785, $p<0.05$). Bonferroni post-hoc analysis revealed that hyperosmotic treatment elevated mRNA levels (t=2.409, $p<0.05$). PBDE-dosed hyperosmotic rats showed

significantly *reduced* PACAP expression as compared to oil-dosed hyperosmotic rats ($t=4.257$, $n=18$, $p<0.001$). PACAP mRNA levels were also significantly *depressed* under PBDE-dosed normosmotic conditions relative to that of corresponding oil-dosed normosmotic controls ($t=2.371$, $p<0.05$)

The changes observed in TH mRNA levels across groups is represented in **Figure 2.7B.** PBDE exposure also caused blunted upregulation of TH mRNA in response to hyperosmotic stimulation. Mean (± s.e.m.) values for TH were 0.89 ± 0.19 for PBDE hyperosmotic rats and 2.45 ± 0.75 for oil-dosed hyperosmotic rats. A two-way ANOVA for adrenal TH expression (**Fig. 2.7B)** revealed significant effects of PBDE dosing ($F_{1,29}=8.803$, $p<0.01$, $n=33$) and hyperosmotic treatment ($F_{1,20}=5.276$, $p<0.05$). Pairwise comparisons using a post-hoc Bonferroni test revealed that hyperosmotic treatment elevated mRNA levels of TH ($t=2.643$, $p<0.05$). Under hyperosmotic conditions, PBDE-dosed hyperosmotic rats showed significantly *reduced* TH mRNA expression when compared to oil-dosed hyperosmotic controls ($t=3.369$, $n=17$, $p<0.01$). A two-way ANOVA for adrenal PNMT expression (**Fig. 2.7C)** showed a statistically significant effect of PBDE exposure ($F_{1,31}=6.078$, $p<0.05$, $n=35$). A student's t-test revealed that PBDE normosmotic animals displayed significantly lower PNMT expression (0.38 ± 0.17) relative to oil-dosed normosmotic animals (1.08 ± 0.15, $n=16$, $p<0.001$).

To determine if stimulated expression of adrenal markers of catecholamine synthesis was sensitive to ganglionic blockade PACAP, TH and PNMT mRNA levels were measured

during hyperosmotic stimulation in the presence of acute GB treatment **(Fig. 2.7 D, E)**. **Fig 2.7D** shows that GB produces a significant reduction in PACAP expression in oil-dosed hyperosmotic rats suggesting that acute GB treatment blunts adrenal PACAP expression to a degree similar to that produced by developmental exposure to DE-71 (**Fig. 2.7A**). A two-way ANOVA comparing these groups shows a significant effect of both GB ($F_{1,29}$=11.12, p<0.01, n=23) and PBDE exposure ($F_{1,29}$=4.399 P, p<0.05). Bonferroni post-hoc comparisons of PACAP mRNA expression reveal that GB has a significant effect on oil-dosed hyperosmotic animals (t=3.889, p<0.01). Adrenal gene expression of TH was not affected by GB treatment. Similarly, GB treatment does not have significant effects on normosmotic groups or on PNMT mRNA in any experimental group (data not shown).

Perinatal DE□71 dosing causes exaggerated plasma CORT responses during adult hyperosmotic stress; similar effect of GB treatment. To examine the impact of PBDE dosing on HPA activity under hyperosmotic stress, we measured plasma corticosterone (CORT) via ELISA. **Figure 2.8A** shows the absolute concentration appears highest in PBDE hyperosmotic rats. A two-way ANOVA for plasma CORT revealed significant effects of hyperosmotic stimulation ($F_{1,12}$=10.61, p<0.01, n=16). Bonferroni post-hoc analysis revealed that PBDE-dosed hyperosmotic rats had significantly elevated levels of plasma CORT relative to PBDE normosmotic rats (t=3.279, n=16, p<0.05). In contrast, oil-dosed rats showed similar CORT levels under normosmotic and hyperosmotic conditions. However, oil-dosed rats also receiving GB treatment displayed markedly

elevated plasma CORT in response to hyperosmotic stimulation **(Fig. 2.8B**). The results of a two-way ANOVA yielded a significant effect of hyperosmotic stimulation on plasma CORT under ganglionic blockade ($F_{1,15}$=179.2, p<0.0001, n=19). Pairwise comparisons of animals receiving GB using a post-hoc Bonferroni test yielded a significant difference between normosmotic and hyperosmotic rats in oil-dosed controls (t=8.582, n=8, p<0.001) and in PBDE-dosed animals relative to PBDE-dosed normosmotic rats (t=10.56, n=11, p<0.001).

Adrenal gene expression of adrenal HPA markers is unaltered by PBDE exposure or GB treatment. Despite elevated plasma CORT in PBDE-dosed animals, mRNA expression of several adrenal HPA markers (StAR, MRAP, MC2R) was unaltered in PBDE-dosed hyperosmotic animals receiving either perinatal PBDE exposure or GB treatment (**Fig 2.9)**.

Discussion:

In this study we tested the hypothesis that PBDEs alter HSA and HPA activity thereby contributing to cardiovascular toxicity (Lind, Örberg et al. 2004; Lema, Schultz et al. 2007). Confirming our prior work, we found that hyperosmotic stress increased systolic blood pressure in rats exposed to perinatal PBDEs even when studied under anesthesia to remove psychogenic stress influences (Shah, Coburn et al. 2011). We confirmed that the pressor responses are unique to physiological activation with hyperosmotic challenge

since PBDE dosing had no effect under normosmotic conditions. Hyperosmotic stimulation, by itself, did not increase systolic blood pressure in oil-dosed controls. Hypertensive systolic BP responses in hyperosmotic-stimulated PBDE rats resembled baseline values reported for SHR used as a model for essential hypertension (Kim et al, 2010). Hypertensive-like activity produced by developmental DE-71 exposure is congruent with published reports of altered heart structure and increased blood pressure in fish larvae exposed to PBDE-47, a major congener represented in DE-71 (Lema et al, 2007). In combination with epidemiological evidence associating high body burdens of similarly-acting organohalogens like PCBs with hypertension, our findings indicate that developmental exposure to PBDEs may increase the risk of cardiovascular abnormalities and/or disease (Kriess et al, 1981; Lind et al, 2004; Lema et al, 2007; Everett et al, 2008). In the current study we show that the hypertensive effects of DE-71 during physiological activation is present as early as 2 months of age but it should be noted that this effect persists into mature to aged adulthood (14-18 months old; Shah et al, 2011).

Mechanistic experiments aimed at evaluating the contribution of sympathetic activity to pressor responses showed that concurrent GB significantly reduced the pressor response in PBDE-exposed animals indicating that exaggerated HSA activity may contribute to elevated pressor responses during hyperosmotic physiological activation. Because hyperosmotic challenges cause a prompt increase in peripheral sympathetic nerve activity (Chen and Toney 2001; Toney and Stocker 2010) local PBDEs may contribute at this level. A recent study by Gump and colleagues (Gump, Yun et al. 2014) indicated that

certain PBDE congeners are associated with a pattern of elevated β-adrenergic activity following psychological stress in children. Alternatively, PBDEs may have endocrine disruptive effects on catecholamine secretion directly on the adrenal gland as noted in *in vitro* release studies (Westerink and Vijverberg 2002; Dingemans, de Groot et al. 2008). In support of this we noted that adrenal CA content was significantly reduced only under acute hyperosmotic conditions suggesting release during the 3 hr experimental period (see below).

Because GB treatment did not eliminate pressor responses in PBDE-exposed hyperosmotic rats we examined the possibility of incomplete GB blockade using our specific protocol. Additional ganglionic blockade with hexamethonium did not lower systolic blood pressure further in PBDE-exposed animals. We, therefore, concluded that our delayed-release pentolinium method was effective at blocking the sympathetic ganglia. Hexamethonium and pentolinium are quaternary ammonium compounds which block transmission in autonomic ganglia by competition with acetylcholine for ganglionic nicotinic Acetylcholine receptors and decrease responses in blood pressure (Mantegazza, Tyler et al. 1958). Neither drug crosses the blood brain barrier and should have no central effects on blood pressure control. The remaining pressor effect seen in PBDE-exposed hyperosmotic rats after GB may be due to an alternate mechanism including a potenital one of central origin.

At the level of the adrenal gland we examined markers of sympathoexcitatory drive and catecholamine synthesis. While hyperosmotic stimulation significantly upregulated mRNA levels of TH and PACAP but not after prior PBDE exposure, suggesting adrenal dysfunction after perinatal PBDE exposure. PACAP and TH are markers of sympathoexcitation contributing to CA synthesis. Interference of PBDEs with cholinergic excitation via sympathetic preganglionic cholinergic nerve fibers to adrenal gland may be partially responsible for this effect (Johansson, Viberg et al. 2008). Impaired cholinergic systems impacting behavior and cognition have been previously demonstrated in adult mice and rats perinatally exposed to PBDEs (Branchi, Alleva et al. 2002; Branchi, Capone et al. 2003; Kuriyama, Talsness et al. 2005; Viberg, Fredriksson et al. 2007; Johansson, Viberg et al. 2008; Kodavanti and Curras-Collazo 2010). Notably, mRNA expression of PACAP, a regulator of catecholamine production and release in the adrenal gland (Vaudry, Gonzalez et al. 2000; Sun, Song et al. 2007; Girard, Wolf-Johnston et al. 2008), is significantly lower in PBDE-exposed animals under both normosmotic control conditions and following hypertonic stress. In pilot work we have found that PACAP content in PBDE-exposed animals is dramatically suppressed in the hyperosmotically activated PVN (Sanchez Jaramillo et al, 2013; (Curras-Collazo, Leon-Olea et al. 2010). The current finding points to further disruption of PACAP signaling in the adrenal gland.

Prior work has indicated that PACAP produced by adrenal chromaffin cells works in an autocrine or paracrine manner within the gland (Shiotani, Kimura et al. 1995; Nussdorfer 1996; Conconi, Spinazzi et al. 2006) and is a potent regulator of catecholamine secretion.

PACAP is produced and released by chromaffin cells (Shiotani, Kimura et al. 1995; Kantor, Heinzlmann et al. 2002; Mazzocchi, Malendowicz et al. 2002; Conconi, Spinazzi et al. 2006) and PACAP works synergistically with neuronal acetylcholine when the adrenal gland is stimulated by the splanchnic nerve (Malhotra and Wakade 1987; Guo and Wakade 1994; Lamouche, Martineau et al. 1999; Lamouche and Yamaguchi 2003). Furthermore, PACAP is critical for adrenal TH and PNMT mRNA induction following restraint stress (Stroth and Eiden 2010). In the present study, adrenal mRNA expression for TH, like that for PACAP, in PBDE-exposed animals is blunted in response to hyperosmotic stimulation. TH expression and activity appears to be a common target of halogenated toxicants since TH activity is inhibited by methyl bromide, a brominated environmental toxicant (Honma, Miyagawa et al. 1991) and by PCBs (Seegal, Bush et al. 1991; Choksi, Kodavanti et al. 1997) and TH mRNA expression is elevated by PCB 126 (Li and Wang 2005; Hinson & Raven, 2006).

Mean adrenal CA concentration was significantly lower in PBDE-dosed animals receiving acute hyperosmotic challenge as compared to PBDE-dosed normosmotic animals. This finding can be interpreted as a depletion of adrenal CA due to excess release without compensatory repletion and is supported by NIBP and qPCR results. Specifically, adrenal dysfunction in PBDE-dosed animals may result in impaired CA synthesis (due to reduced PACAP and TH mRNA expression). This hypothesis can be used to argue that high body burdens in humans may contribute to adrenal insufficiency

especially under conditions of chronic physiological stress leading to exaggerated cardiovascular and endocrine ailments.

The finding that LF/HF ratios are decreased in our heart rate variability analysis in PBDE animals suggests that under resting conditions, HSA activity in PBDE-dosed animals is attenuated rather than enhanced. A decreased LF/HF ratio suggests a shift from sympathetic to parasympathetic drive to the heart (Task Force and Electrophysiology 1996). Blunted SNS synaptic transmission may, therefore, result in decreased PACAP signaling in the adrenal gland of PBDE-exposed animals and subsequently blunted TH mRNA transcription following hyperosmotic stimulation. In humans some PBDE congeners are associated with elevated sympathetic markers of stress and rats subjected to chronic stress show decreased adrenal hormone synthesizing enzyme mRNAs in response to subsequent stressors (Aguilera, Kiss et al. 1995). The balance between parasympathetic and sympathetic drive appeared similar in PBDE-exposed rats under hyperosmotic and normosmotic conditions suggesting little contribution to raised blood pressure responses. Taken together, it appears that HSA activity and adrenal function are blunted at rest, yet under hyperosmotic stress the sympathetic nervous system produces an exaggerated response which can be attenuated via ganglionic blockade, likely due to catecholamine release.

In the current study we observed that the combination of PBDE dosing and hyperosmotic challenge also elevated diastolic blood pressure. A significant effect on diastolic blood

pressure had not been seen in conscious animals in our prior report perhaps because of the contribution of psychogenic stress or age-related factor to the diastolic blood pressure (Shah et al, 2011). In the present study diastolic blood pressure was measured in rats under light anesthesia which would remove possible psychogenic effects of restraint. Indeed, mean baseline values for diastolic (and systolic) blood pressure under isoflurane were lower than in the previous report as expected and unmasked this additional effect we have reported in hyperosmotically challenged PBDE-dosed animals. Elevated diastolic blood pressure as a function of PBDE exposure and acute hyperosmotic challenge may reflect increased peripheral resistance in PBDE-dosed animals receiving hyperosmotic stimulation. Diastolic and mean arterial pressures were not affected by GB. The differential sensitivity of elevated systolic pressure vs. diastolic blood pressure to ganglionic blockade is consistent with a more pronounced effect of PBDEs on cardiac output rather than on peripheral resistance. Alternatively, the underlying mechanism of action disrupting diastolic blood pressure may be more dependent on altered organization of responsible physiological systems that may be targeted by PBDEs over the course of prenatal and postnatal development.

Emerging evidence suggests that the HPA plays a significant role in human hypertension and associated cardiovascular abnormalities seen in Cushing's disease (Whitworth, Brown et al. 1995; Sundaram, Carluccio et al. 2013). In children and adolescents, altered HPA-axis functioning is associated with cardiovascular risk factors (Prodam, Ricotti et al.). Furthermore, osmotic stimulation has pronounced and rapid effects on mechanisms

of corticosterone release (Watts 1992). PBDE animals had significantly elevated plasma CORT in response to hyperosmotic stimulation. Plasma CORT levels are also elevated in female C57Bl6 mice that are subchronically dosed with DE-71 at a cumulative dose of 250 mg/kg after a 14 day regimen (Fowles, Fairbrother et al. 1994). One difference between our study and previous work is that in our rats CORT levels were elevated only in PBDE-dosed rats with baseline CORT levels showing no significantly changes. PCBs can alter corticosterone responses to stress in rats (Orito, Gotanda et al. 2007), indicating that exposure to organohalogens seems to disrupt the hypothalamo-pituitary-adrenal axis under physiological challenge. Organochlorines and brominated flame retardants have been associated with HPA dysfunction in birds leading to either elevated (Sapolsky, Romero et al. 2000; Verboven, Verreault et al. 2010) or blunted (Lorenzen, Moon et al. 1999; Martinovic, Lean et al. 2003) plasma CORT. Our work in adult rats suggests that HPA disruption during development may lead to increased HPA responsiveness in adulthood.

Despite elevated plasma CORT levels, expression of adrenal HPA markers were not impacted by PBDE exposure suggesting that neuroendocrine disruption of the HPA may occur through a central mechanism or by disrupting glucocorticoid receptor activity (Bovee, Helsdingen et al. 2011) thereby reducing feedback inhibition. Preliminary findings, generated in collaboration with Drs. Sanchez Jaramillo and Leon-Olea, support a disruption of central HPA activity following developmental exposure to PBDEs (Sanchez Jaramillo et al, 2013). A caveat to this interpretation is that acute treatment with

the ganglionic blocker pentolinium tartrate, which does not penetrate centrally (Mantegazza, Tyler et al. 1958), resulted in a significant increase in plasma CORT in oil-dosed hyperosmotic rats, indicating that increased CORT levels seen in these experiments could be partially due to peripheral pituitary-adrenal signaling. This interpretation is supported by our finding that sympathoadrenal suppression via GB reduces PACAP mRNA expression in oil-dosed hyperosmotic animals to a degree that approximates expression levels seen in PBDE-dosed animals. In both instances, developmental exposure to DE-71 appears to produce an effect that is similar to acute ganglionic blockade. Prior work with rats supports this notion as treatment with alpha-methyl-p-tyrosine, an inhibitor of catecholamine synthesis, causes elevated plasma CORT and ACTH levels (Švob Štrac, Muck-Šeler et al. 2012). This unusual effect is reversed when ascending catecholamine projections are removed, indicating that HPA is under feedback regulation by SNS. Specifically, catecholaminergic systems may facilitate negative feedback within the HPA (Kaminski and Watts 2012) and because PBDEs appear to blunt CA synthesis to a degree that approximates ganglionic blockade, elevated plasma CORT in PBDE-dosed animals may be due to impaired CA synthesis at the level of the adrenal gland. Interestingly, cortisol in humans may produce its hypertensive effects through a reciprocal mechanism, by increasing responsiveness to catechols (Whitworth, Brown et al. 1995). These potential interactive mechanisms as targets of endocrine disruption are unexplored for organohalogen pollutants and merits further study.

Because GB only partially decreased pressor responses to hyperosmotic stimulation tonic saline in PBDE-dosed animals it is likely that other factors also contribute to these pressor responses. PCBs, which are structurally and functionally similar to PBDEs also produce cardiovascular toxicity (Lind, Örberg et al. 2004). Some PCBs alter the activity of aryl hydrocarbon receptors (AhR) shown to be involved in blood pressure regulation and are linked to renin-angiotensin system (RAS) activity (Zhang, Agbor et al. 2010). However, unlike PCBs PBDEs are not particularly effective at activating AhRs due to their non-coplanarity (Peters, van Londen et al. 2004; Peters, Nijmeijer et al. 2006). Nevertheless, angiotensin, a potent vasoconstrictor and stimulant for cardiovascular-relevant hormones, aldosterone and vasopressin (Kobori, Nangaku et al. 2007; Saavedra, Sánchez-Lemus et al. 2010) may be involved though nothing is currently known about the effects of PBDEs (or PCBs) on RAS. Alternatively, it is possible that PBDEs could be altering aquaporin (AQ) water channel expression in the kidneys in a manner similar to that seen in uterine AQ channels (Tewari, Kalkunte et al. 2009). PBDE-induced downregulation of AQ transporter expression could result in hypovolemia leading to elevated SNS and RAS compensatory actions (Nielsen, Kwon et al. 1999; Chagnon, Vaidya et al. 2008; Versteilen, Heemskerk et al. 2008). This possibility may explain reduced osmoregulatory capacity seen in mature adult rats previously exposed to DE-71 perinatally (Shah, Coburn et al. 2011). Future work will focus on exploring these possibilities.

Our current findings reveal novel mechanisms that may underlie the pressor effects of DE-71 under conditions of osmotic activation. These include disrupted sympathoadrenal and hypothalamo-pituitary-adrenal activity in PBDE-exposed rats. Specifically, we found enhanced autonomic ganglionic transmission, depleted adrenal catecholamine content, blunted responsivity of sympathoadrenal markers of catecholamine production and activation and elevated plasma CORT. The latter may also contribute to the disruption of cardiovascular responses, based on its association with hypertension in Cushing's disease and on experimental studies showing a cortisol-induced blood pressure rise (Whitworth, Brown et al. 1995; Sundaram, Carluccio et al. 2013). The combination of HSA and HPA disruption seen in DE-71-exposed animals may have additional physiological consequences and merits further study. The degree to which these effects originate in central vs systemic compartments is important to understand in our quest to offset the adverse health effects of cumulative action of organohalogenated pollutants.

We note that cardiovascular toxicity persists long after the developmental exposure period. The Barker hypothesis proposes a developmental basis for adult disease (Barker, Eriksson et al. 2002; Barker, Bagby et al. 2006) and a variation of this hypothesis specifically involves cardiovascular reactivity to stressors in childhood as a predictor of adult hypertension (Falkner, Kushner et al. 1981; Parker, Croft et al. 1987). Our work involves modifying this "reactivity hypothesis" (Light 2001) to include developmental PBDE exposure as a risk factor that may predispose individuals to stress-related diseases later in adulthood. By adding 'history of BFR exposure' to the clinical decision-making

process, it should enable physicians, clinical and social workers to predict which treatment strategies and lifestyle modifications are most likely to benefit high-risk patients with cardiovascular and stress-related ailments.

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Table 2.1: Sequences of qPCR primers used to probe for adrenal genes markers involved in regulating glucocorticoid/mineralocorticoid and catecholamine biosynthesis and induction. Primers listed are for HPA-relevant genes encoding steroidogenic acute regulatory protein (StAR), melanocortin 2 receptor (MC2R), and melanocortin receptor accessory protein (MRAP). qPCR primers for adrenomedullary markers include tyrosine hydroxylase (TH), the first synthetic and rate-limiting enzyme in the biosynthesis of catecholamines, and phenylethanolamine N-methyltransferase (PNMT) which methylates norepinephrine to epinephrine.

Gene	Accession #	Primer	Sequence (5'-3')	Annealing Temp (°C)
β actin	NM_031144	forward	TTCTTGCAGCTCCTCCGTCGC	61.3
		reverse	CACCATCACACCCTGGTGCCTA	
PACAP	NM_016989	forward	TTCGGTGTCACGCTCCCTCCT	61.9
		reverse	GCTACACATGGTCATTCGCGGCT	
StAR	NM_031558	forward	AGCTCTCTACTTGGTTCTCAACTG	58.3
		reverse	CTCCAGTCGGAACACCTTGC	
MRAP	NM_001135834	forward	GATGCCTCTGTCCCGTTCAC	58.1
		reverse	GGGGACTATGCCTTACCTGTG	
MC2R	NM_001100491	forward	TGAGGTTGCACACAGAGCGA	59.2
		reverse	TGTACTTTCCAAACTGCCACG	
TH	NM_012740	forward	GGCTGTCACGTCCCCAAGGTT	63.2
		reverse	GCCCGAGACAAGGAGGAGGGTT	
PNMT	NM_031526.1	forward	TGTCTGGACAGGTCCTCATTGACA	60.0
		reverse	TGAGGCAGACATGCTGGCTATACA	

Table 2.2 Baseline blood pressure measurements under isoflurane anesthesia No significant differences were noted between groups prior to the experimental period in blood pressure or heart rate. Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71.

Baseline NIBP values	Oil	PBDE
Systolic blood pressure (mmHg)	111.9 ± 2.24	109.8 ± 1.457 n=26, P=0.42
Diastolic blood pressure (mmHg)	83.18 ± 1.41	81.85 ± 1.18 n=26, p=0.47
Heart rate (bpm)	385.2 ± 8.48	387.0 ± 7.91 n=26, p=0.88

Fig. 2.1. Long-Evans rats perinatally dosed with DE-71 displayed normal weight gain during development. Throughout the course of development and until the day of sacrifice (PND 59-60), there were no statistically significant differences in absolute body weight between control Long-Evans males and Long-Evans rats perinatally dosed with DE-71. PND = post-natal day, Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71.

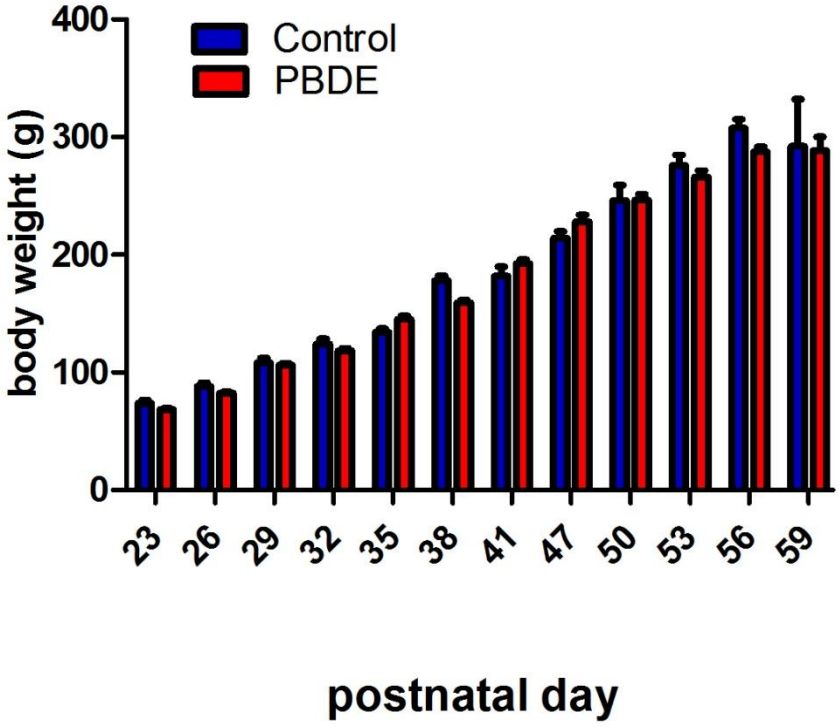

Control
PBDE
body weight (g)
400
300
200
100
0
23
26
29
32
35
38
41
47
50
53
56
59
postnatal day

Fig. 2.2 Acute hyperosmotic stimulation increases plasma osmolality in presence and absence of ganglionic blockade. Blood from anesthetized rats was taken via cardiac puncture at sacrifice three hours post-injection. Hyperosmotic stimulation increased plasma osmolality in all groups including those treated with the ganglionic blocker, pentolinium tartrate. Therefore, osmosensory stimulation should be similar in all groups. Asterisks indicate statistical difference between PBDE Hyper vs PBDE Norm at $p<0.001$(***). ### indicate statistical difference between PBDE Hyper +GB vs PBDE Hyper at $p<0.001$. Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71, GB = ganglionic blockade with pentolinium tartrate

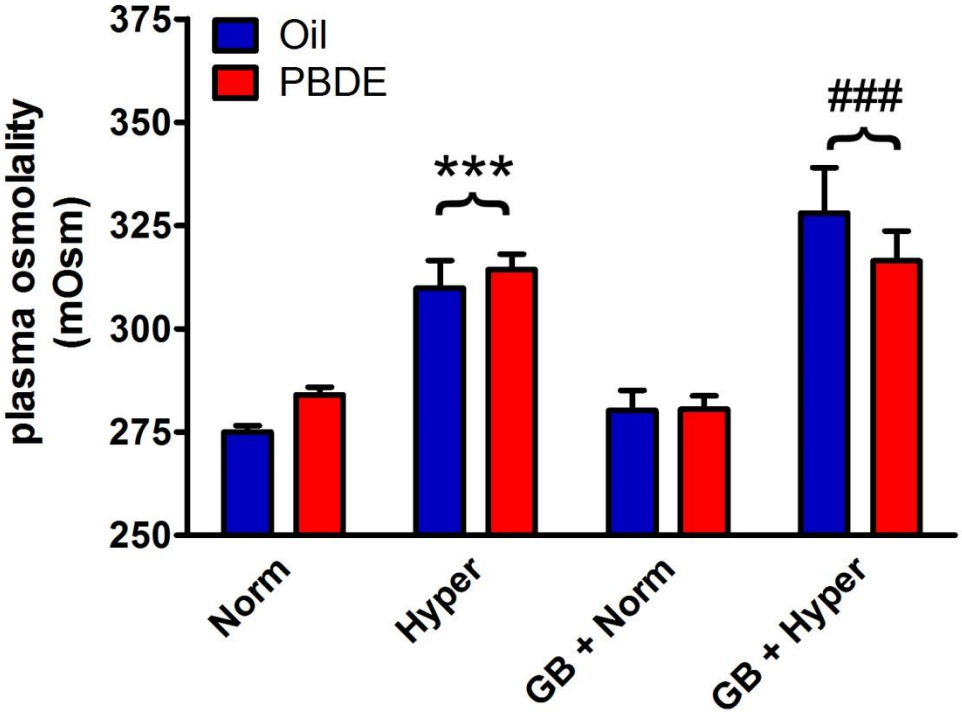

plasma osmolality (mOsm)
375
350
325
300
275
250
Oil
PBDE
***
###
Norm
Hyper
GB + Norm
GB + Hyper

Fig. 2.3 Perinatal DE-71 exposure exaggerates systolic blood pressure responses to hyperosmotic stress in adult rats; sensitivity to ganglionic blockade. Rats were dosed perinatally with DE-71 and in adulthood were treated with either hyperosmotic or normosmotic stimulation in the presence or absence of the ganglionic blocker, pentolinium (GB). After 3 hours of stimulation blood pressure was measured via NIBP. **A:** PBDE-dosed hyperosmotic animals displayed a significant increase in systolic blood pressure compared to oil-dosed hyperosmotic controls. Ganglionic blockade (GB) significantly reduced the stimulated rise in systolic blood pressure seen in PBDE hyperosmotic rats. **B:** Acute treatment with an additional ganglionic blocker (hexamethonium; Hex) produced no further decrease in systolic blood pressure in PBDE-dosed hyperosmotic animals receiving GB. Asterisks indicate statistical significance as compared to PBDE-dosed normosmotic rats $p<0.001$ (***). Hashtags indicate statistical significance as compared to PBDE-dosed hyperosmotic rats $p<0001$(###). GB = ganglionic blockade using pentolinium tartrate, Hex = hexamethonium, Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71

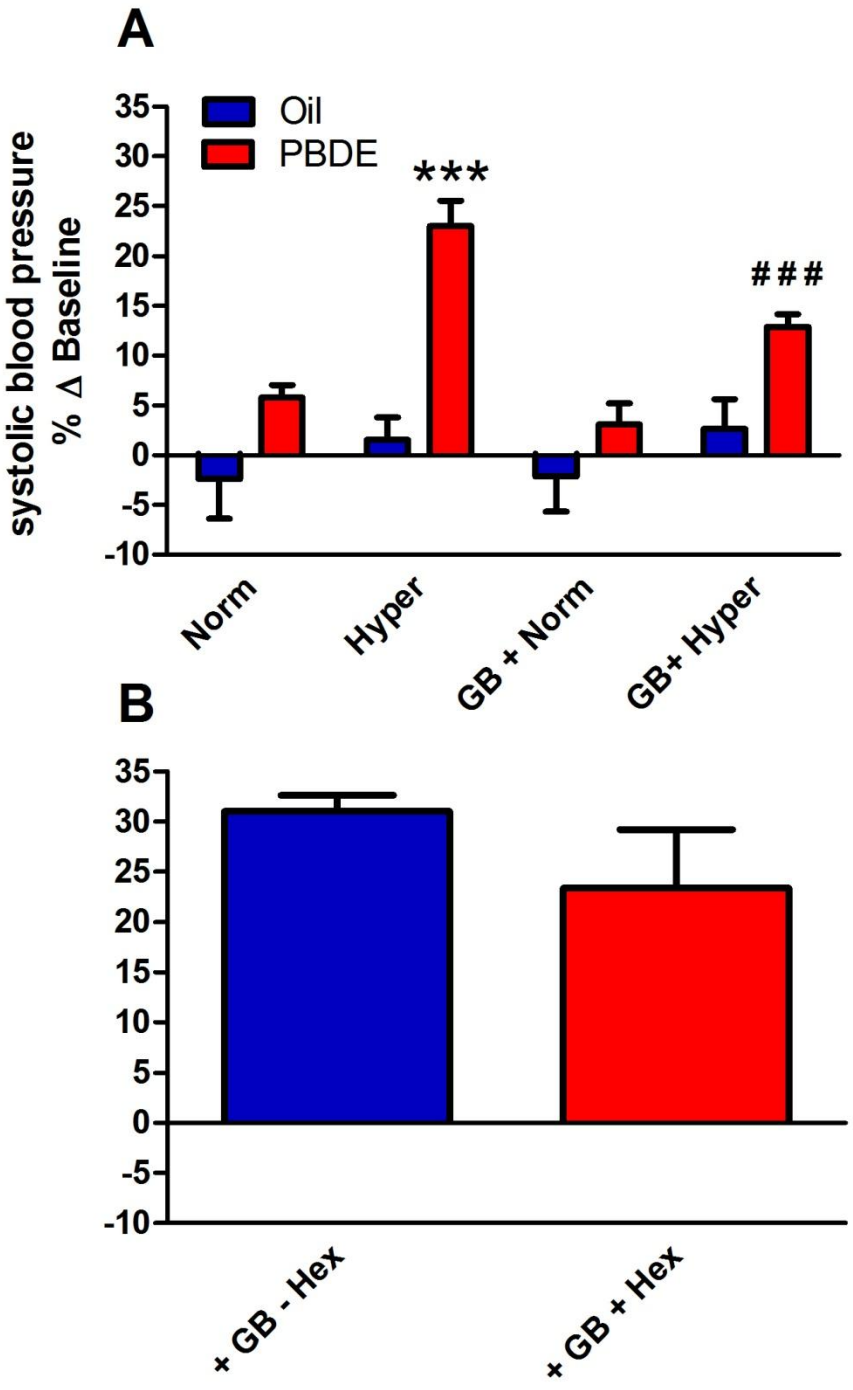

A
Oil
PBDE
***
###
systolic blood pressure
% Δ Baseline
35
30
25
20
15
10
5
0
-5
-10
Norm
Hyper
GB + Norm
GB+ Hyper
B
35
30
25
20
15
10
5
0
-5
-10
+ GB - Hex
+ GB + Hex

Fig. 2.4 Perinatal DE-71 exposure significantly elevates diastolic and mean arterial pressure during hyperosmotic stimulation in adulthood. Adult rats perinatally exposed to DE-71 received hyperosmotic or normosmotic injection with (+GB) or without ganglionic blockade. After 3 hours of stimulation blood pressure was measured via NIBP under anesthesia. **A:** PBDE-dosed hyperosmotic rats showed a significant increase in their mean arterial blood pressure (MAP) compared to PBDE-dosed normosmotic controls ($t= 5.034$, $p<0.001$). **B**: PBDE-dosed hyperosmotic rats displayed a significant increase in their diastolic blood pressure compared to PBDE-dosed normosmotic controls ($t= 5.133$, $p<0.001$). Ganglionic blockade (GB) with pentolinium did not significantly reduce average values of mean arterial or diastolic blood pressure. Asterisks indicate a statistically significant reduction as compared to the corresponding normosmotic controls, $p<0.01$ (**), $p<0.001$ (***). GB = ganglionic blockade using pentolinium tartrate, Oil = Oil-dosed controls, PBDE = rats perinatally dosed

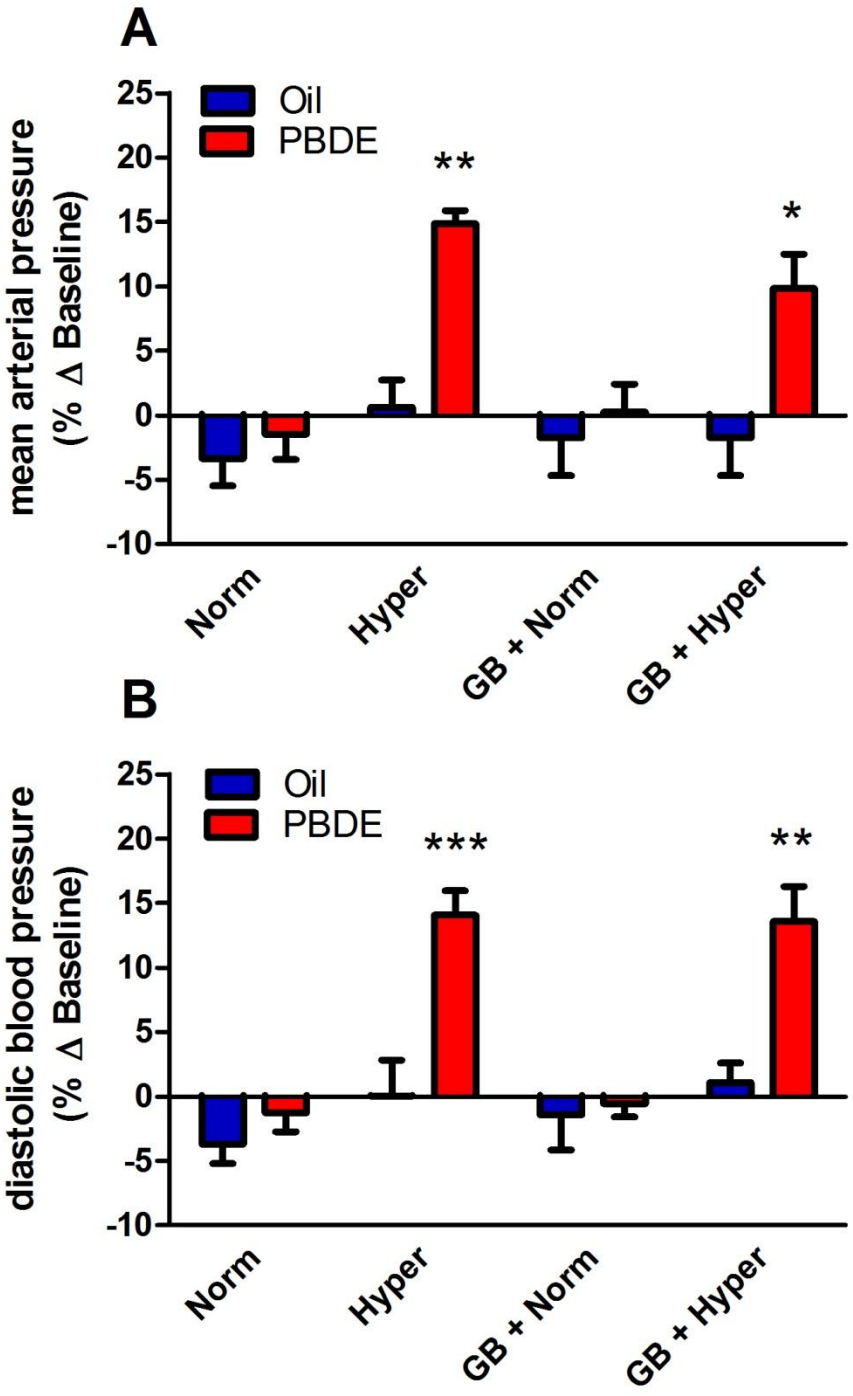

A
mean arterial pressure
(% Δ Baseline)
Oil
PBDE
25
20
15
10
5
0
-5
-10
**
*
Norm
Hyper
GB + Norm
GB + Hyper
B
diastolic blood pressure
(% Δ Baseline)
Oil
PBDE
25
20
15
10
5
0
-5
-10
***
**
Norm
Hyper
GB + Norm
GB + Hyper

Fig. 2.5 Effects of perinatal dosing with DE-71 on heart rate variability as measured by LF/HF ratio. PBDE hyperosmotic rats failed to show a higher LF/HF ratio indicative of sympathetic overactivity when compared to PBDE-dosed normosmotic rats. In contrast, PBDE-dosed normosmotic rats displayed significantly lower LF/HF ratio compared to oil-dosed normosmotic controls. Asterisks indicate statistical significance via student's t-test at $p<0.05$(^). LF = low frequency, HF = high frequency, Oil= oil-dosed controls, PBDE = rats perinatally dosed with DE-71

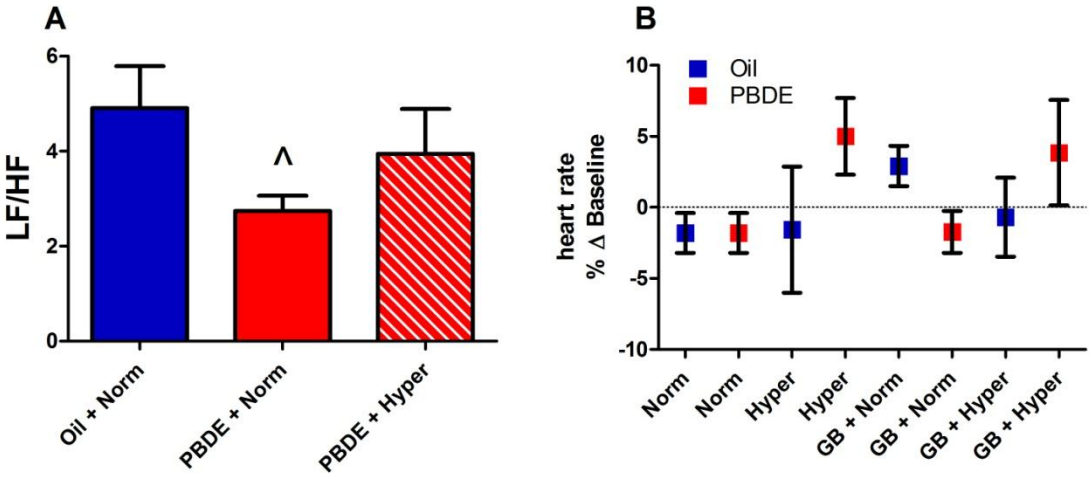

A
LF/HF
6
4
2
0
^
Oil + Norm
PBDE + Norm
PBDE + Hyper
B
heart rate
% Δ Baseline
10
5
0
-5
-10
Oil
PBDE
Norm
Norm
Hyper
Hyper
GB + Norm
GB + Norm
GB + Hyper
GB + Hyper

Fig. 2.6 Developmental exposure to DE-71 markedly reduces adrenal catecholamine content during hyperosmotic stimulation in adulthood. PBDE-exposed animals had significantly lower adrenal catecholamine (CA) content after a 3 hr hyperosmotic challenge when compared to PBDE-dosed animals receiving normosmotic treatment. Asterisk indicate statistical significance at $p<0.05$ (*). GB = ganglionic blockade using pentolinium tartrate, Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71.

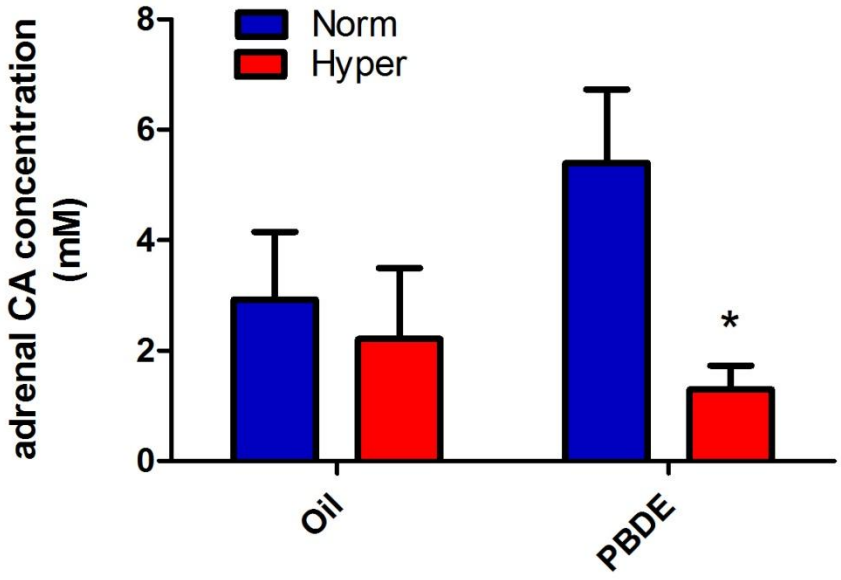

adrenal CA concentration (mM)
8
6
4
2
0
Norm
Hyper
*
Oil
PBDE

Fig. 2.7 Upregulated adrenal PACAP and TH expression in response to hyperosmotic stimulation is blunted in stimulated DE-71 exposed animals. Offspring perinatally exposed to DE-71 were hyperosmotically challenged in adulthood. To examine the effect of PBDEs on adrenal markers of catecholamine biosynthesis and signaling we used qPCR analysis of adrenals collected at sacrifice. Hyperosmotic treatment upregulated adrenal PACAP and TH mRNA levels (A and B). A: PACAP: PBDE-dosed rats showed significantly *reduced* PACAP gene expression under normosmotic conditions and in response to hyperosmotic stimulation. **B:** TH: PBDE-dosed rats showed significantly *reduced* TH mRNA expression under hyperosmotic conditions when compared to oil-dosed hyperosmotic controls. **C:** Adrenal PNMT expression was significantly *depressed* in PBDE-dosed normosmotic relative to oil-dosed normosmotic rats. **D and E**: Ganglionic blockade blunts stimulated PACAP mRNA levels to a degree that is similar to that achieved in PBDE-exposed rats. Adrenal PACAP and TH expression in GB-treated hyperosmotic rats. GB significantly reduces PACAP mRNA (D) but not TH expression (E) in oil-dosed hyperosmotic animals. Asterisks indicate statistical significance as compared to oil controls receiving the normosmotic stimulation at $p<0.05$(^). Hashtags indicate statistical significance relative to oil-dosed hyperosmotic controls $p<0.01$ ($\alpha\alpha$) and $p<0.001$ ($\alpha\alpha\alpha$). Carets indicate statistical significance relative to oil-dosed normosmotic controls at $p<0.05$ (^), and $p<0.001$(^^^). Symbols indicate statistical significance as compared to oil controls receiving the same osmotic stimulation at $p<0.01$ ($\alpha\alpha$).

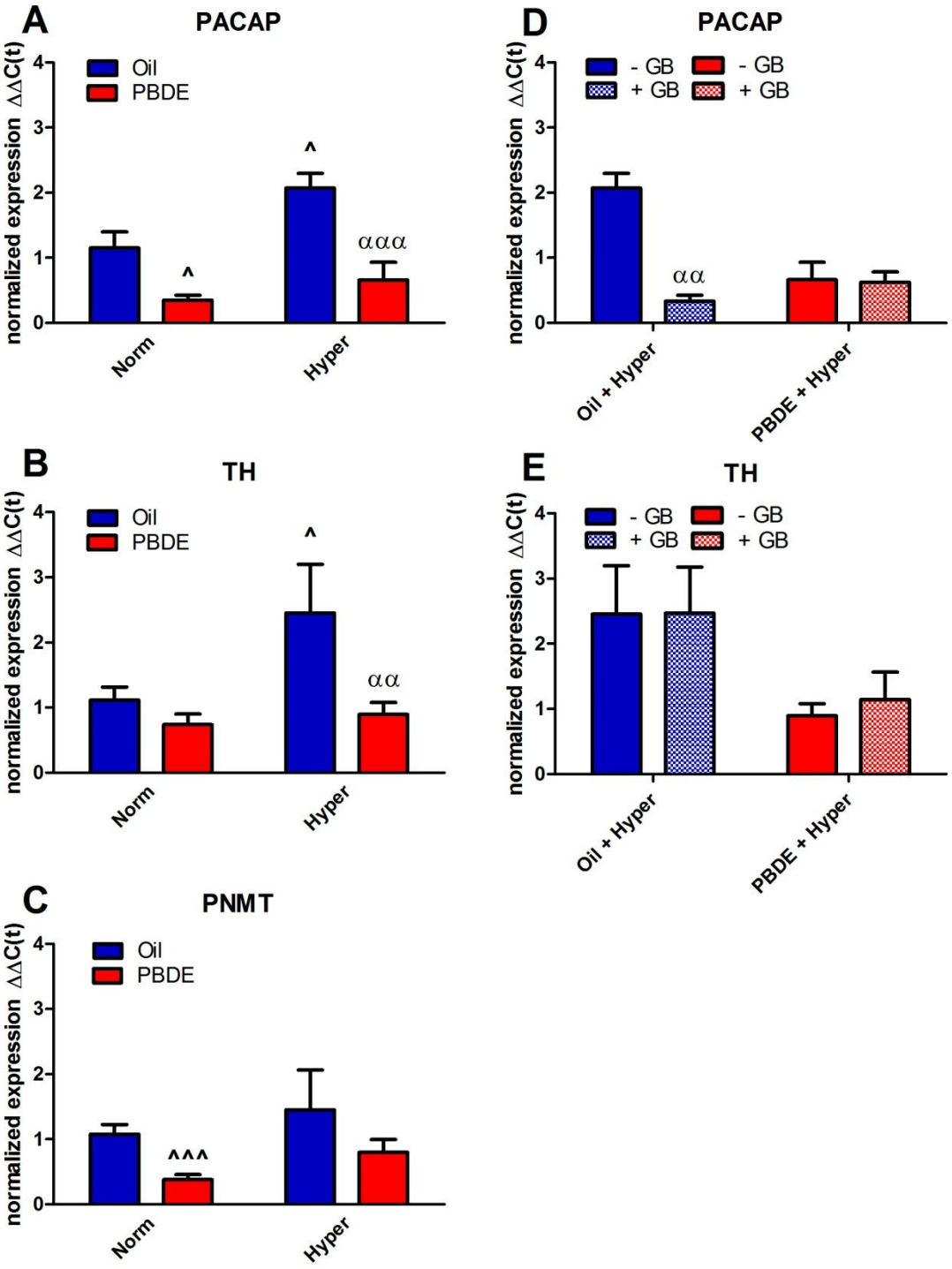

A
PACAP
Oil
PBDE
normalized expression ΔΔC(t)
0
1
2
3
4
^
^
ααα
Norm
Hyper
B
TH
Oil
PBDE
normalized expression ΔΔC(t)
^
αα
Norm
Hyper
C
PNMT
Oil
PBDE
normalized expression ΔΔC(t)
^^^
Norm
Hyper
D
PACAP
- GB
+ GB
- GB
+ GB
normalized expression ΔΔC(t)
αα
Oil + Hyper
PBDE + Hyper
E
TH
- GB
+ GB
- GB
+ GB
normalized expression ΔΔC(t)
Oil + Hyper
PBDE + Hyper

Fig. 2.8 Perinatal DE-71 dosing causes exaggerated plasma CORT responses during adult hyperosmotic stress; similar effect of GB treatment. To examine the impact of early life exposure to stress axis plasma was collected at sacrifice by cardiac puncture in male Long Evans rats. Plasma was assayed for corticosterone using an enzyme-linked immunoassay. **A**: Hyperosmotic stimulation triggered a significant rise in plasma CORT only in DE-71 exposed rats. **B:** Animals also treated with the ganglionic blocker, pentolinium tartrate displayed elevated plasma CORT responses to hyperosmotic stimulation in both oil-dosed controls and DE-71-dosed animals. Asterisks indicate statistical difference relative to PBDE-normosmotic group, $p<0.05$ (*). Symbols indicate statistical difference relative to corresponding normosmotic control receiving GB, $p<0.001$ (βββ). Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71 , GB: ganglionic blockade using pentolinium tartrate

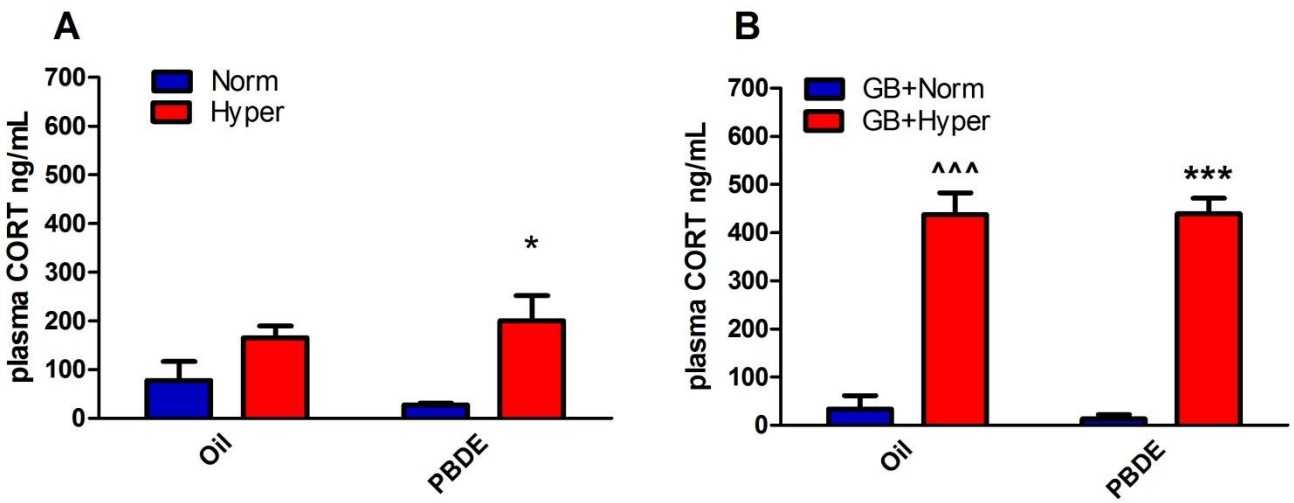
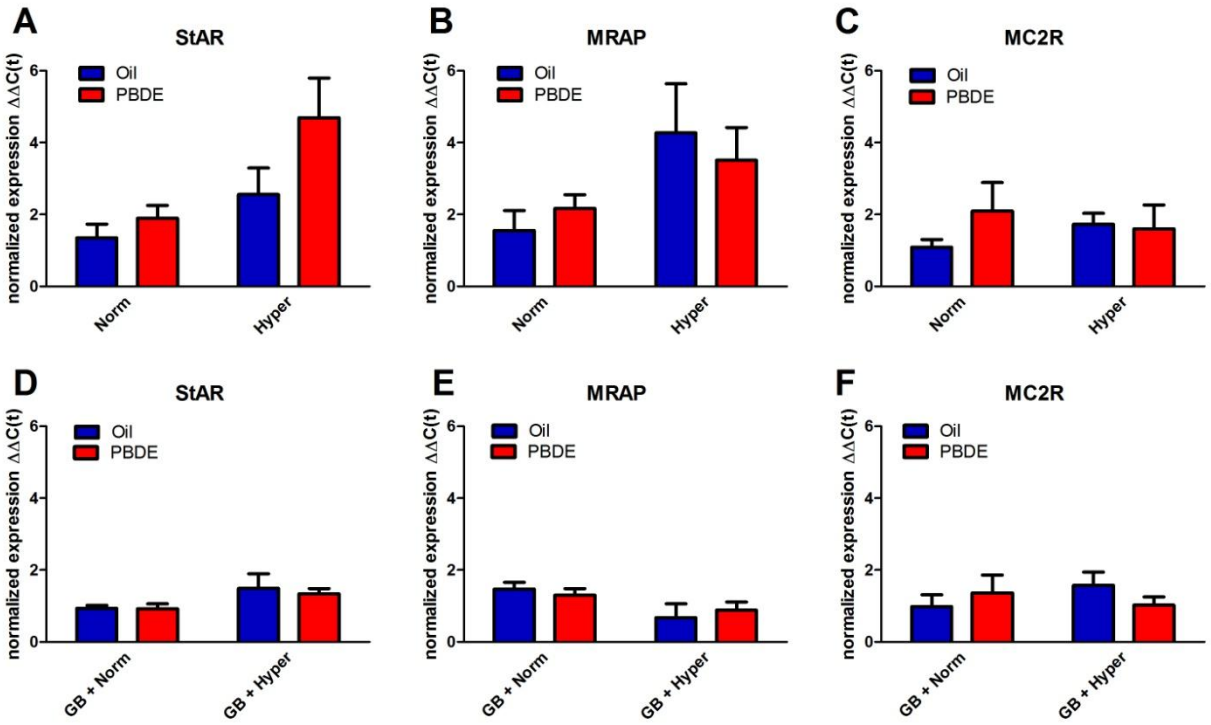

A
Norm
Hyper
plasma CORT ng/mL
700
600
500
400
300
200
100
0
*
Oil
PBDE
B
GB+Norm
GB+Hyper
plasma CORT ng/mL
700
600
500
400
300
200
100
0
^^^
***
Oil
PBDE

Fig 2.9. Adrenal markers for HPA activity are unaffected by perinatal dosing with PBDEs or treatment with GB. A-C: Mean mRNA expression of StAR, MC2R, and MRAP genes associated with CORT production in the adrenal gland were not affected by PBDE-dosing. D-F: Mean mRNA expression of StAR, MC2R, and MRAP genes associated with CORT production in the adrenal gland were not affected by PBDE-dosing. Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71, GB: ganglionic blockade using pentolinium tartrate, StAR = steroidogenic acute regulatory protein, MC2R = adrenal melanocortin 2 receptor, MRAP= melanocortin receptor accessory protein


A
StAR
B
MRAP
C
MC2R
D
StAR
E
MRAP
F
MC2R
Oil
PBDE
normalized expression ΔΔC(t)
0
2
4
6
Norm
Hyper
GB + Norm
GB + Hyper

Chapter 3

Exaggerated pressor responses in adult rats exposed to PBDEs during development are blocked by the anti-hypertensive drug captopril

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Abstract

PBDE organohalogens are common flame retardants used in indoor consumer products including electronics, furnishings, and baby products. We have previously shown that perinatal dosing with PBDEs causes adult rats to exhibit pressor responses to acute hyperosmotic stress. A likely contributor to this response is the renin-angiotensin system which has long been implicated in the pathogenesis of essential hypertension. The abnormal pressor responses to hyperosmotic stress displayed in PBDE-exposed animals (22.99 ± 2.5% Δ baseline) can be abolished by pretreatment with the angiotensin-converting enzyme (ACE) inhibitor, captopril (CAP; -0.085 ± 2.2% Δ baseline, $p<0.001$). PBDE exposed rats display reduced plasma levels of AngII following hyperosmotic stimulation in PBDE-dosed animals (1861.15 ± 440.90 pg/μL) when compared to oil-dosed hyperosmotic animals (7526.30 ± 1407.86 pg/μL, $p<0.01$). Pretreatment with CAP increased plasma AngII responses to hyperosmotic stimulation. PBDE-dosed hyperosmotic animals also had blunted adrenal AT1R expression (0.421 ± 0.08) compared to oil-dosed hyperosmotic animals (1.01 ± 0.18, $p<0.01$) suggesting that CAP may work via mechanisms other than direct inhibition of the RAS to produce this effect. In combination, these results suggest that developmental exposure to PBDEs results in disruption of the renin-angiotensin system contributing to elevated pressor responses to hyperosmotic stimulation.

Introduction:

PBDE organohalogens are used as flame retardants in indoor consumer products including electronics, furnishings, and baby products (WHO 1994; Darnerud, Eriksen et al. 2001; Mariussen and Fonnum 2006). Functionally, PBDEs are similar to polychlorinated biphenyls (PCBs) which are well-documented as endocrine disruptors and neurotoxins (Costa and Giordano 2007; Kodavanti and Curras-Collazo 2010). The production of PCBs has been banned since 1970's, but the global manufacture and use of PBDEs has continued at an increasing rate (WHO 1994; 2000). Infants and children are at risk for high body burdens of PBDEs (and PCBs) due to decreased capacity for detoxification as well as high metabolic demands required during development (Johnson-Restrepo and Kannan 2009). While animal studies on organohalogens such as PCBs and PBDEs have focused on cognitive deficits and endocrine disruption few studies have examined the ability of these toxicants to alter physiological processes, including those related to cardiovascular function. In humans, PCBs have been associated with hypertension, diabetes, and metabolic disease (Kreiss, Zack et al. 1981; Kreiss 1985; Stehr-Green, Welty et al. 1986; Lim, Lee et al. 2008; Goncharov, Bloom et al. 2010) but the mechanisms underlying this association is unknown. Furthermore, cardiovascular risk factors have been documented in PCB-exposed rats (Lind, Örberg et al. 2004), and PBDE-exposed zebrafish (Lema, Schultz et al. 2007). In agreement with the results of these animal studies Gump and others have found an association between cardiovascular responses to stress in adulthood and childhood exposure to PBDEs (Gump, Yun et al.

2014). However, more study is required to determine other potential targets of PBDE actions leading to cardiovascular toxicity.

The treatment of cardiovascular disease and hypertension has focused on the renin-angiotensin system (RAS) and sympathetic nervous system (SNS) as dominant players (Esler, Rumantir et al. 2001; Navar, Harrison-Bernard et al. 2002; Esler, Lambert et al. 2010; Navar 2011). In previous studies our lab has shown that perinatal dosing with PBDEs causes adult rats to exhibit pressor responses to acute hyperosmotic stress, a stimulus which does not typically cause an elevation in blood pressure (Shah, Coburn et al. 2011). We examined the role that the SNS plays in this process and found that the pressor response seen in PBDE-dosed animals is partially attenuated by ganglionic blockade suggesting a general increase in sympathetic nervous system reactivity to hyperosmotic stress. However, a significant increase in systolic blood pressure remained after sympathetic ganglion blockade indicating that other systems likely contribute to this response.

A likely contributor to this response is the RAS which has long been implicated in the pathogenesis of essential hypertension (Esler, Rumantir et al. 2001; Navar, Harrison-Bernard et al. 2002; Esler, Lambert et al. 2010; Navar 2011). The renin-angiotensin cascade begins with renin synthesized by juxtaglomerular cells in response to changes in renal perfusion. Renin converts circulating angiotensinogen into Angiotensin I which gets converted to Angiotensin II (Ang II) by angiotensin-converting

enzyme (ACE) which is present on the plasma membranes of various cell types (Atlas 2007). The RAS also recruits pressor actions of aldosterone and vasopressin (VP) secretion for long-term regulation of blood pressure (Guyton 1991; Navar and Hamm 1999; Navar, Harrison-Bernard et al. 2002; Kobori, Nangaku et al. 2007; Navar 2011). A variety of acute stressors have been shown to enhance peripheral RAS activity such as psychological stress (Kosunen 1977), physiological stress (Kosunen, Pakarinen et al. 1976), restraint stress (Armando, Tjurmina et al. 2003), and cold-restraint stress (Saavedra and Benicky 2007). Circulating AngII constricts vascular smooth muscle, enhances myocardial contractility. (Kobori, Nangaku et al. 2007; Saavedra, Sánchez-Lemus et al. 2010). Ang II also stimulates aldosterone production from the adrenal gland which, and increases sympathetic nervous system (SNS) activity while stimulating adrenal catecholamine (CA) release As a result, the most commonly used treatments for hypertension involve inhibition of RAS by way of angioitensin-converting enzyme (ACE) and the primary receptor for AngII, AT1R. ACE inhibitors and AT1R blockers are effective antihypertensive drugs, prolong the lifespan of spontaneously hypertensive rats, and reduce stress-induced release of catecholamines, vasopressin, and glucocorticoids (Rosendorff 1996; Linz, Heitsch et al. 2000; Armando, Carranza et al. 2001; Baiardi, Bregonzio et al. 2004; Armando, Carranza et al. 2007 ; Saavedra, Sánchez-Lemus et al. 2010).

We examined the role of the peripheral RAS in the pressor responses we have observed in PBDE-exposed rats. Acute hyperosmotic stress does not typically alter serum renin

activity or AngII levels (Yamaguchi, Sakaguchi et al. 1982; Sladek, Chen et al. 1987). However, because PBDE animals exhibit unusual pressor responses to this stimulus, we hypothesized that developmental PBDE exposure may alter RAS functioning by altering adrenal sensitivity to AngII and increasing plasma AngII concentrations. Our results show that abnormal pressor responses to hyperosmotic stress displayed only in PBDE-exposed animals can be abolished by pretreatment with the ACE inhibitor, captopril. Surprisingly PBDE exposed rats display reduced plasma levels of AngII as well as blunted adrenal AT1R expression, suggesting that CAP may work via mechanisms other than direct inhibition of the RAS to produce this effect.

Methods:

Animals: Male Long-Evans rats (2 months old) were housed under a 12/12 h light/dark cycle in standard cages with heat-treated pine bedding. Animals had access to water and food (Purina Lab Diet) *ad libitum* except during experiments. Temperature and relative humidity in the vivarium was kept at 21 ± 2 °C and 50 ± 10%, respectively. All experiments were performed between 13:00- 18:00h). All animals were maintained and experiments designed in accordance with the guidelines in National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of California, Riverside's Institutional Animal Care and Use Committee.

PBDE exposure: PBDE dosing was accomplished using the commercial pentaPBDE mixture, DE-71 (supplied by Great Lakes Chemical Corporation, El Dorado, AR, lot# 1550OI18A) which contains the individual congeners PBDE 47, 99, 100, 153 and 154 (Kodavanti and Ward 2005; Kodavanti and Curras-Collazo 2010) DE-71 was dissolved in corn oil and loaded (2ml/kg b.w.) into cheese puffs (Cheetohs, Frito Lay). Pregnant dams were weighed daily and received cheese puffs with the appropriate dose (30.6 mg/kg) from gestational day (GD) 6 through postnatal day (PND) 21, except on the day of birthing (PND 0). Control dams received cheese puffs loaded with corn oil vehicle only (2ml/kg b.w.). Rat litters were reduced to 8 pups for consistent lactational transfer between groups. Pup weight and size were monitored daily and no significant differences were found in pup size between PBDE and oil control groups as described previously (Shah, Coburn et al. 2011). The dose of DE-71 was selected to match with our previous report showing pressor responses to hyperosmotic stimulation in PBDE- but not oil-dosed control rats (Shah, Coburn et al. 2011). This dosing regimen matches doses of Aroclor 1254 (18 mg/kg) on a molar basis (for review, see Kodavanti and Ward 2005; Kodavanti and Curras-Collazo 2010) and yields PBDE levels in milk and fat of pregnant dams ~1000 μg/g lipid (Kodavanti, Coburn et al. 2010). This concentration is one to two orders of magnitude higher than reported for human milk from the U.S. (Johnson-Restrepo and Kannan 2009). However, the concentration used is realistic with regard to levels in infants and toddlers since dust samples from low income California households contained PBDE levels up to 20 times higher than those reported elsewhere in US (Quirós-Alcalá, Bradman et al. 2011).

In vivo hyperosmotic challenge. Normosmotic and hyperosmotic groups were injected with either 0.15 M or 3.5 M NaCl (0.6 cc/100g b.w., i.p.) using 10 mM HEPES to achieve neutral pH of 7.4 (Shah, Coburn et al. 2011). After injection, water was withheld to prevent the animal from normalizing plasma osmolality by drinking (Gillard, Leon-Olea et al. 2006). A period of 3 hours was allowed before the start of anesthesia and NIBP recordings, when exaggerated pressor responses have been documented in PBDE-exposed rats (Shah, Coburn et al. 2011).

Non-Invasive Blood Pressure (NIBP): For NIBP measurements animals were lightly anesthetized (1.75% isoflurane in O_2,; 2L/min) after a brief induction (5% isoflurane in O_2; 2L/min). Body temperature was maintained using a heat lamp and monitored via infrared thermometer. Tail-cuff plethysmography using a blood pressure monitoring system with manual deflation (IITC) was used to measure blood pressure (Shah, Coburn et al. 2011). Systolic blood pressure was recorded as the highest pressure at which the heart pulses return while mean arterial pressure was recorded as the peak amplitude pulse (Rodriguez-Iturbe, Zhan et al. 2003; Kamba, Tam et al. 2006). Diastolic blood pressure was determined using the formula MAP = diastolic BP + 1/3 PP, where PP is calculated as systolic BP − diastolic BP. Blood pressure values were normalized to baseline NIBP measurements (an average of 3 recordings) obtained 5-7 days prior to manipulation. Previous studies have determined a strong correlation (0.98) between the pulse based tail-cuff method and radiotelemetry measurements (Whitesall, Hoff et al. 2004).

Quantitative Polymerase Chain Reaction (qPCR): Phenol-chloroform extraction was used to isolate total RNA from snap-frozen adrenal glands and kidneys. qPCR was used to determine expression levels for the primary receptor for angiotensin II (AT1R). PCR primers were first checked via standard RT-PCR and gel electrophoresis. After this initial confirmation, trial qPCR was performed with a melt curve to confirm amplification and to rule out primer-dimers. qPCR was performed on the Bio-Rad CFX 96 Real-Time PCR Detection System using a Bioline SensiFAST™ SYBR No-ROX One-Step Kit. A temperature gradient from 52-59^{o} C was used to capture all ideal annealing temperatures and the reaction was run for 40 cycles. Results are presented as relative expression normalized to the housekeeping gene β-actin using the equation described in Pfaffl et al (Pfaffl 2001). Accession numbers, primers sequences, annealing temperatures are as follows: AT1R (Agtr1), accession NM_031009, F: CAGACACACACAGCCTTTCCA, R: GTAATTGTGCCTGCCAGCCT; β-actin accession NM_031144 F: TTCTTGCAGCTCCTCCGTCGC, R: CACCATCACACCCTGGTGCCTA.

ACE Inhibition with captopril (CAP): Inhibition of Angiotensin-converting enzyme (ACE) was achieved via captopril (CAP) (C-4042, Sigma) added to tap drinking water (15 mg/liter) for a period of two weeks (Ashab, Peer et al. 1995) prior to experimentation. Oral administration of captopril at this dose has been shown to lower the blood pressure of hypertensive rats while having no significant effect on the blood pressure of normotensive rats (Hutchinson, Mendelsohn et al. 1980). In humans, captopril is a commonly prescribed anti-hypertensive drug which inhibits the conversion of angiotensin

I to angiotensin II, i.e., an angiotensin-converting enzyme (ACE) inhibitor, thereby lowering vascular resistance, decreasing cardiac output, and increasing natriuresis (Dezsi 2014).

Plasma Angiotensin II (AngII) concentrations and plasma osmolality: Following NIBP measurements, anesthesia was increased (4% isoflurane in O_2; 2L/min) and an incision was made in the chest to expose the heart. A 16g needle was inserted in to the left ventricle of the heart and blood was removed and immediately centrifuged at 4^{o}C. Plasma was stored at -20^{o}C until assay. AngII was measured using a commercially available competitive enzyme-linked immunosorbent assay (ELISA) kit (Enzo, ADI-900-204). This assay is species independent and is sensitive to 4.6 pg/ml in a range of 3.9 pg/ml-10,000 pg/ml. The same plasma samples were brought to room temperature for plasma osmolality measurements using a vapor-pressure osmometer (Wescor). Values for each rat were expressed as mOsm/L.

Statistical analysis. Two-way ANOVA was used for multifactor analysis where data met normal distribution/equal variance assumptions. Pairwise comparisons were done using a post-hoc Bonferroni test following general linear model ANOVA. Two-group comparisons were determined using Student's t-test. Statistical significance was acknowledged at an alpha level of 0.05.

Results:

Plasma osmolality is elevated in all groups subjected to hyperosmotic stimulation. Captopril did not significantly alter plasma osmolality. A two-way ANOVA comparing oil-dosed and PBDE-dosed animals revealed a significant effect of hyperosmotic challenge on plasma osmolality ($F_{1,31}$=78.93, n=35, p<0.0001) as expected (**Fig.3.1**). Pairwise comparisons revealed a significant rise in mean plasma osmolality in hyperosmotic rats when compared to normosmotic controls. Mean values (± s.e.m.) for oil-dosed normosmotic and oil-dosed hyperosmotic were 281.60 ± 1.44 and 309.83 ± 6.7, respectively (n=11, p<0.01). Corresponding values for PBDE-dosed rats were 274.60 ± 2.25 and 302.75 ± 6.95, respectively (n=9, p<0.01). No significant differences were noted between PBDE-dosed and oil-dosed hyperosmotic animals. Similarly, no significant effect of captopril administration was seen on plasma osmolality.

Exaggerated pressor responses to hyperosmotic stress displayed in rats developmentally exposed to DE□71 is abolished by ACE□inhibitor captopril. To determine if elevated pressor responses in DE-71-dosed hyperosmotic rats were due to altered RAS activity we administered the ACE-inhibitor captopril (CAP) in the tap water for 2 weeks preceding hyperosmotic treatment. This treatment has been previously shown to an effective anti-hypertensive treatment (REF). **Figure 3.2** shows that CAP treatment completely abolished the rise in systolic blood pressure seen 3 hrs post-injection with hyperosmotic NaCl. A two-way ANOVA of systolic blood pressure in PBDE-dosed animals receiving normosmotic or hyperosmotic injections ± CAP showed significant effects of interaction

($F_{1,14}$= 15.30, p<0.01, n=18), hyperosmotic treatment ($F_{1,14}$=16.51, p<0.01), and CAP therapy ($F_{1,14}$=46.64, p<0.0001, **Fig 3.2A**). Comparisons using Bonferroni post-hoc test revealed a statistically significant reduction in mean arterial blood pressure (MAP) in PBDE-dosed hyperosmotic rats receiving CAP as compared to PBDE hyperosmotic rats receiving hypertonic saline without CAP (t=5.639 p<0.001). A two-way ANOVA of MAP in PBDE-dosed animals studied under normosmotic or hyperosmotic conditions ± CAP showed significant effects of hyperosmotic treatment ($F_{1,19}$=20.03, p<0.001, n=23) and interaction ($F_{1,19}$=4.582, p<0.05, n=23) and , **Fig 3.2B**). Bonferroni tests revealed that PBDE-dosed animals subjected to CAP pre-treatment had significantly lower MAP than animals that did not receive CAP treatment (t=5.056 p<0.001).

Similar results were found for diastolic blood pressure (**Fig. 3.2C)**. A two-way ANOVA yielded significant effects of hyperosmotic treatment ($F_{1,17}$=24.53, p<0.001) and interaction ($F_{1,17}$=9.089, p<0.01, n=22). Pairwise comparisons made using a post-hoc Bonferroni test revealed that PBDE-dosed animals without CAP had significantly higher diastolic blood pressure than animals receiving CAP (t=4.660 p<0.001). Interestingly, CAP produced a significant decrease in diastolic blood pressure in oil-dosed normosmotic control animals. A two-way ANOVA of MAP values in oil-dosed animals receiving normosmotic or hyperosmotic injections ± CAP showed significant effects of hyperosmotic treatment ($F_{1,15}$=7.305, p<0.05), and CAP therapy ($F_{1,14}$=9.085, p<0.01, n=19). A Bonferroni post-hoc test revealed a statistically significant reduction in diastolic

blood pressure in oil-dosed normosmotic rats receiving CAP as compared to oil-dosed normosmotic rats receiving hypertonic saline without CAP ($t=3.165$ $p<0.05$)

Plasma AngII is blunted following hyperosmotic stimulation in DE□71□dosed animals. Pretreatment with CAP increases plasma AngII responses to hyperosmotic stimulation. Because pretreatment with CAP was highly effective at eliminating the pressor responses seen in PBDE-dosed animals, we expected that plasma AngII levels would be elevated in PBDE-dosed hyperosmotic animals. Interestingly, we found that plasma AngII levels were blunted in PBDE-dosed animals (**Fig. 3.3A**). A two-way ANOVA for plasma AngII revealed significant effects for PBDE dosing ($F_{1,19}=12.14$, $n=23$, $p<0.01$). Bonferonni post-hoc analysis revealed that plasma AngII levels were significantly *reduced* in PBDE-dosed animals stimulated with hyperosmotic treatment as compared to oil-dosed hyperosmotic controls ($t=3.72$ $n=12$, $p<0.01$). A two way ANOVA was performed to evaluate the effect of CAP pre-treatment on plasma AngII concentrations for both oil-dosed and PBDE dosed animals. A statistically significant treatment effect was measured for hyperosmotic stimulation in both oil-dosed ($F_{1,15}=9.599$, $n=19$, $p<0.01$) and PBDE-dosed animals ($F_{1,18}=5.347$, $n=24$, $p<0.001$). In both oil-dosed and PBDE-dosed animals CAP pre-treatment resulted in significantly *increased* plasma AngII concentrations in response to hypertonic stimulation (**Fig 3.3B,C**).

Adrenal AT1R expression is blunted in DE□71□exposed animals. In spite of significantly lower circulating AngII in hypertensive PBDE-dosed rats excessive RAS

activity could be produced by upregulated AngII receptors which regulate aldosterone production (Mazzocchi, Gottardo et al. 1998; Atlas 2007). To determine if PBDE-dosed hyperosmotic rats showed altered adrenal expression of AngII type 1 receptor (AT1R) we measured mRNA levels for AT1R. **Fig 3.4A** shows a marked reduction in adrenal AT1R in PBDE-dosed hyperosmotic rats. A two-way ANOVA that examined PBDE- and oil-dosed animals receiving normosmotic or hyperosmotic stimulation revealed a significant effect of PBDE dosing ($F_{1,24}$=8.705, n=30, p<0.01). PBDE-dosed animals had significantly lower adrenal AT1R expression (0.421 ± 0.17) following hypertonic saline injection relative to oil-dosed hyperosmotic controls (1.01 ±0.18, n=13, t-test p<0.01). When oil-dosed animals are pre-treated with CAP for two weeks they show significantly reduced AT1R expression following hyperosmotic stimulation (0.10 ± 0.02) as compared to hyperosmotic stimulation without CAP treatment (1.01 ±0.18, **Fig 3.4B**). A Two way ANOVA of oil dosed animals ± CAP revealed a significant effect of CAP ($F_{1,14}$=9.419, n=18, p<0.01). Pairwise comparisons made using a post-hoc Bonferroni test revealed significant differences of hyperosmotic stimulation in the +CAP condition (t=2.8398, p<0.05). CAP did not produce a similar reduction in AT1R expression in with hyperosmotic stimulation in PBDE-dosed animals (**Fig 3.4C**) as AT1R expression was already blunted in PBDE-dosed animals.

Discussion:

As expected, hyperosmotic stimulation was effective at increasing plasma osmolality even after drinking captopril-containing water. These results indicate that the significant

captopril effects we observed did not reduce PBDE-induced pressor responses to hyperosmotic stress by reducing osmosensory activation.

In spite of the fact that hyperosmotic stress should not alter serum renin activity or AngII levels (Yamaguchi, Sakaguchi et al. 1982; Sladek, Chen et al. 1987), we hypothesized that with the added nephrotoxic effects of PBDEs that the PBDE-mediated pressor responses were due to altered RAS activity. Moreover, AngII is highly potent vasoconstrictor (Taubman 2003) and can contribute significantly to many forms of hypertension directly and via vasopressin (VP) secretion (Guyton 1991; Navar and Hamm 1999; Navar, Harrison-Bernard et al. 2002; Kobori, Nangaku et al. 2007; Navar 2011). As expected, NIBP recordings in PBDE-exposed hyperosmotic rats taken after pre-treatment with the ACE inhibitor, CAP, showed that abnormal pressor responses were eliminated. These results suggested that pressor responses to a 3 hr hyperosmotic challenge were mediated by hyperactivity of the peripheral RAS, a likely target of PBDEs either at the level of the kidney or lungs.

Because of the effective CAP actions on elevated blood pressure we examined whether PBDE-dosed animals show an exaggerated AngII response coincident with exaggerated pressor responses to 3 hours of hyperosmotic activation. Surprisingly, PBDE-exposed rats displayed *reduced* not elevated plasma levels of AngII, suggesting other RAS components may be responsible. This is an unexpected but important result since plasma AngII contributes to sodium reabsorption both directly and via stimulating aldosterone

secretion (Taubman 2003). These effects increase blood volume and blood pressure, are a compensatory means of raising blood pressure in response to hemorrhage and can occur within 3 hrs of the rise in AngII (Catt, Mendelsohn et al. 1984; Taubman 2003). Equally surprising was the finding that CAP administration elevated RAS markers in PBDE-dosed rats examined after 3 hours of hyperosmotic stimulation. Still if gene expression of AT1R were elevated, lowered levels of circulating AngII could still be effective in contributing to PBDE-mediated pressor response during hyperosmotic conditions. This result was not observed and again an opposite finding was achieved, i.e., adrenal AT1R expression was reduced in PBDE-dosed animals, indicating disruption of the peripheral RAS. Adrenal AT1R are critical for catecholamine and aldosterone production, two important endpoints in sympathetic and RAS control of blood pressure (Hinson and Raven 2006).

This begs the question: if the RAS is blunted in PBDE-exposed animals, what is causing the pressor responses in PBDE-exposed hyperosmotic animals and how is CAP effective at lowering these pressor responses? CAP administration *increased* plasma levels of AngII in PBDE-dosed hyperosmotic animals to a level similar to that seen in oil-dosed controls. In aggreement with our findings other studies report that CAP reduces the *tissue* levels of angiotensin II and aldosterone, although sometimes this is not reflected in the plasma (Mooser, Nussberger et al. 1990). Consistent with inhibition of RAS function CAP blunts plasma aldosterone responses (Ramirez, Ganguly et al. 1988) perhaps by increasing renal plasma flow (Fisher, Price et al. 1999). Related to this other

organohalogens such as PCB126 has been reported to alter (increase) gene expression of CYP11B2 which encodes for the requisite enzyme leading to aldosterone production at the level of adrenal (Hinson and Raven 2006). Therefore, this is a possible explanation for the apparent discordance between elevated AngII and reduced pressor responses after CAP administration seen in PBDE hyperosmotic rats. CAP can also decrease circulating levels of vasopressin (Thomsen, Danielsen et al. 1986) as well as vasopressin immunoreactivity in the hypothalamic supraoptic nucleus (Berecek, Wyss et al. 1991). However, we discount this as a viable explanation since pilot data from our lab (in collaboration with Drs. Sanchez Jamarillo and Leon-Olea) shows that a) perinatal exposure to DE-71 decreases vasopressin content in the magnocellular neuroendocrine cell system within the hypothalamus and b) that acute DE-71 treatment of supraoptic nucleus punches *reduces* not enhances vasopressin release (Coburn et al, 2007). Alternatively, it is known, for instance, that ACE inhibitors like CAP also increase bradykinin levels (Baram, Kommuri et al. 2013) which may contribute to the inhibition of pressor responses. CAP has also been shown to increase plasma renin activity (Usberti, Di Minno et al. 1986; Wilcox and Dzau 1992) and, therefore, increased plasma renin activity by CAP could account for a normalization of plasma AngII concentrations in PBDE-exposed animals displaying disrupted RAS activity.

Alternatively, the effectiveness of CAP in this study could be due to elevated plasma atrial natriuretic peptide (ANP) levels which have been shown to increase significantly with CAP administration (Wilkins, Lewis et al. 1987). ANP is typically released when

the atria become distended by high extracellular fluid and blood volume (which increases blood pressure directly) is important for renal excretion of sodium which can normalize blood pressure (Singer, Shore et al. 1987). ANP is also a powerful vasodilator which increases glomerular filtration rate and reduces blood pressure (Saito 2010). Therefore, pretreatment of rats with CAP in this study could reduce pressor responses via increased plasma ANP independently of the RAS. This possibility is supported by our finding of decreased diastolic blood pressure following normosmotic injection in oil-dosed CAP treated rats. If circulating ANP levels were elevated by two weeks of CAP treatment, we might expect hypovolemia which may present as reduced diastolic blood pressure (Ruskoaho 1992; Sabrane, Kruse et al. 2005). Future research will explore this possibility.

Here we show that adrenal gene expression of Angtr1 is decreased in PBDE-exposed hyperosmotic rats. We speculate that this may be due to altered sympathoadrenal regulators of AT1R (i.e., reduced PACAP, TH and PNMT) which coincidently occur in PBDE-exposed hyperosmotic rats (Spurgin et al, submitted). Unlike PBDEs, PCB 126 increases on adrenal AT1R (see (Hinson and Raven 2006). This may be due to the divergent activity of the non-coplanar structure of PBDEs relative to dioxin-like PCBs (such as PCB 126) that have major effects on Aryl hydrocarbon receptors (AhR) in human hepatoma cells (Peters, van Londen et al. 2004; Peters, Nijmeijer et al. 2006). Aryl hydrocarbon receptors (AhR) are involved in BP regulation and are linked to RAS activity (Zhang, Agbor et al. 2010) via the induction of CYP1A1 and CYP1B1 in several

tissues (Harrigan, McGarrigle et al. 2006). Additionally, it is possible that PBDEs could be altering aquaporin (AQ) water channel expression, the associated V2-type vasopressin receptor and/or the NOS signaling in the kidneys in a manner similar to that seen in uterine AQ channels (Tewari, Kalkunte et al. 2009).

The present study provides evidence that the renin-angoiotensin system is adversely affected by developmental PBDE exposure. However, the precise nature of this disruption and the mechanisms underlying the effectiveness on CAP in this study are in need of further examination. Future work will focus on exploring these questions.

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Fig 3.1 Plasma osmolality is elevated in all groups stimulated with hypertonic saline injection. Captopril did not significantly alter plasma osmolality. Blood from anesthetized rats was taken via cardiac puncture at sacrifice three hours post-injection. Hyperosmotic stimulation increased plasma osmolality in all groups including those treated with captopril. Therefore, osmosensory stimulation should be similar in all groups. Asterisks indicate statistical difference between PBDE Hyper vs PBDE Norm at $p<0.01$(**). Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71, CAP = captopril

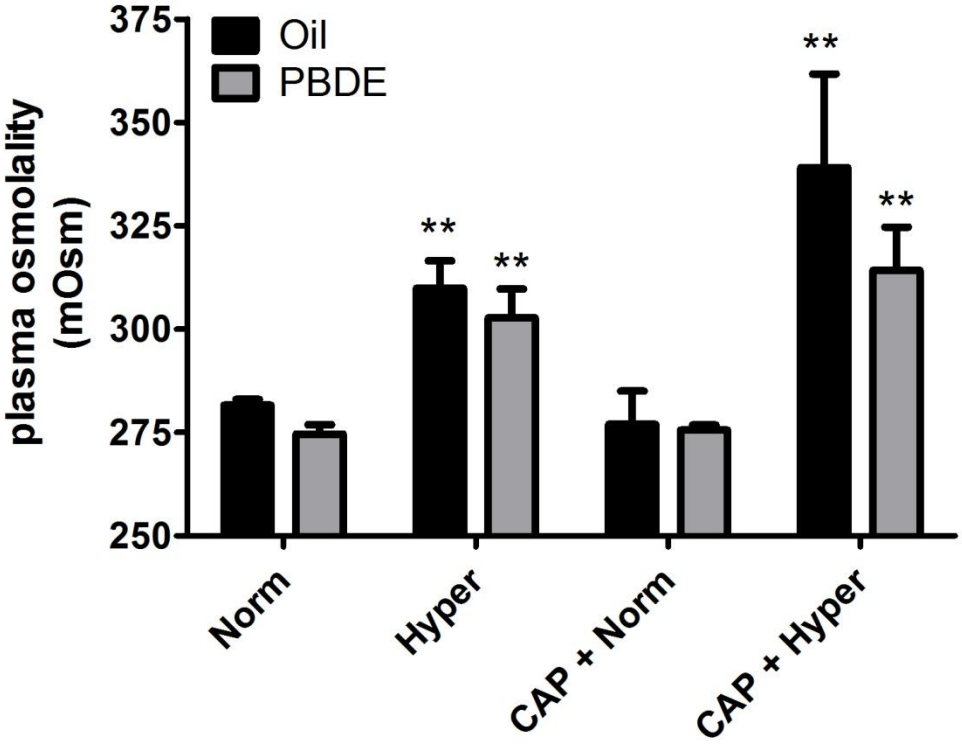
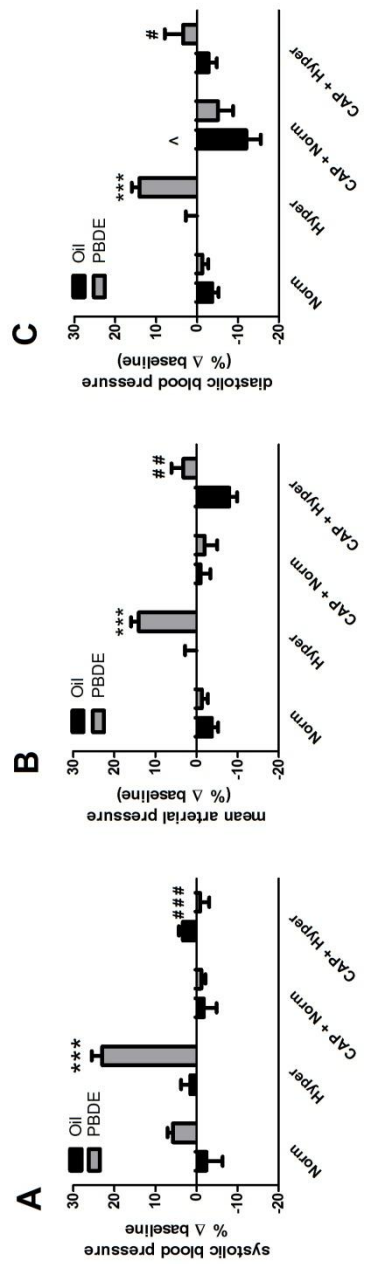

375
350
325
300
275
250
plasma osmolality (mOsm)
Oil
PBDE
**
**
**
**
Norm
Hyper
CAP + Norm
CAP + Hyper

Fig 3.2 Exaggerated pressor responses to hyperosmotic stress displayed in rats developmentally exposed to DE-71 is abolished by ACE-inhibitor captopril. Rats were dosed perinatally with DE-71 and in adulthood were treated with either hyperosmotic or normosmotic stimulation in the presence or absence of the ACE-inhibitor captopril (CAP). After 3 hours of stimulation blood pressure was measured via NIBP. **A:** PBDE-dosed hyperosmotic animals displayed a significant increase in systolic blood pressure compared to oil-dosed hyperosmotic controls. CAP significantly reduced the stimulated rise in systolic blood pressure seen in PBDE hyperosmotic rats. **B:** PBDE-dosed hyperosmotic animals displayed a significant increase in mean arterial pressure compared to oil-dosed hyperosmotic controls. CAP significantly reduced the stimulated rise in mean arterial pressure seen in PBDE hyperosmotic rats. **C:** PBDE-dosed hyperosmotic animals displayed a significant increase in diastolic pressure compared to oil-dosed hyperosmotic controls. CAP significantly reduced the stimulated rise in mean arterial pressure seen in PBDE hyperosmotic rats. CAP also produced significantly reduced diastolic pressure in oil-dosed normosmotic animals. Asterisks indicate statistical significance as compared to PBDE-dosed normosmotic rats $p<0.001$ (***). Hashtags indicate statistical significance as compared to PBDE-dosed hyperosmotic rats at $p<0.001$(###), $p<0.01$ (##), and $p<0.05$(#). Caret indicates statistical significance as compared to oil-dosed normosmotic rats at $p<0.05$(^). CAP = captopril, Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71


A
systolic blood pressure
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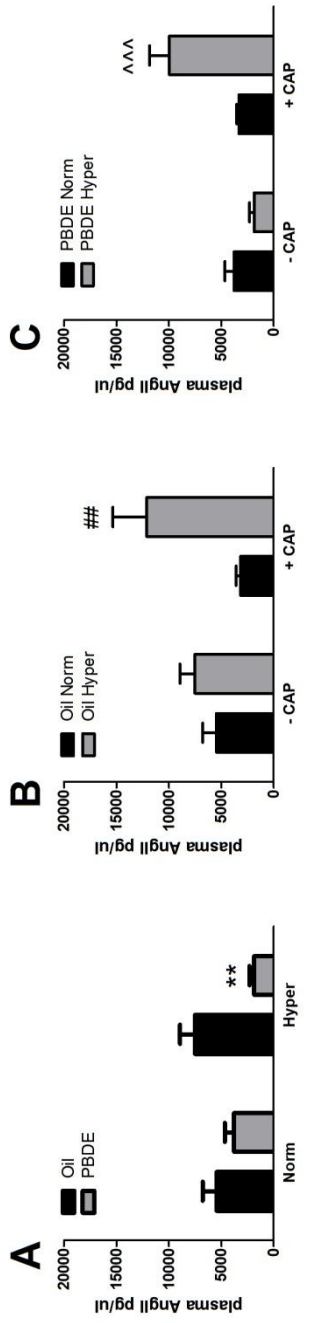
Oil
PBDE
***
###
B
mean arterial pressure
(% Δ baseline)
***
##
C
diastolic blood pressure
(% Δ baseline)
***
^
#
Norm
Hyper
CAP + Norm
CAP+ Hyper

Fig. 3.3 Plasma AngII is blunted following hyperosmotic stimulation in PBDE-dosed animal. Pretreatment with CAP increases plasma AngII responses to hyperosmotic stimulation. A: Plasma AngII levels are blunted in PBDE-dosed animals. **B:** In oil dosed animals CAP pre-treatment resulted in significantly increased plasma AngII concentrations in response to hypertonic stimulation **C:** In PBDE-dosed animals CAP pre-treatment resulted in significantly increased plasma AngII concentrations in response to hypertonic stimulation. Asterisks indicate statistical significance as compared to PBDE-dosed normosmotic rats at $p<0.01$(**). Hashtags indicate statistical significance as compared to oil-dosed normosmotic rats at $p<0.01$ (##). Carets indicate statistical significance as compared to PBDE-dosed normosmotic rats at $p<0.001$ (^^^). CAP = captopril, Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71


A
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C
PBDE Norm
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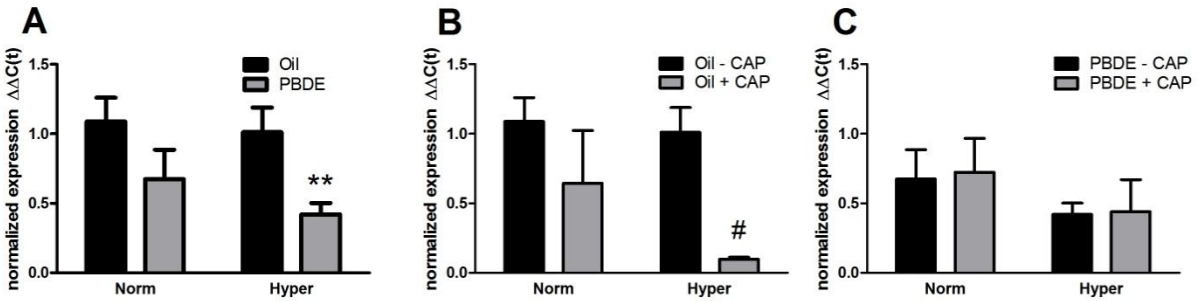
Fig 3.4 Adrenal AT1R expression is blunted in PBDE-exposed animals. **A:** AT1R are markedly reduced in PBDE-dosed hyperosmotic rats. **B:** When oil-dosed animals are pre-treated with CAP for two weeks they show significantly reduced AT1R expression following hyperosmotic stimulation as compared to hyperosmotic stimulation without CAP treatment. **C:** CAP did not produce a similar reduction in AT1R expression in with hyperosmotic stimulation in PBDE-dosed animals as AT1R expression was already blunted in PBDE-dosed animals. Asterisks indicate statistical significance as compared to oil-dosed hyperosmotic rats at $p<0.01$(**). Hashtag indicates statistical significance as compared to oil-dosed hypersmotic rats not receiving CAP at $p<0.05$(#). CAP = captopril, Oil = Oil-dosed controls, PBDE = rats perinatally dosed with DE-71


A
B
C
normalized expression ΔΔC(t)
1.5
1.0
0.5
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Oil
PBDE
Oil - CAP
Oil + CAP
PBDE - CAP
PBDE + CAP
**
#
Norm
Hyper

Chapter 4

Chronic Psychogenic Stress Increases Blood Pressure and Alters Stress Axis Markers in the Adrenal Gland and Hypothalamus

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Abstract:

The hypothalamo–sympatho–adrenal (HSA) axis, responsible for regulating blood pressure via excitatory drive to the heart and resistance blood vessels and via adrenal catecholamine secretion, plays an important role in the etiology of hypertension. Pre-autonomic neurons within the hypothalamic paraventricular nucleus project to the ventrolateral medulla where preganglionic neurons send fibers via the splanchnic nerve. The latter contain acetylcholine (ACh) which stimulates catecholamine synthesis and release from adrenal chromaffin cells. Neuroendocrine cells within the PVN also regulate hypothalamo-pituitary-adrenal (HPA) activity. Within the adrenal gland locally produced pituitary adenylate cyclase-activating polypeptide (PACAP) may positively modulate sympathoadrenal and CORT endocrine functions independently. In order to test this possibility, we examined mRNA expression of PACAP, and gene markers associated with HPA responsiveness and CORT production. We also examined pressor responses that have been associated with upregulated central PACAP signaling. Male Sprague-Dawley rats were then exposed to daily sound stress for eight weeks and blood pressure measurements were taken under anesthesia using NIBP recordings. Systolic, mean arterial and diastolic blood pressure and heart rate were measured and expressed as a percent of the pre-stress baseline value. Rats subjected to sound stress showed a significant increase in resting systolic blood pressure of 8.45% over pre-stress baseline (± 1.96 n=14; $p<0.01$) whereas age-matched controls showed a slight decrease of 1.01% from pre-stress baseline (-1.01 ± 1.93, n=28). Adrenal glands and PVN were collected at sacrifice for CA content assay and qPCR analysis. CA content was not significantly

different in stress and sham controls. We found significant changes in gene markers PACAP and MC2R, markers for sympathoadrenal and HPA axes. Elevated expression of PACAP mRNA was noted in the adrenal glands of sound stress animals (2.24 ± 0.26 N=16; $p<0.001$) versus controls (1.07 ± 0.13, n=13). In contrast, sound stress significantly *blunted* adrenal mRNA expression for the adrenocorticotropic hormone receptor MC2R in the adrenal gland (0.81 ± 0.11) vs controls (1.13 ± 0.16, $P<0.05$, n=23). The PVN of rats exposed to chronic sound stress showed increased mRNA expression of the Grin 2B gene (which encodes the NR2B subunit of NMDA receptors) relative to sham controls (2.42 ± 0.29 vs. 1.32 ± 0.22; n=13; p= 0.01). This caused an increase in the relative expression of NR2B:NR2A, likely leading to changes in glutamatergic transmission in the CRH-containing parvocellular neurons within the PVN that drive the HPA axis. These results suggest that chronic psychogenic stress dramatically increases adrenal PACAP gene expression and this may regulate CORT levels independently of HPA activation. In support of this adrenal MC2R mRNA levels were blunted, indicating reduced responsiveness to ACTH, and changes in the relative expression of NR2A:NR2B are not consistent with enhanced PVN activation. It is unclear, however, what alternative mechanisms may underlie elevated pressor responses following chronic stress. These data may also indicate that changes in adrenal gene expression may be permanent since they outlast the duration of the actual stimulus.

Introduction

There is evidence that psychological stressors may contribute significantly to elevated blood pressure (Henry 1991; Henry, Liu et al. 1993; Sparrenberger, Cichelero et al. 2008; Spruill 2011). Stress undoubtedly involves activation of the HPA axis (Raab, Dantzer et al. 1986; Heinrichs, Pich et al. 1992; Berton, Durand et al. 1999; Buwalda, Felszeghy et al. 2001; Razzoli, Carboni et al. 2007); however, stress also influences blood pressure in part via activation of the sympathetic nervous system (SNS) (Sherwood, Allen et al. 1986; Goldstein 1995; Lohmeier 2001; Grassi, Seravalle et al. 2010). The SNS has featured prominently in the debate over the etiology of hypertension (Esler, Rumantir et al. 2001; Navar, Harrison-Bernard et al. 2002; Esler, Lambert et al. 2010; Navar 2011) and the success of renal sympathetic nerve ablation procedures in humans (Krum, Schlaich et al. 2009; Ressler, Mercer et al. 2011) supports a role for the SNS in the etiology of hypertension. During SNS activation, norepinephrine (NE) released by cardiac sympathetic nerve terminals increase heart rate and cardiac contractility while catecholamines (CA) released into the circulation from adrenal chromaffin cells elevate heart rate, cardiac output, and blood pressure (Lymperopoulos 2013). SNS activation induces mRNA transcription of CA biosynthetic enzymes in the adrenal gland: tyrosine hydroxylase (TH), the rate-limiting enzyme of CA biosynthesis, and phenylethanolamine N-methyltransferase (PNMT), which methylates norepinephrine to epinephrine (Wong and Tank 2007). Sympathetic activation of the kidney increases sodium and water retention (ultimately increasing blood volume and blood pressure) (Ritz, Amann et al. 1998; Grisk and Rettig 2004) as well as activation of the renin-angiotensin system (RAS).

The latter contributes to long-term regulation of blood pressure via several routes of action including angiotensin-mediated stimulation of adrenal aldosterone production (Atlas 2007). The pituitary adenylate cyclase-activating polypeptide (PACAP) system (involving central and peripheral origins) has received significant attention in recent years (Stroth and Eiden 2010; Ressler, Mercer et al. 2011; Stroth, Holighaus et al. 2011; Tsukiyama, Saida et al. 2011; Stroth, Kuri et al. 2013) as a major player in chronic stress responses. The adrenal medulla contains abundant PACAP (Shioda, Shimoda et al. 2000) where it is synthesized in adrenal chromaffin cells (Kantor, Heinzlmann et al. 2002; Mazzocchi, Malendowicz et al. 2002; Conconi, Spinazzi et al. 2006). Within the adrenal gland exist intrinsic PACAPergic fibers (Frodin, Hannibal et al. 1995; Dun, Miyazaki et al. 1996; Holgert, Holmberg et al. 1996; Moller and Sundler 1996; Nielsen, Hannibal et al. 1998); and PACAPergic fibers innervate the adrenal gland via splanchnic nerves. PACAP binds to VPAC receptors in the adrenal cortex (Mazzocchi, Malendowicz et al. 2002; Conconi, Spinazzi et al. 2006) and PAC1 receptors in the adrenal medulla (Watanabe, Masuo et al. 1992; Shiotani, Kimura et al. 1995). PACAP receptors coupled to Gs proteins lead to activation of second messengers which influence gene expression, and CA synthesis (Ohtaki, Watanabe et al. 1990; Ohtaki, Masuda et al. 1993).

Both acetylcholine (ACh) (Milner, Pickel et al. 1989; Arneric, Giuliano et al. 1990) and PACAP (Farnham, Li et al. 2008; Farnham, Inglott et al. 2011) work synergistically to stimulate CA release by the adrenal gland when stimulated by the splanchnic nerve

(Malhotra and Wakade 1987; Guo and Wakade 1994; Lamouche, Martineau et al. 1999; Lamouche and Yamaguchi 2003). PACAP is also critical for induction of adrenal TH and PNMT mRNA in response to restraint stress (Stroth and Eiden 2010). PACAP produced by chromaffin cells may act on adrenomedullary and adrenocortical endocrine processes through local catecholamine release (Nussdorfer 1996; Nussdorfer and Malendowicz 1998; Vaudry, Gonzalez et al. 2000). Local PACAP may also influence corticosterone production and release. For example, in rats PACAP-38 elicits glucocorticoid secretion by stimulating an intra-adrenal CRH-ACTH system or by way of adrenomedullary catecholamines (Nussdorfer 1996; Nussdorfer and Malendowicz 1998).The paraventricular nucleus of hypothalamus (PVN) is an important locus for the integration of autonomic and cardiovascular responses to stress.

Glutamatergic input to the PVN arrives from regions such as the bed nucleus of the stria terminalis, brainstem nuclei via the peri-PVN region (Ziegler, Cullinan et al. 2005; Busnardo, Crestani et al. 2012; Mastelari, de Abreu et al. 2012; Busnardo, Alves et al. 2013; Martins-Pinge, Mueller et al. 2013) as well as the periaqueductal gray, caudal portions of the z*ona incerta* and subparafascicular nucleus, and the lateral parabrachial nucleus (Mastelari, de Abreu et al. 2012) Recent work using animal models of hypertension and heart failure have reported a shift toward increased excitatory (glutamatergic) drive in the PVN (Ziegler, Cullinan et al. 2005; Ye, Li et al. 2013). Recently, Busnardo et al. showed that non-NMDA glutamate receptors facilitate stress

evoked autonomic response in the PVN whereas NMDA receptors in the PVN appear to play an inhibitory role on the cardiovascular responses to stress (Busnardo, Alves et al. 2013). This excitatory shift enhances the activation of pre-autonomic neurons in the PVN and may contribute to persistently elevated blood pressure seen in essential hypertension (Biancardi, Campos et al. 2010).

The PVN is also the origin of the HPA axis, containing CRH-rich neurons within the parvocellular subregion of the PVN (Ziegler and Herman 2002). CRH (as well as vasopressin) regulate ACTH release from the portal circulation surrounding the anterior pituitary. Circulating ACTH then stimulates CORT production from the adrenal gland. Interestingly, previous reports have demonstrated that PACAP regulates both peptides. Central PACAP induces CORT release from the HPA in a CRH-dependent fashion (Nussdorfer and Malendowicz 1998; Kageyama, Hanada et al. 2007). Stress-induced PACAP Our lab has shown that osmotic stress triggers endogeneous PACAP secretion into the blood and that vasopressin responses to hyperosmotic stress requires PAC1R activity (Gillard, Leon-Olea et al. 2006).

Psychogenic stress in the form of alternating frequencies and pitch of sound is effective in elevating blood pressure in experimental animals. Chronic noise stress with a frequency of 2640 Hz and a power of 30w performed 15 min daily over a period of 30 consecutive days has been shown to elevate blood pressure in rats (Alario, Gamallo et al.

1987). Plasma epinephrine levels have been shown to remain elevated as long as 28 days after a 4-day chronic sound stress protocol (Khasar, Burkham et al. 2008; Khasar, Dina et al. 2009). Prior work has shown that epinephrine levels in the adrenal medulla are increased after brief noise exposure, but are reduced when rats are exposed 6 h daily for 21 days (repeated exposure) as epinephrine cells in the adrenal gland become habituated to the stress (Gesi, Lenzi et al. 2002). Large non-homogeneous vesicles were also found in epinephrine cells of animals exposed to repeated noise (Gesi, Lenzi et al. 2002), indicating structural and biochemical remodeling within the adrenal gland in response to chronic stress. Other studies have shown elevated plasma epinephrine following an initial sound stress exposure, but no significant epinephrine response with subsequent noise exposure (de Boer, Slangen et al. 1988; De Boer, Van Der Gugten et al. 1989). Therefore, adrenal responses to noise stress appear to differ depending on the duration of exposure. Chronic stress may produces changes in adrenal parameters that outlast the duration of the actual stressful stimulus, indicating potential permanent effects on stress axes that converge on the adrenal gland.

We hypothesized that persistent elevations in blood pressure would be associated with altered adrenal CA content and expression of genes responsible for ssympathoadrenal and HPA activity within the adrenal gland. Furthermore, we expected that chronic sound stress would be associated with altered expression of NMDA receptor subunits in the PVN. We examined these predictions by using NIBP for cardiovascular recordings, an

enzymatic assay for CA content analysis and PCR analysis for gene expression determination. Rats subjected to 8 weeks of chronic sound stress showed significantly elevated mean systolic blood pressure values concurrent with elevated levels of adrenal PACAP (but not nAChR and PAC1R) mRNA relative to that of sham stress rats. In contrast, adrenal expression of MC2R, adrenocorticotropic hormone receptor, was *blunted* in sound stress rats relative to sham controls. These results indicate local expression of PACAP in the adrenal gland is regulated by chronic stress and that this may impact the sensitivity of adrenal CORT production to activation by ACTH. In support of this the PVN stress rats also showed increased relative expression of NR2B to NR2A subunits, suggesting increased excitation of central components within the HPA.

Methods

Animals: All animals were maintained in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines. Male Sprague-Dawley rats were group-housed 3-4 per cage after weaning in standard cages with heat-treated pine shavings as bedding. Water and food (Purina Lab Diet) were provided *ad libitum*. Temperature and relative humidity were maintained at 21 ± 2 °C and 50 ± 10% under a 12/12 h light/dark cycle. Experiments were performed between 13:00- 18:00h). All experiments were approved by the IACUC on animal care and use at the University of California, Riverside and designed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Chronic Sound Stress Sprague-Dawley rats housed 3 per cage were placed in a sound chamber 25 cm from two powered studio speakers (Berhinger) that emitted 4 pure tones (5, 11, 15, and 19 kHz), with amplitudes varying in intensity between 20 and 110 dB (Khasar, Green et al. 2005; Khasar, Dina et al. 2009). The sound chamber was kept in an isolated room and was further insulated from outside sounds foam board. A small fan provided ventilation to prevent animals from overheating. Control animals were placed in the sound chamber with no sound emanating from the speakers. Sound stress was performed 30 min daily for 8 wks.

Non□Invasive Blood Pressure (NIBP): Immediately following the final sound stress session, animals were anesthetized and NIBP was measured by tail-cuff plethysmography using a manual blood pressure monitoring system (IITC) as previously described (Shah, Coburn et al. 2011). Parameters recorded were: systolic blood pressure (highest pressure at which the heart pulses return) and mean arterial pressure (highest amplitude pulses) (Rodriguez-Iturbe, Zhan et al. 2003; Kamba, Tam et al. 2006). All measures were obtained under isoflurane anesthesia (1.75% isoflurane in O_2; 2L/min) after a brief induction (5% isoflurane in O_2; 1L/min). A heat lamp was used to maintain the animal's temperature. Baseline NIBP recordings were made for each animal prior to beginning the sound stress protocol and values were averaged over three NIBP readings. Diastolic blood pressure was determined using MAP = diastolic BP + 1/3 PP, where PP is

calculated as systolic BP − diastolic BP. The pulse-based tail-cuff method used here provides values with a strong correlation (0.98) to those readings generated via radiotelemetry (Whitesall, Hoff et al. 2004). Following the final NIBP reading, animals were sacrificed and adrenal glands and brains were snap-frozen and kept at -80^{o}C until processing.

Quantified Polymerase Chain Reaction (qPCR): For qPCR, snap-frozen brains were sliced on a cryostat at 300 μm. The PVN was identified via a stereotaxic atlas and PVN tissue was punched out using a specially modified 16g needle. PVN tissue and adrenal glands were then homogenized in Trizol and total RNA was prepared using phenol-chloroform extraction. qPCR was employed to examine genes of interest that are involved in regulating CORT and CA production in the adrenal gland. Tyrosine hydroxylase (TH) is the rate-limiting enzyme of CA biosynthesis, and phenylethanolamine N-methyltransferase (PNMT) methylates norepinephrine to epinephrine (Wong and Tank 2007). Also examined were markers for peptidergic and cholinergic signaling in the adrenal gland, pituitary-adenylate-cyclase activating polypeptide (PACAP), the primary PACAP receptor, PAC1R, and nicotinic Acetylcholine receptors (nAChR). NMDA receptor subunits Steroidogenic acute regulatory protein (STAR) plays a key role in the acute regulation of steroid hormone synthesis by enhancing the conversion of cholesterol into pregnenolone. Melanocortin 2 receptor (MC2R) is the adrenocorticotropic hormone receptor in the adrenal gland and melanocortin receptor accessory protein (MRAP) is essential for the cell surface

trafficking and signaling of MC2R receptors. PVN tissue was evaluated for mRNA expression of NMDA NR2A and NR2B subunits. The nucleotide sequences and accession numbers of qPCR primers are listed in **Table 4.1**. PCR primers were initially tested via standard RT-PCR with gel electrophoresis and then checked via qPCR with a melt curve to rule out primer-dimers. qPCR was performed using Bioline SensiFAST™ SYBR No-ROX One-Step Kit on a Bio-Rad CFX 96 Real-Time PCR Detection System. Genes of interest were normalized to the housekeeping gene β-actin and results are presented as relative expression using the equation described in Pfaffl et al (Pfaffl 2001).

CA assay: Catecholamine content in adrenal glands was measured via fluorometric assay using a modification of published protocols (Wakade 1981; Wakade and Wakade 1983; Wakade 1988; Guo and Wakade 1994). The assay is a non-specific catecholamine assay that detects dopamine, epinephrine and epinephrine. A standard curve was obtained by dissolving norepinephrine in 0.05N perchloric acid (PCA) with serial dilutions. For samples, the right adrenal gland homogenized in 400 µl PCA. Eighty-five µL of each blank, standard, or sample was added to each well of a sterile 96 well plate. Using a multi-well pipette 3.7 µL of $K_3Fe(CN)_6$ solution was added to each well and the plate was put on a plate shaker. Thirty-four µL of ascorbic acid solution was added to each well to stop the reaction after 3 minutes. Absorbance was measured at 450nm and 560nm. Blank well absorbances obtained at 560nm and 450nm were subtracted from sample absorbances. The best-fit four-power polynomial equation was used to convert absorbance into catecholamine concentration. All curves had a correlation coefficient greater than

0.99. Data are expressed in mM units. This assay is capable of detecting changes at the adrenal level with a sensitivity of 0.125 mM.

Statistical analysis. Multifactor analysis was carried out using two-way ANOVA where data met normal distribution/equal variance assumptions. Post-hoc comparisons were made using Bonferroni test. Statistical significance was acknowledged at an alpha level of 0.05.

Results

Chronic sound stress increases systolic blood pressure in rats. **Figure 4.1** shows that animals subjected to eight weeks of daily sound stress had a significant increase in their resting systolic blood pressure. A two way ANOVA revealed a significant effect of stress $F_{2,26}$=7.897, n=82 P<0.01). Systolic blood pressure was elevated by 8.45 ± 1.9 % in stressed animals vs -1.01 ± 1.9% in control animals (p<0.01, t-test, n=28) No significant effect of stress was noted in diastolic pressure or mean arterial pressure between groups.

Adrenal PACAP expression is elevated in rats subjected to chronic sound stress. Adrenal catecholamine content was unaffected by chronic sound stress. SNS activation of the adrenal gland was monitored via qPCR for mRNA gene expression. A two-way ANOVA

revealed a significant effect of stress on the SNS genes of interest in the adrenal gland ($F_{4,98}$=4.222, n=106, p<0.05, **Fig 4.2A**). Bonferroni post-hoc analysis revealed a significant effect of stress on PACAP gene expression (t=3.741, p<0.01). Expression of PACAP mRNA increased almost three-fold in stressed animals (2.90 ± 0.51) vs. controls (1.05 ± 0.09, t-test, p<0.01, n=41). No significant differences were noted in gene expression of nicotinic acetylcholine receptors (nAChR) or the primary PACAP receptor PAC1R. The catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT) were also unaltered by sound stress. Adrenal CA content was unaltered by 8 weeks of daily sound stress **(Fig 4.2B)**

Adrenal MC2R expression is blunted in rats subjected to chronic sound stress. Peripheral HPA activation of the adrenal gland was monitored via qPCR for mRNA gene expression (**Fig 4.3**). A two way ANOVA examining the effects of stress on the expression of HPA relevant genes, MRAP, MC2R and StAR revealed a significant effect of stress ($F_{2,62}$=11.79,p<0.01, n=79). Bonferroni post-hoc test revealed that expression of MC2R mRNA was significantly decreased in stressed animals (0.61 ± 0.09) vs. controls (1.10 ± 0.164, t=2.918, p<0.05, n=22). No significant differences were noted in gene expression of MRAP or StAR.

Swith in the expression of NMDA subunits is altered in the PVN of rats exposed to chronic sound stress. In unstressed sham animals the expression levels of NR2A and

NR2B subuints are similar (**Fig. 4.4)**. In contrast, stressed rats show a different profile in the relative expression of these subunits. A two-way ANOVA was performed to evaluate the relative expression of NR2A and NR2B NMDA subunits of rats receiving chronic sound stress (**Fig.4.4**). There was a significant effect of interaction ($F_{1,24}$=12.03, $p<0.01$, n=28) and Bonferroni post-hoc tests revealed a significant difference in NMDA subunit expression following stress (t=5.817, $p<0.001$, n=16). Chronic sound stress increased mRNA expression of the NR2B subunit (2.42 ± 0.29) relative to the NR2A subunit (0.67 ± 0.12).

Discussion

Blood pressure elevations have been described following a variety of chronic stress paradigms (Dobrakovova, Oprsalova et al. 1984; Herrmann, Hecht et al. 1984; Lawler, Barker et al. 1984; Mills and Ward 1984). Our results are consistent with prior work demonstrating that chronic sound stress results in elevated blood pressure in rats (Alario, Gamallo et al. 1987; Gamallo, Alario et al. 1988; Gamallo, Alario et al. 1992). However, the mechanism underlying this is still unclear.

In the SNS, different types of stressors may drive adrenal catecholamine synthesis and release via different mechanisms. The relative contribution of ACh and PACAP to CA synthesis and release in the adrenal gland has been debated. Several early papers

indicated that ACh mediates catecholamine secretion during high neuronal activity reminiscent of moderate to severe stress, whereas non-cholinergic signaling predominates during low neuronal activity reminiscent of mild stress (Malhotra and Wakade 1987; Wakade 1988; Guo and Wakade 1994; Kuri, Chan et al. 2009). More recent work has indicated that PACAP may regulate catecholamine secretion in response to high-frequency splanchnic nerve stimulation (Smith and Eiden 2012; Stroth, Kuri et al. 2013) or in response to acute stress (Stroth and Eiden 2010; Stroth, Liu et al. 2011; Smith and Eiden 2012). Nevertheless, the course of the PACAP involved in these effects is controversial. We have recently shown that PACAP mRNA is expressed locally at the level of the adrenal gland and responds differentially to acute and chronic restraint stress in the mouse (Sprugin et al, submitted). In the present study we found that chronic sound stress resulted in a significant increase in PACAP mRNA expression in the *rat* adrenal gland. This finding is consistent with evidence that PACAP-dependent mechanisms within the adrenal gland itself sustain sympathoadrenal responses to prolonged but not acute stress (Stroth, Holighaus et al. 2011). Prior studies have identified a diffuse presence of PACAP in adrenal chromaffin cell cytoplasm but not in dense cored vesicles in splanchnic nerve terminals (Shiotani, Kimura et al. 1995). Adrenal PACAP mRNA in the present study is likely being transcribed and released by chromaffin cells in an autocrine or paracrine manner (Shiotani, Kimura et al. 1995; Nussdorfer 1996; Conconi, Spinazzi et al. 2006).

Habituation to chronic or repeated stressors appears to be stressor-specific. Some prior work has indicated that repeated exposure to stressors such as handling and novel environment (Bassett, Cairncross et al. 1973), sound (Borrell, Torrellas et al. 1980), and water restraint stress (Murison, Bruce Overmier et al. 1986) eventually result in adaptation with a corresponding decrease in stress axis activity. Other studies utilizing prolonged periods of immobilization (Taché, Du Ruisseau et al. 1976; Baron and Brush 1979; Jean Kant, Eggleston et al. 1985) foot shock (Jean Kant, Eggleston et al. 1985) or a combination of restraint, auditory and visual stress (Vogel and Jensh 1988) do not appear to result in habituation to these stressors over time. It does appear that one trend in the literature is that animals are more likely to become desensitized to milder stressors (even if chronic) as opposed to more severe stressors. In the current study animals receiving chronic sound stress displayed a decrease in adrenal MC2R mRNA expression. This is consistent with prior work in which chronic mild psychosocial stress in mice decreased adrenal MC2R mRNA expression and adrenal corticosterone responses to ACTH administration i*n vitro* (Uschold-Schmidt, Nyuyki et al. 2012). In our study the elevated PACAP with blunted MC2R gene expression in adrenal may indicate a reduced ACTH responsiveness to HPA activation but with normal production of adrenal CORT production. Indeed, other CORT production markers, such as StAR (and CYP11B1 left to do), were not significantly altered in our animals. This mechanism may act independently of hypothalamic PACAPergic processes that trigger activity in the HPA axis. The possibility of double PACAP-mediated processes acting at both hypothalamic and adrenal levels must be tested through further work. For example, PACAP stimulates

the activity of both CRF and AVP promoter via protein kinase A pathway within the hypothalamus (Kageyama et al, 2007). PACAP also regulates the HPA axis via a direct effect on pituitary corticotrophs in addition to stimulation of CRH gene expression in the PVN (Grinevich et al, 1997).

In our study we found no evidence for an overactive sympathoadrenal system. In animals exposed to four days of sound stress, plasma epinephrine levels remain persistently elevated for up to 21 days (Khasar, Burkham et al. 2008; Khasar, Dina et al. 2009). Indeed, repeated bouts of restraint stress do not increase TH mRNAs in the nucleus of the solitary tract (NTS) or in the dorsal motor nucleus of vagus (Tóth, Zelena et al. 2008). Xu and colleagues showed that after 7 days of restraint stress, the percentage of TH mRNA molecules in the adrenal medulla actively being translated were similar to control levels (Xu, Chen et al. 2007). In the present study we utilized a more prolonged period of chronic sound stress. Under these conditions, we found no effect of chronic sound stress on TH or PNMT mRNA induction in the adrenal gland. Because CA synthesizing enzymes mRNA levels did not increase following repeated stress, as is typical with acute stress (Nankova, Kvetnanský et al. 1994; Xu, Chen et al. 2007; Stroth and Eiden 2010), our results suggests that the CA synthesizing enzymes habituate to the repeated SNS activity associated with chronic sound stress. This is also supported by our finding that CA concentrations were not altered by chronic sound stress. In combination with elevated PACAP expression levels, our results provide further evidence that PACAP-dependent

mechanisms are important for regulating and maintaining adrenal responses to chronic stress.

Prior studies have documented changes in NMDA NR2B subunit mRNA expression in stressed animals in both the BNST (Ventura-Silva, Pêgo et al. 2012) and the PVN (Ziegler, Cullinan et al. 2005). To our knowledge, our study is the first to demonstrate a switch in the relative expression of NR2A and NR2B receptors in the PVN of rats following chronic sound stress, making NMDA receptors less effective in stimulating synaptic transmission. The role of NMDA receptors in PVN regulation of cardiovascular activity is still being studied. Consistent with our findings application of a selective NMDA receptor antagonist (LY235959) in the PVN increases acute stress-evoked pressor responses (Busnardo, Alves et al. 2013). These findings are controversial since injection of NMDA into the PVN increases blood pressure (Martins-Pinge, Mueller et al. 2013) while microinjection of kynurenic acid in the PVN of rats decreases blood pressure (Mastelari, de Abreu et al. 2012). These data suggest that NMDA receptors may downregulate these responses normally. In contrast, Ziegler and colleagues demonstrated decreased (not elevated) NR2B expression in the PVN of rats subjected to chronic variate stress (Ziegler, Cullinan et al. 2005) and hypothesized that this change was consistent with increased glutamatergic drive to the PVN via increased Ca^{2+} conductance and faster deactivation kinetics. Our work provides further evidence that chronic stress alters glutamate receptor expression and drive in the PVN. Related to this it is unclear what alternative mechanisms may underlie elevated pressor responses following chronic stress.

More research is necessary to determine the importance of this shift and the differential regulation of NMDA subunits in response to various stressors.

The present study provides evidence that sound stress leads to significant changes in local PACAP signaling in the adrenal gland. Adrenal markers of CA synthesis were unaltered. However, increased adrenal PACAP occurred in spite of reduced HPA activity (glutamategric responses and blunted ACTH responsiveness in the adrenal), suggesting independent regulation of adrenal CORT production. In fact, unaltered levels of StAR mRNA confirmed normal (desensitized) CORT production during chronic stress. Evidence for an independent PACAPergic system within the adrenal gland will need to be examined in further studies. The current findings provide important insight into how stress axes adapt to chronic mild stressors.

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Table 4.1: Sequences of qPCR primers used to probe for adrenal genes markers involved in regulating glucocorticoid/mineralocorticoid and catecholamine biosynthesis and induction. Listed is the primer used for housekeeper β gnidocne seneg tnaveler-APH rof era detsil osla sremirP .nitca-steroidogenic acute regulatory protein (StAR), melanocortin 2 receptor (MC2R), and melanocortin receptor accessory protein (MRAP). qPCR primers for adrenomedullary markers include tyrosine hydroxylase (TH), the first synthetic and rate-limiting enzyme in the biosynthesis of catecholamines, and phenylethanolamine N-methyltransferase (PNMT) which methylates norepinephrine to epinephrine. Also listed are markers for peptidergic and cholinergic signaling in the adrenal gland, pituitary-adenylate-cyclase activating polypeptide (PACAP), the primary PACAP receptor, PAC1R, and nicotinic Acetylcholine receptors (nAChR). NMDA receptor subunits NR2A and NR2B are encoded by the GRIN1A and GRIN2B genes respectively.

Gene	Accession #	Primer	Sequence (5'-3')	Annealing Temp (°C)
β actin	NM_031144	forward	TTCTTGCAGCTCCTCCGTCGC	61.3
		reverse	CACCATCACACCCTGGTGCCTA	
PACAP	NM_016989	forward	TTCGGTGTCACGCTCCCTCCT	61.9
		reverse	GCTACACATGGTCATTCGCGGCT	
StAR	NM_031558	forward	AGCTCTCTACTTGGTTCTCAACTG	58.3
		reverse	CTCCAGTCGGAACACCTTGC	
MRAP	NM_001135834	forward	GATGCCTCTGTCCCGTTCAC	58.1
		reverse	GGGGACTATGCCTTACCTGTG	
MC2R	NM_001100491	forward	TGAGGTTGCACACAGAGCGA	59.2
		reverse	TGTACTTTCCAAACTGCCACG	
TH	NM_012740	forward	GGCTGTCACGTCCCCAAGGTT	63.2
		reverse	GCCCGAGACAAGGAGGAGGGTT	
PNMT	NM_031526.1	forward	TGTCTGGACAGGTCCTCATTGACA	60.0
		reverse	TGAGGCAGACATGCTGGCTATACA	
GRIN2A	NM_012573	forward	TCATCGTCTCAGCCATTGCTGTCT	60.1
		reverse	ACACCATGATCTTGCTGGTTGTGC	
GRIN2B	NM_012574	forward	TATCTCGCAGCAATGGGACTGTGT	60.2
		reverse	TGCTTTGCCGATGGTGAAAGATGG	
PAC1R	NM_001270583	forward	ATCAAAGGCCCCGTGGTTGGC	63.0
		reverse	AGCGGGCCAGCCGTAGAGTAA	
nAChR	NM_024354	forward	ACGTGTGGGTGAAGCAGGAGTG	61.7
		reverse	GGGGGTGTCCACTGCACCCTT	

Figure 4.1 Animals subjected to eight weeks of daily sound stress had a significant increase in their resting systolic blood pressure. In sound stress animals, systolic blood pressure was elevated by 8.45 ± 1.9 % in stressed animals vs -1.01 ± 1.9% in control animals. No significant effect of stress was noted in diastolic pressure or mean arterial pressure between groups. Asterisks indicate statistical significance as compared control rats $p<0.01$ (**). Control = control rats, Stress = rats subjected to 8 weeks of daily sound stress. SYS = systolic blood pressure, MAP = mean arterial pressure, DIA = diastolic blood pressure

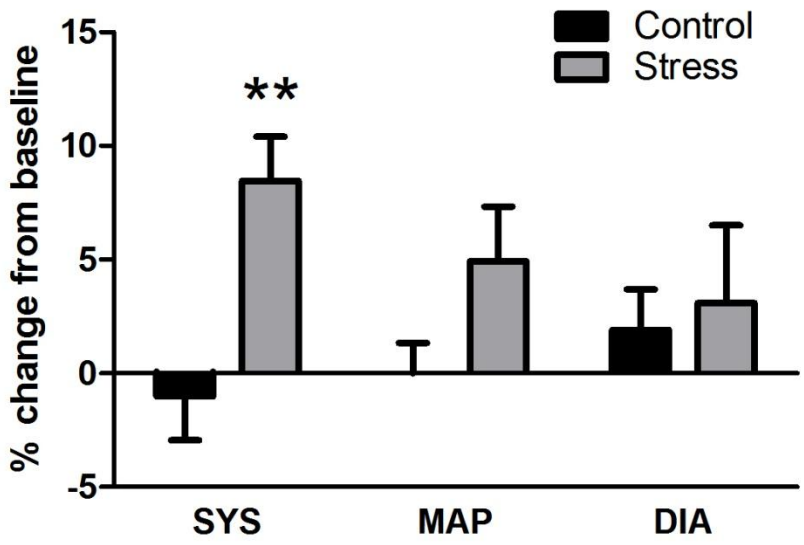

Control
Stress
15
10
5
0
-5
% change from baseline
**
SYS
MAP
DIA

Figure 4.2 Adrenal PACAP expression is elevated in rats subjected to chronic sound stress. Adrenal catecholamine content was unaffected by chronic sound stress. SNS activation of the adrenal gland was monitored via qPCR for mRNA gene expression. **A:** Expression of PACAP mRNA increased almost three-fold in stressed animals (2.90 ± 0.51) vs. controls (1.05 ± 0.09, t-test, $p<0.01$, n=41). No significant differences were noted in gene expression of nicotinic acetylcholine receptors (nAChR) or the primary PACAP receptor PAC1R. The catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT) were also unaltered by sound stress. **B:** Adrenal CA content was unaltered by 8 weeks of daily sound stress**.** Asterisks indicate statistical significance as compared control rats $p<0.01$ (**). Control = control rats, Stress = rats subjected to 8 weeks of daily sound stress.

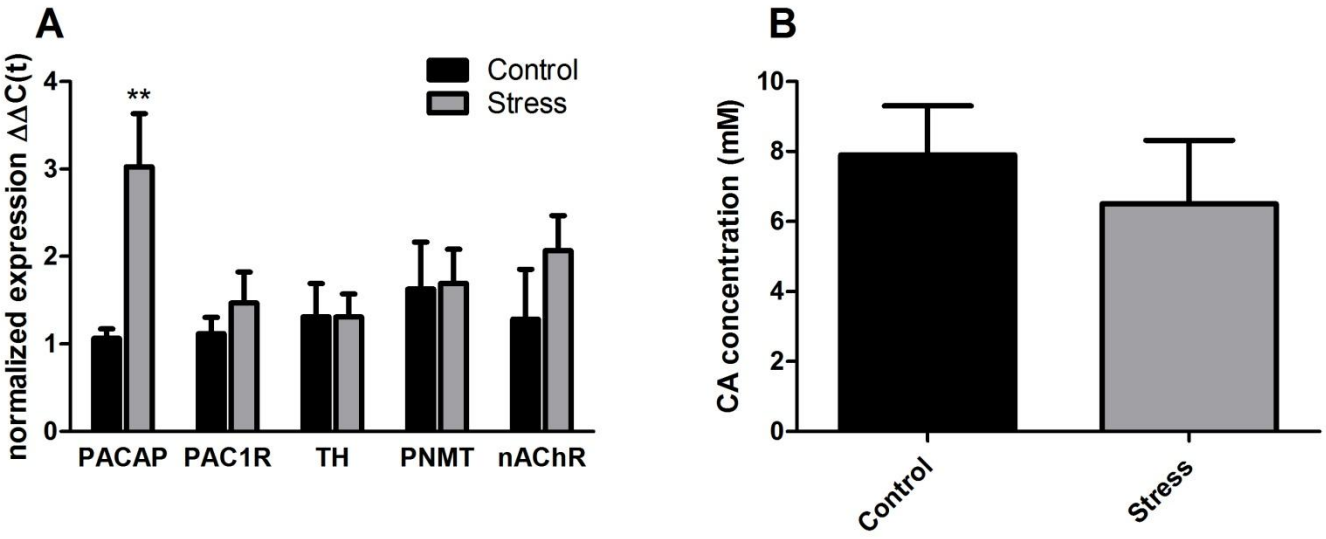

A
Control
Stress
**
normalized expression ΔΔC(t)
0
1
2
3
4
PACAP
PAC1R
TH
PNMT
nAChR
B
CA concentration (mM)
0
2
4
6
8
10
Control
Stress

Figure 4.3 Adrenal MC2R expression is blunted in rats subjected to chronic sound stress. Peripheral HPA activation of the adrenal gland was monitored via qPCR for mRNA gene expression. Adrenal xpression of MC2R mRNA was significantly decreased in stressed animals (0.61 ± 0.09) vs. controls (1.10 ± 0.164, $t=2.918$, $p<0.05$, $n=22$). No significant differences were noted in gene expression of MRAP or StAR. Asterisks indicate statistical significance as compared control rats $p<0.05$ (*). Control = control rats, Stress = rats subjected to 8 weeks of daily sound stress.

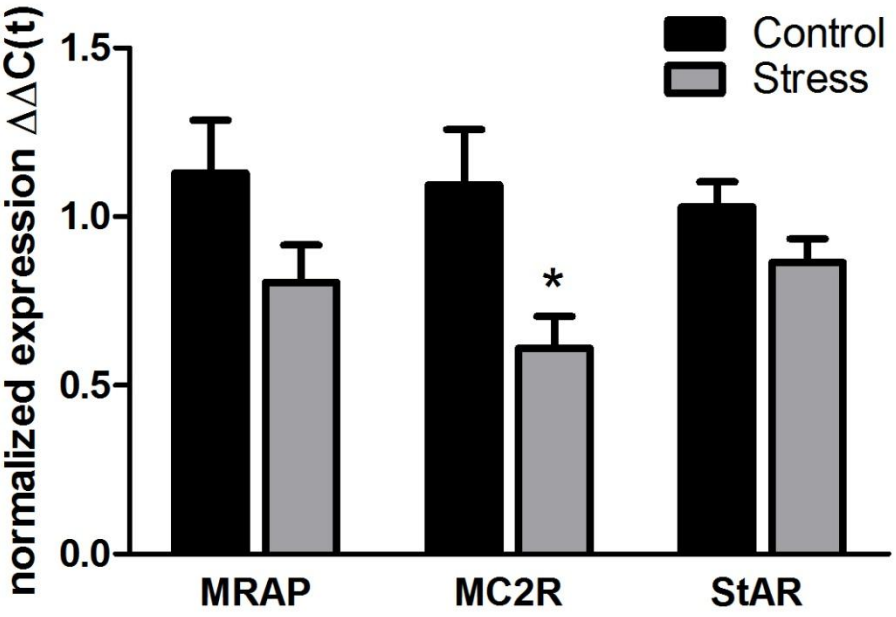
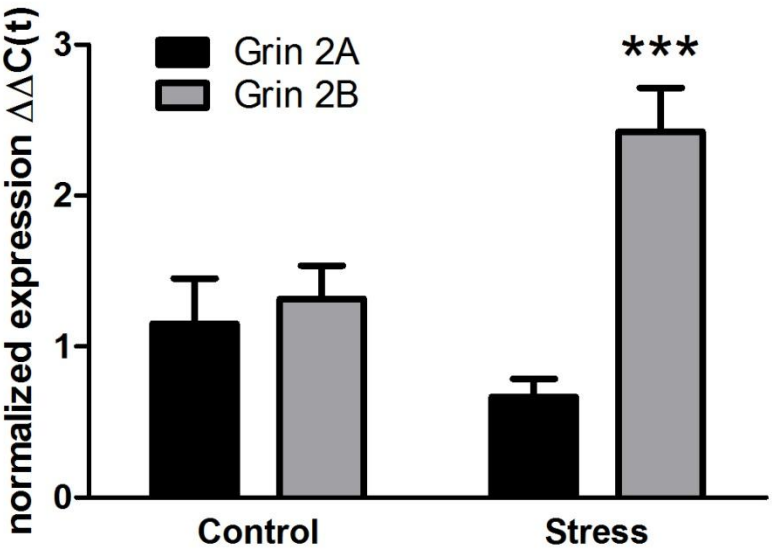

normalized expression ΔΔC(t)
1.5
1.0
0.5
0.0
Control
Stress
*
MRAP
MC2R
StAR

Figure 4.4 Expression of NMDA subunits is altered in the PVN of rats exposed to chronic sound stress. Chronic sound stress increased PVN mRNA expression of the NR2B subunit (2.42 ± 0.29) of NMDA receptors relative to the NR2A subunit (0.67 ± 0.12). Asterisks indicate statistical significance as compared control rats $p<0.05$ (*). Control = control rats, Stress = rats subjected to 8 weeks of daily sound stress.


normalized expression ΔΔC(t)
3
2
1
0
Grin 2A
Grin 2B
***
Control
Stress

Chapter 5

A Calibrated Method of Manual Therapy Decreases Systolic Blood Pressure Concomitant with Changes in Heart Rate Variability in Male Rats

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Abstract

Manual therapies such as massage have recently been associated with significant impacts on the autonomic nervous system. In humans, moderate pressure massage decreases heart rate and blood pressure to a greater degree than light pressure massage. Massage-like stroking of the abdomen in rats can reduce both blood pressure and heart rate to a greater degree than stroking either the dorsal back or lateral abdomen. To better exploit this alternative form of clinical therapy a reproducible method for the application of variable pressures during massage-like stroking in rats is needed. Massage pressures were monitored using a modified neonatal blood pressure cuff (Trimline Tempa-Cuff) attached to a clinical aneroid gauge (Tycos). Lightly anesthetized rats (1.75% isoflurane) were stroked on the ventral abdomen for 5 min at pressures of 20 mmHg and 40 mmHg at 60 strokes per minute. Systolic blood pressure (BP) and mean arterial pressure (MAP) were monitored via non-invasive blood pressure (NIBP) using sphygmomanometry for 20 minutes following massage therapy at 5 minute intervals. In Long-Evans Rats, systolic blood pressure dropped by an average of 9.86 ± 0.27% following application of 40 mmHg massage pressure. Similar effects were seen following 20 mmHg pressure (6.52 ± 1.7%) though latency to effect was greater than at 40 mmHg. Sprague-Dawley rats behaved similarly to Long-Evans rats except for increased latency to significant effect. Heart rate variability, measured via electrocardiogram, was reduced after 40 mmHg manual therapy suggesting increased parasympathetic tone. The calibrated manual

therapy method provided a reproducible method for applying manual massage pressures in rodents that lowered blood pressure and heart rate variability.

Introduction:

Nearly 31% of U.S. adults have high blood pressure (CDC 2008) resulting in an annual cost of more than $76.6 billion in health care services and missed work (Lloyd-Jones, Adams et al. 2009). The etiology of essential hypertension is still unclear, though both the renin-angiotensin system (RAS) (Jezova, Ochedalski et al. 1998; Saavedra and Benicky 2007; Saavedra, Sánchez-Lemus et al. 2010) and sympathetic nervous systems (SNS) (Esler, Rumantir et al. 2001; Carter, Durocher et al. 2008; Esler, Lambert et al. 2010) appear to play important roles the development of high blood pressure.

While pharmacological approaches to the management of hypertension via the RAS and SNS have proven to be effective, side effects such in as angioedema, headache, hypotension, and dizziness are common (Calhoun, Lacourciere et al. 2009; Campo, Fernandez et al. 2013). There are relatively few well-studied, non-pharmacological options for the prevention and treatment of hypertension. One potential non-pharmacological treatment option, manual therapy, has been associated with significant impacts on blood pressure and autonomic nervous system activity. In humans, moderate pressure massage can decrease heart rate and blood pressure while increasing vagal afferent activity as measured by heart-rate variability (Delaney, Leong et al. 2002; Diego and Field 2009). Lumbar spine manipulation has been shown to increase lumbar

parasympathetic nervous system output in patients with pain (Roy, Boucher et al. 2009). Moderate pressure massage in humans has also been shown to decrease self-reported stress while increasing electroencephalogram (EEG) delta activity and decreasing alpha and beta activity, suggesting a relaxation response after only 10 min of moderate pressure massage (Diego, Field et al. 2004). Massage-like stroking of the skin in rats has been reported to increase withdrawal latency to noxious stimuli (Agren, Lundeberg et al. 1995), a sedative response (Uvnas-Moberg, Alster et al. 1996), indicating a benefit for pain modulation. Similar manipulation was shown to produce an inhibitory effect on the cardiovascular excitatory response (Akio 1987), and a reduction in both blood pressure and heart rate (Kurosawa, Lundeberg et al. 1995; Lund, Lundeberg et al. 1999). Studies on the effects of manual therapies have emphasized that the responses vary significantly depending on the type, location and strength of the manual stimulation procedures (Araki, Ito et al. 1984; Akio 1987; Kurosawa, Lundeberg et al. 1995; Lund, Lundeberg et al. 1999). Importantly, negative side effects from manual therapy are exceedingly rare (Ernst 2003).

Relatively few studies have utilized animal models to examine the specific mechanisms underlying how manual therapies impact blood pressure. One possible mechanism is revealed from studies using related stimulation (cutaneous brushing of the chest in rats), which significantly decreases adrenal efferent nerve activity and catecholamine secretion (Araki, Ito et al. 1984; Akio 1987). The lack of mechanistic studies performed in animals

may be due to the lack of an inexpensive, precise, and repeatable technique for applying calibrated massage pressures in small rodents. Several studies were published in the early 1990's that performed massage-like stroking of the skin in rats. Unfortunately, the massage pressures used were either not calibrated (Agren, Lundeberg et al. 1995) or were estimated and compared to pressures subsequently applied to a balloon connected to a pressure gauge (Kurosawa, Lundeberg et al. 1995; Lund, Lundeberg et al. 1999). These past studies produced interesting results and demonstrated significant differences between the applications of mild and moderate estimated pressures. However, we saw a need to develop a technique for applying calibrated massage pressures to rodents with improved inter- and intra-examiner reliability for our own studies.

In this study we developed a method for applying calibrated massage pressures using commonly available, inexpensive sphygmomanometer parts. This technique can be employed in a laboratory to reliably quantify the amount of pressure applied during manual massage in small animals and should enable a wider range of laboratories to examine mechanisms underlying the effects of massage and manual therapy in rodents. Our purpose was to develop this methodology and validate its use as a quantitative method of applying manual stimuli to rodents. Another aim of this study was to determine if specified pressures of manual therapy could alter blood pressure and parasympathetic/sympathetic neural drive to the heart.

Methods

Animals: All animals were maintained in accordance with the guidelines in National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley and Long-Evans rats (300–500g) were group-housed 3 per cage in standard polycarbonate plastic cages with heat-treated pine shavings as bedding. Food pellets (Purina Lab Diet) and water were provided ad libitum except during the experimental period. Temperature was maintained at 21 ± 2 °C and relative humidity at 50 ± 10% under a 12/12 h light/dark cycle (lights on from 7:00–19:00 h). Experiments were performed between 13:00- 18:00h). All experiments were designed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the IACUC, University of California, Riverside.

Manual Therapy: A neonatal blood pressure cuff (**Fig 5.1A**) was modified via a midline cut that retained only the inflatable bladder. The cuff was attached to a standard aneroid gauge via luer connector (**Fig 5.1B**) and the bladder was inflated to 100 mmHg and held lightly by the edges. The hook and loop side of the neonatal cuff was positioned at the distal index finger to provide appropriate friction in anesthetized rats while the smooth side of the cuff was used to contact the rats' fur (**Fig 5.2A**). Though not needed in this study, an investigator could wrap his or her index finger with a "loop surface" which would attach to the hook surface on the neonatal cuff to secure the cuff to the index

finger. Rats were stroked on the ventral abdomen at a rate of 60 strokes per minute for 5 minutes (**Fig 5.2B**). Massage pressures were monitored as net pressure above 100 mmHg baseline setting and therapy was delivered at pressures of 40 mmHg and 20 mmHg. A "no therapy" session was performed 5–7 days prior to manipulation of the treated rats to demonstrate the effect of anesthesia alone. Therefore, each rat received three sessions (no therapy, massage therapy at 20 mmHg, massage therapy at 40 mmHg) in randomized order. Each session lasted 30 min and subsequent therapy sessions were spaced 2 days or more apart. Equal numbers of 20 mmHg and 40 mmHg treatments were performed on each day. Baseline systolic blood pressure and ECG (see below) values were established after 5 minutes of maintenance anesthesia. Post-massage data collection was performed on each subject for 20 minutes. Data is expressed as percentage change from the baseline measurement obtained at the beginning of each session. The neonatal cuff was cleaned with 75% ethanol between therapy sessions. Blood pressure (BP) was monitored via non-invasive blood pressure (NIBP) using sphygmomanometry at 5 minute intervals.

Non□Invasive Blood Pressure (NIBP): NIBP was measured by tail-cuff plethysmography using a manual blood pressure monitoring system with manual deflation (IITC) as described (Shah, Coburn et al. 2011). Each NIBP reading was recorded on digital video and systolic blood pressure (highest pressure at which the heart pulses return) and mean arterial pressure (MAP, peak amplitude pulses) were measured over a minimum of 20 cardiac cycles for each recording. Diastolic blood pressure was calculated using the

formula MAP = diastolic BP + 1/3 pulse pressure (PP), where PP is the difference between systolic BP and diastolic BP. All measures were obtained under light anesthesia (1.75% isoflurane, 1L/min oxygen) after a brief induction (5% isoflurane: 95% O2; 2L/min). A heat lamp was used to maintain the animal's temperature. When compared to simultaneous measurements with an invasive catheter and transducer method, NIBP values vary no more than 7%. Moreover, previous studies have determined a near perfect correlation (0.98) between radiotelemetry measurements and the pulse based tail-cuff method (Whitesall, Hoff et al. 2004).

Heart rate variability: Heart rate variability was recorded via electrocardiogram (ECG) under light anesthesia (1.75% isoflurane, 1L/min oxygen) following a brief induction (5% isoflurane: 95% O2; Flow = 2L/min). ECG recordings were obtained using adhesive gel electrodes placed on the paws and data was recorded using PowerLab 15T data acquisition hardware (ADInstruments) running LabChart8 software (ADInstruments). Data from the ECG recording was divided into 5 minute intervals and was exported and smoothed with a Savitzky-Golay filter (Savitzky and Golay 1964) using Python software customized in our lab. Data was analyzed via the open source gHRV software (Milegroup) using time-domain parameters, non-linear analysis, and frame-based analysis. Band limits for HRV were low frequency 0.050–0.150 (LF), and high frequency 0.150–0.400 (HF). Data was calculated using LF/HF ratio and reported as a percentage of pre-treatment baseline plotted over time.

Statistical analysis: Data was analyzed via one-way repeated measures ANOVA and two-way ANOVA to evaluate significant differences at individual time points. Multiple group comparisons were made post-hoc using Student–Newman–Keuls and Bonferroni tests, respectively. Statistical significance was acknowledged at an alpha level of 0.05 or lower.

Results:

A calibrated method for applying manual therapy to rodents was developed using a modified neonatal blood pressure cuff. A neonatal blood pressure cuff (Trimline Tempa-Cuff, size 1) was modified via a midline cut that retained only the inflatable bladder **(Fig 5.1A**). The cuff was attached to a standard clinical aneroid gauge (Tycos) via luer connector (**Fig 5.1B**). These components were chosen because of their durability, ease of cleaning, and ready availability. Early prototypes were tested on co-workers to determine if a difference could be felt between 20mmHg and 40mmHg. During this early testing, these pressures were estimated to be appropriate for adult male rats; however, the pressures used could be adjusted to suit larger or smaller animals.

The calibrated manual therapy method provided a reproducible method for applying manual pressures in rodents. This method was used on adult male rats within a weight

range of 300–500g that were lightly anesthetized and immobile. The blood pressure cuff was inflated to 100 mmHg, held lightly by the edges, and positioned mid-abdomen in preparation for massage therapy procedure. The hook and loop side of the neonatal cuff was positioned at the distal index finger to provide appropriate friction while the smooth side of the cuff was used to contact the rats' fur. During each stroke, pressure was precisely monitored relative to 100 mmHg baseline (**Fig 5.2 A,B**) and applied at a rate of 60 strokes per minute (1 stroke per second) for 5 minutes. The procedure can be performed alone with the use of a digital timer to monitor stroke rate and a skilled experimenter could easily monitor stroke pressure with minimal effort. Gloves are used as a safety precaution but are not necessary for the procedure to be effective. The apparatus used was inexpensive with an approximate cost of $160 per unit, though the apparatus could be re-created using an inexpensive aneroid gauge.

Calibrated manual therapy reduced systolic blood pressure and MAP in male Long□Evans rats in a dose□dependent manner. **Figure 5.3** shows the changes in systolic blood pressure (**5.3A**), MAP (**5.3B**) and diastolic blood pressure (**5.3C**) displayed by male Long Evans rats following manual therapy of the lower abdomen at 0, 20, and 40 mmHg. A two-way ANOVA revealed significant effect of manual therapy treatment ($F_{2,109}$=19.57, p<0.0001, n=27) and time ($F_{4,109}$=3.038, p<0.05, n=27) on systolic pressure. Multiple post-hoc comparisons via Bonferroni test revealed significant differences between no therapy vs. 20 mmHg pressure at 10 min post-treatment (t=3.246,

p <0.01) and at 20 min post-treatment (t=2.779, p <0.05). At a pressure of 40 mmHg therapy significantly reduced systolic blood pressure at all time points: 5 min (t=3.492, p <0.01), 10 min (t=3.782, p <0.01), 15 min (t=2.780, p < 0.05) and 20 min (t=3.657, p <0.01). A two-way ANOVA revealed significant effect of pressure on MAP in Long-Evans rats ($F_{2,104}$=5.413, p=0.0058, n=27). A Bonferroni post-hoc test revealed significant differences between no therapy and 20mmHg at 10 minutes (t=2.890, p<0.05) and between no therapy and 40 mmHg at 10 minutes (t=2.960, p<0.05) and 15 minutes (t=2.891, p<0.05, Fig. **5.3B**). No effect of therapy was seen on diastolic pressure measurements at any time point following therapy (n=27, Fig. **5..3C**).

The calibrated manual therapy method reduced systolic blood pressure and MAP in male Sprague□Dawley rats. **Figure 5.4** shows the changes in systolic blood pressure (**5.4A**), MAP (**5.4B**) and diastolic blood pressure (**5.4C**) displayed by male Sprague-Dawley rats following manual therapy of the lower abdomen at 0, 20 and 40 mmHg. A two-way ANOVA revealed a significant effect of pressure ($F_{2,89}$=24.10, p<0.0001), time following treatment ($F_{4,89}$=10.66, p<0.0001) and interaction ($F_{8,89}$=3.20, p=0.003) on systolic blood pressure in Sprague-Dawley rats. A Bonferonni post-hoc test revealed significant differences between no pressure vs 20 mmHg at 15 min (t=2.662, p<0.05), no pressure vs 20 mmHg at 20 min on systolic blood pressure (t=4.614, p<0.001). Comparisons between no pressure vs 40 mmHg yielded significant differences at 10 min (t=3.561, p<0.01), 15 min (t=5.060, p <0.001) and 20 min following treatment (t=5.223, p<0.001). A two-way

ANOVA performed on the pooled MAP data indicated a significant effect of pressure ($F_{2,89}$=5.413; p=0.0058). Bonferonni multiple comparison post-hoc test revealed significant differences in MAP between no pressure vs 20 mmHg at 10 min (t=2.890, p<0.05), no pressure vs 40 mmHg at 10 min (t=2.960, p <0.05), and 15 min (t=2.891, p <0.05). No effect of therapy was seen on diastolic pressure measurements at any time point following therapy (n=29, Fig. **5.4C**).

Calibrated manual therapy reduced heart rate variability in Long□Evans rats as measured by LF/HF ratio. In our last set of experiments we measured HRV to estimate the relative contribution of parasympathetic and sympathetic tone to the changes provoked by manual therapy on blood pressure. The same rats as shown in **Figure 3** were equipped with ECG leads and heart rate monitored for 5 min to acquire baseline reading. After sham or manual therapy ECG readings were taken at 5, 10, 15 and 20 min post-treatment. **Fig. 5.5** shows the changes in HRV over time following massage therapy. A one-way repeated measures ANOVA of LF/HF ratio indicated significant differences between groups ($F_{2,8}$=4.692, R square =0.539, p<0.05, n=16). A Newman-Keuls multiple comparison post-hoc test revealed significant differences between no pressure and 40 mmHg pressure (q=4.420, p < 0.05), and no pressure and 20 mmHg (q=4.214, p <0.05, **Fig 5.5**). To determine the time points at which manual therapy pressure produced a significant effect we ran a two-way ANOVA. A significant effect of pressure was found

($F_{2,63}$=3.820, p<0.05). A Bonferroni post-hoc test revealed a significant effect of manual therapy at 40 mmHg at 10 min post-treatment (t=2.843, p <0.05).

Discussion:

Our study demonstrates a method for applying calibrated massage pressures in small rodents. The described technique utilizes readily available and inexpensive sphygmomanometer parts that can be configured in such a way as to provide for consistent and calibrated pressures to improve inter- and intra-examiner reliability. Utilizing this technique we were able to confirm reports that that manually stroking the abdomen of rats in rodents can lower blood pressure (Kurosawa, Lundeberg et al. 1995; Lund, Lundeberg et al. 1999). Our research demonstrated that blood pressure and heart rate decreased with little delay following the application of the massage procedure. In the case of systolic blood pressure, manual therapy at 40 mmHg was able to produce significant reduction within 5 min in Long Evans rats. This was the most striking effect of our study. Importantly, this effect persisted for the duration of the 20 minute post-massage monitoring period. Both Long Evans and Sprague-Dawley rats were responsive to massage therapy, suggesting that the beneficial effect is due to conserved physiological traits.

Using our calibrated pressure technique, therapy-induced reduction in blood pressure recorded from Long Evans rats manifested with less latency (at 10 min post-treatment) than those displayed by Sprague-Dawley (at 15 min post-treatment). This occurred at the lowest pressure applied (20 mmHg). This differential in latency was maintained when applying 40 mmHg pressure but the absolute latency times were reduced to 5 and 10 min post-treatment, respectively. Long-Evans rats have been shown to have higher blood pressure in response to stressors such as psychosocial stress (Henry, Liu et al. 1993) and caloric restriction (Evans, Messina et al. 2005). This finding demonstrates that Long-Evans rats may be more sensitive to manual therapy application and highlights the need for accurately calibrating the application of manual therapies in rodents to optimize responses and also for comparing results across studies.

Our results also agree with prior work in humans that indicates that massage can impact heart rate variability and decrease LF/HF ratio (Delaney, Leong et al. 2002; Diego and Field 2009). The effect in humans on HRV occurred with very little latency and persisted for approximately 15 minutes. We showed that both massage pressures decreased LF/HF ratio suggesting a shift from sympathetic to parasympathetic drive to the heart (Task Force and Electrophysiology 1996). The effect is similar to effects on blood pressure decrease, though the time course for changes in HRV have a latency of ~ 5min and do not persist as long as blood pressure changes.

Prior studies have demonstrated that massage-like stroking of the abdomen produces a more robust decrease in blood pressure than does stroking of the sides or back (Lund, Lundeberg et al. 1999), therefore it is likely that abdomen activates visceral in addition to somatosensory afferents. Though this technique likely produces some mechanical compression of the vena cava, prior studies have demonstrated that decreased blood pressures following stroking of the abdomen in rats are most likely due to a reduction of the efferent sympathetic nerve activity and associated decreases in adrenal catecholamine secretion (Akio 1987; Kurosawa, Lundeberg et al. 1995; Lund, Lundeberg et al. 1999). These effects are thought to involve central somato-sympathetic reflex pathways (Araki, Ito et al. 1984; Akio 1987). However it is likely that endocrine, in addition to neural pathways, contribute to the reduced systolic blood pressure following therapy since the effect persisted for 20 min post-treatment. Alternatively, long-acting peptide modulatory systems released from sympathetic nerves may participate. These mechanisms are likely to be functional in spite of the light anesthesia. Studying the degree to which various massage pressures impact these variables should provide a more complete understanding of the mechanisms underlying autonomic effects of manual therapies.

Manual therapies have also been shown to impact behavioral manifestations associated with chronic activation of the hypothalamic-pituitary-adrernal (HPA) axis such as anxiety and depression (Field, Hernandez-Reif et al. 2005; Garner, Phillips et al. 2008). Manual therapies decrease plasma, urinary, and salivary cortisol (Hernandez-Reif, Ironson et al.

2004; Field, Hernandez-Reif et al. 2005; Moraska, Pollini et al. 2008; Stringer, Swindell et al. 2008) as well as urinary corticotropin releasing factor-like immunoreactivity (CRF-LI) (Lund, Lundeberg et al. 2006). Manual stimulation in rats has been shown to significantly increase glucocorticoid receptor gene expression which enhanced negative feedback inhibition of HPA activity and reduced post-stress secretion of ACTH and glucocorticoid (Jutapakdeegul, Casalotti et al. 2003). Reduced HPA activity at the level of CRF may, in turn, reduce sympathetic efferent nerve activity and adrenal catecholamine secretion (Akio 1987). The described method for applying manual therapy in rodents is expected to provide a valuable tool for studying how manual therapy impacts the complex relationships between the HPA and SNS.

Considering the broad socioeconomic impact of hypertension and related diseases, there is a need to evaluate the efficacy of safe, widely available, and minimally invasive alternatives to popular pharmacological treatments. Manual therapies such as manipulation and massage show promise in this arena because of the relationship between hypertension and the SNS and the ability of manual therapies to impact the SNS. Importantly, recurring manual therapy could prove to be a valuable tool in the prevention of stress-related disorders. Consider, for instance, that nearly 1 in 5 U.S. soldiers returning from Iraq and Afghanistan suffer from post-traumatic stress with an estimated societal cost of ~$2–3 billion per year (Adamson, Burnam et al. 2008). Combat-deployed soldiers also exhibit a higher incidence of hypertension (Granado, Smith et al. 2009). In

this context, a hypertension prevention strategy that includes manual therapy could be incredibly valuable. Further, if a preventive strategy is to be broadly employed, safety is of vital importance. Current treatment strategies carry significant side-effects and are not suited for prophylactic treatment. Our novel calibrated method of applying massage therapy offers an improved strategy that is non-invasive and inexpensive. Further improvements could involve automation for application of massage pressures and measurement of blood pressure. Improving techniques for examining the effects of manual therapies in animal models should assist in a better understanding of the mechanisms underlying manual therapy effects and the possible long-term persistence of benefits. Further research is needed to develop guidelines for the appropriate use of this treatment strategy in the treatment and/or prevention of stress related disorders and hypertension.

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Fig 5.1 Apparatus used for applying calibrated manual therapy to rodents was developed using a modified neonatal blood pressure cuff. A: A neonatal blood pressure cuff (Trimline Tempa-Cuff, size 1) was modified via a midline cut that retained only the inflatable bladder. **B:** The neonatal cuff was attached to a standard clinical aneroid gauge (Tycos) via luer connector.

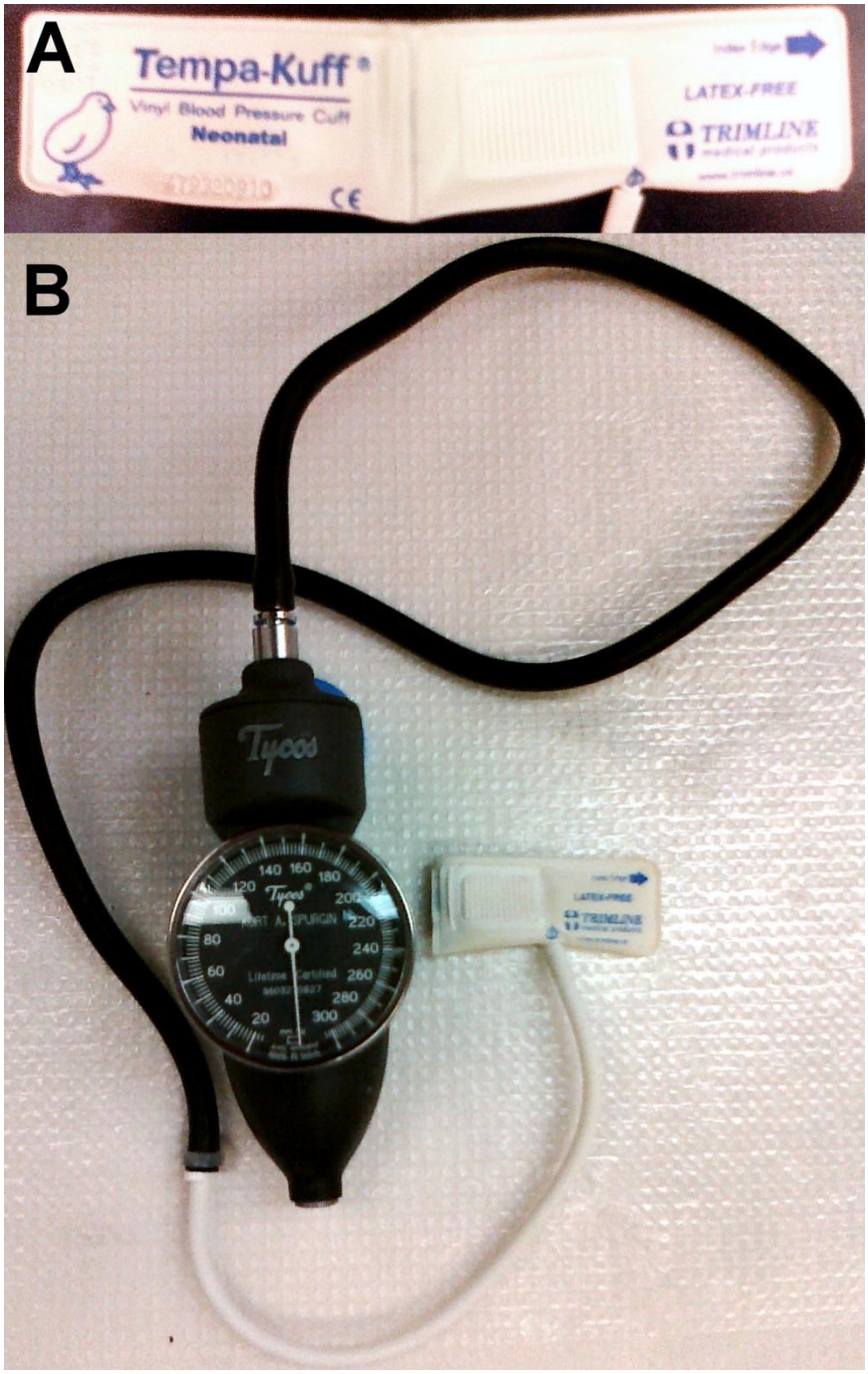

A
Tempa-Kuff
Vinyl Blood Pressure Cuff
Neonatal
LATEX-FREE
TRIMLINE
B
Tycos

Fig 5.2 The calibrated manual therapy method provided a reproducible method for applying manual therapy pressures in rodents. A: The blood pressure cuff was inflated to 100 mmHg, held lightly by the edges, and positioned mid-abdomen in preparation for massage therapy procedure. The hook and loop side of the neonatal cuff was positioned at the distal index finger to provide appropriate friction while the smooth side of the cuff was used to contact the rats' fur. During each stroke, pressure is precisely monitored relative to 100 mmHg baseline. **B**: demonstrates a lightly anesthetized animal receiving treatment at a massage pressure of 20 mmHg over the 100mmHg baseline.

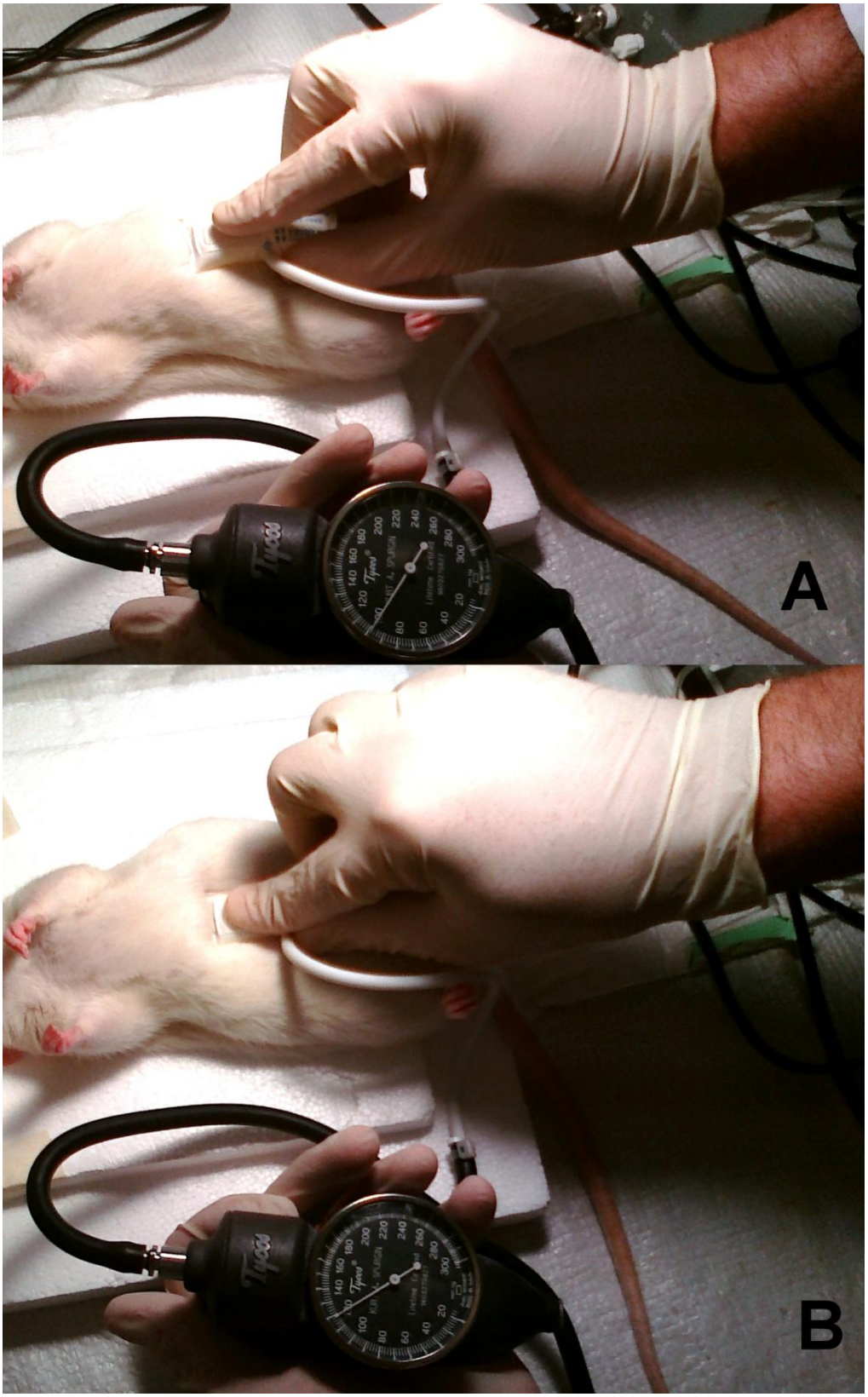

A
B

Fig. 5.3 The calibrated manual therapy method reduced blood pressure in male Long-Evans rats in a dose-dependent manner. Male Long-Evans rats were treated with no therapy, manual therapy at 20mmHg, and manual therapy at 40mmHg on separate days under light anesthesia (isoflurane, 1.75%). **A:** Manual therapy reduced systolic blood pressure in a dose-dependent manner at all time points following treatment vs no therapy control session. **B:** Manual therapy reduced mean arterial pressure (MAP) at 10 and 15 min following treatment vs no therapy control session. **C:** No changes were seen in diastolic blood pressure following manual therapy. Each data point represents mean change from baseline ± s.e.m. Asterisks indicate statistical significance in comparison to no therapy control session via two-way ANOVA at $p<0.05$ (*) and $p<0.01$ (**).

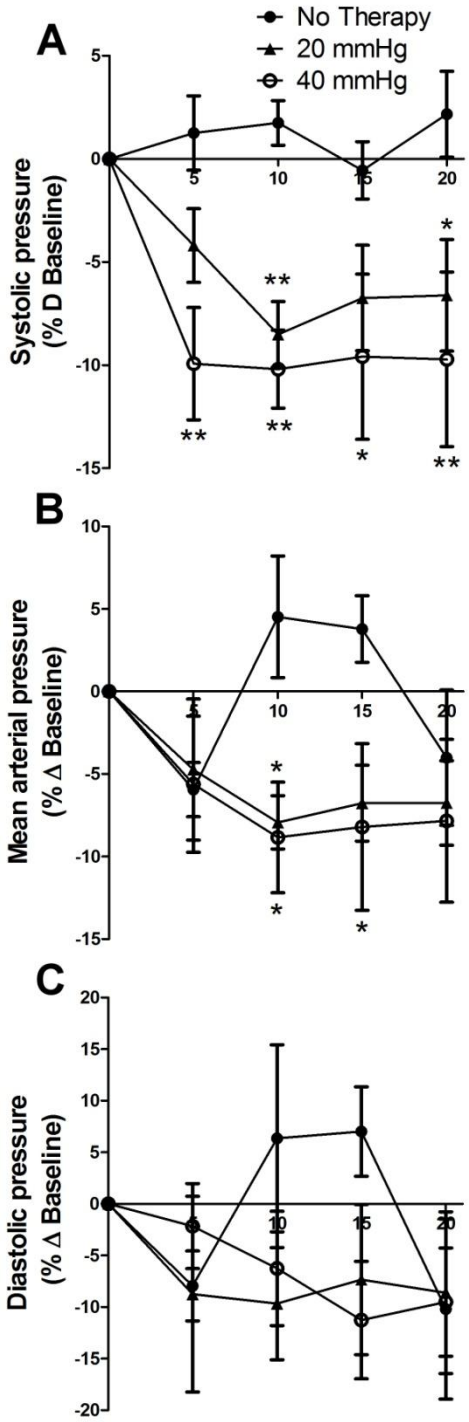

A
No Therapy
20 mmHg
40 mmHg
Systolic pressure
(% D Baseline)
5
0
-5
-10
-15
5
10
15
20
**
**
**
*
*
B
Mean arterial pressure
(% Δ Baseline)
10
5
0
-5
-10
-15
10
15
20
*
*
*
C
Diastolic pressure
(% Δ Baseline)
20
15
10
5
0
-5
-10
-15
-20
10
15
20

Fig. 5.4 The calibrated manual therapy method reduced blood pressure in male Sprague-Dawley rats. Male Sprague-Dawley rats were treated with no therapy, manual therapy at 20mmHg, and manual therapy at 40mmHg on separate days under light anesthesia (isoflurane, 1.75%). **A**: Manual therapy at 20mmHg and 40mmHg both reduced blood pressure at 10, 15 and 20 min following treatment vs no therapy control session. **B:** Manual therapy reduced mean arterial pressure (MAP) at 15 and 20 min following treatment vs no therapy control session. **C:** No changes were seen in diastolic blood pressure following manual therapy. Each data point represents mean change from baseline ± s.e.m. Asterisks indicate statistical significance in comparison to no therapy control session via two-way ANOVA at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$(***)

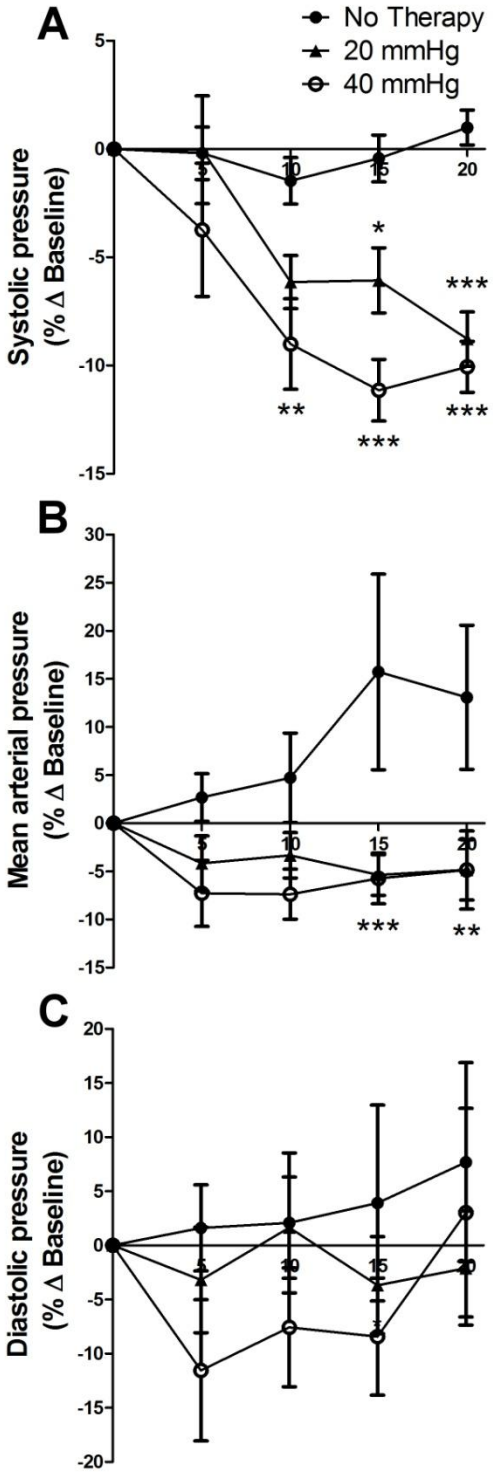
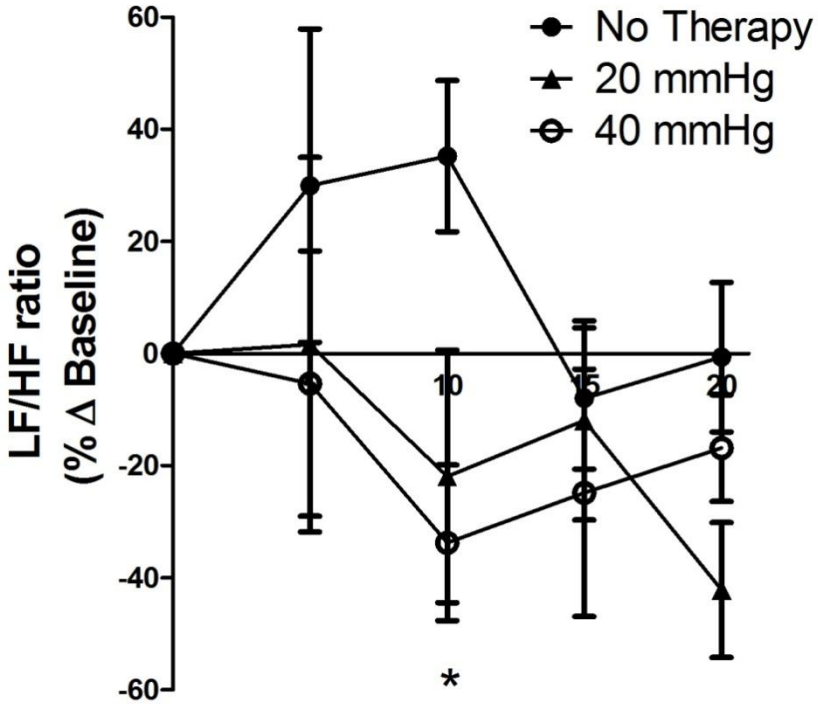

A
No Therapy
20 mmHg
40 mmHg
Systolic pressure
(% Δ Baseline)
5
0
-5
-10
-15
10
15
20
*
***
**
***
***
B
Mean arterial pressure
(% Δ Baseline)
30
25
20
15
10
5
0
-5
-10
-15
***
**
C
Diastolic pressure
(% Δ Baseline)
20
15
10
5
0
-5
-10
-15
-20

Fig. 5.5 Calibrated manual therapy reduced LF/HF ratio. Male Long Evans rats were treated with no therapy, manual therapy at 20mmHg, and manual therapy at 40mmHg on separate days under light anesthesia (isoflurane, 1.75%). Pooled data for each treatment group is represented as mean change from baseline ± s.e.m. A repeated measures ANOVA of LF/HF ratio revealed that manual therapy at 20 mmHg and 40mmHg significantly reduced heart rate following treatment vs no therapy control session. Asterisk indicates statistical significance in comparison to no therapy control session via two-way ANOVA at $p<0.05$ (*)


No Therapy
20 mmHg
40 mmHg
LF/HF ratio
(% Δ Baseline)
60
40
20
0
-20
-40
-60
10
15
20
*

Chapter 6

Conclusion

Through my dissertation research I have examined how the major stress axes respond to physiological, psychological, and environmental stressors. The hypothalamus receives input regarding various environmental, psychological, and physiological stressors and generates appropriate responses via the hypothalamo–pituitary–adrenal (HPA) and hypothalamo–sympatho–adrenal (HSA) axes, as well as via the sympathetic nervous system (SNS) generally. As I have shown, the adrenal gland plays a critical role in coordinating output for these major stress axes.

The adrenal cortex and medulla have generally been considered as distinct functional units with an outer steroid-producing cortex and an inner catecholamine-producing medulla. However, more recent evidence suggests that there may be significant interaction and between these two different endocrine tissues. Adrenal chromaffin cells can be found in the adrenal cortex (Gallo-Payet, Pothier et al. 1987) while cortical cells are dispersed throughout the medulla (Bornstein, Ehrhart-Bornstein et al. 1991). This co-mingling of chromaffin and cortical cells provides numerous opportunities for paracrine interactions. Furthermore, a dense network of intra-adrenal nerve fibers is present at corticomedullary junction (Rundle, Canny et al. 1988) providing significant potential for communication between these regions. My research provides important clues for how interactions within the adrenal gland impact HPA and HSA responses to various stressors. Furthermore, I have provided some important evidence for the role that PACAP plays in coordinating these responses

Several studies have demonstrated the involvement of effects of PACAP on the behavioral, endocrine and cellular responses to stress. PACAP-containing neuronal projections innervate the bed nuclei of the stria terminalis and portions of the amygdala, central areas involved in anxiety and stress responses (Kozicz and Arimura 2002; Kageyama, Hanada et al. 2007). Within the PVN PACAP fibers arrive from pain and cardiovascular centers such as midbrain periaqueductal gray and RVLM (Das, Vihlen et al. 2007; Farnham, Li et al. 2008). Intracerebroventricular (icv) injections of PACAP have been shown to activate the HPA and stimulate the release of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol (Vaudry, Gonzalez et al. 2000; Agarwal, Halvorson et al. 2005; Norrholm, Das et al. 2005; Conconi, Spinazzi et al. 2006) by stimulating adenylates cyclase, PKA and CREB phosphorylation in CRF-containing neurons in the parvocellular PVN (Agarwal, Halvorson et al. 2005; Kageyama, Hanada et al. 2007; Kageyama and Suda 2009). PACAP treatment also reproduces the behavioral effects of stress such as stereotypic face washing, body grooming, rearing, and wet dog shakes (Agarwal, Halvorson et al. 2005). PACAP may work to stimulate CRF promoter activity through, IL-6, which is participates in interactions between immune and neuroendocrine systems (Kageyama, Hanada et al. 2007; Kageyama and Suda 2009). Further, exogenous PACAP treatment has been shown to amplify the effects of stress and fear; increasing aversive behaviors in the shock probe fear test (Legradi, Das et al. 2007).

PACAP also has wide ranging effects on the HSA axis. Recent work suggests that PACAP, rather than acetylcholine (ACh), may be primary regulator of adrenomedullary function during stress (for review see (Mustafa and Lee 2013). PACAP has been shown to increase heart rate, elevate arterial blood pressure, and stimulate breathing (Runcie, Hulman et al. 1995; Seki, Suzuki et al. 1995). PACAP also causes relaxation of tracheal smooth muscles and bronchodilation; and PACAP is a potent vasodilator (Vaudry, Gonzalez et al. 2000). The adrenal medulla has the second highest concentration of PACAP among peripheral tissues and endogenous PACAP is released in response to direct splanchnic nerve stimulation in isolated, perfused adrenal glands (Guo and Wakade 1994; Tornoe, Hannibal et al. 2000). Co-localization of ACh and PACAP in these fibers permits the concurrent and synergistic activities of ACh and PACAP during splanchnic nerve stimulation (Holgert, Holmberg et al. 1996; Lamouche, Martineau et al. 1999). PACAP can facilitate neuronal depolarization and excitability, increase catecholamine release from sympathetic neurons, and induce second-messenger production in sympathetic neurons (Braas 1995; Beaudet, Braas et al. 1998; Beaudet, Parsons et al. 2000; Vaudry, Gonzalez et al. 2000).

PACAP also plays an important role in potentiating the actions of other neurotransmitters. For instance, PACAP has been shown to potentiate cholinergic signaling (Cuevas and Adams 1996; Liu, Cuevas et al. 2000). A growing body of research suggests that PACAP may be linked to alterations in glutamate signaling.

PACAP can modulate both pre-synaptic release as well as post-synaptic sensitivity to glutamate (Michel, Itri et al. 2006). PACAP enhances NMDA receptor activity and enhances synaptic NMDA currents via activation of the PAC1R (Yaka, He et al. 2003; Macdonald, Weerapura et al. 2005). Specifically as it relates to our findings, PACAP induces tyrosine phosphorylation of NR2B (Yaka, He et al. 2003). PACAP also increases NMDA-receptor-mediated responses in preganglionic sympathetic neurons of rats (Wu and Dun 1997).

In chapter one, I presented findings that demonstrate that acute and chronic restraint stress as well as isolation stress differentially impacts the intra-adrenal PACAPergic system. Furthermore, changes in local PACAP signaling in the adrenal gland appear to influence steroid endocrine function by association with steroid biosynthesis and/or ACTH receptor responsiveness. Specifically I discovered that acute restraint stress upregulates adrenal PACAP and gene markers associated with MC2R trafficking whereas chronic stress upregulates adrenal genes associated with the synthesis of glucocorticoids concomitant with reduced PACAP gene expression. In a PACAP gene deletion model, chronic social isolation stress upregulates MC2R and MRAP concomitant with upregulation of TH mRNA. Taken together, finding presented in chapter one suggest that local intra-adrenal PACAP signaling may influence adrenal HPA and HSA responses to stress. The intra-adrenal PACAPergic system appears to respond differentially to acute and chronic restraint stress and sometimes in a manner exclusive of sympathoadrenal

responses; and PACAP gene deletion results in disrupted CORT activation during chronic social isolation.

In chapter two I examined how a highly-prevalent environmental stressor impacts adrenal function. I presented novel mechanisms that may underlie the propensity of PBDE exposed rats to have elevated blood pressure in response to hypertonic saline injection. Attenuation of this response by ganglionic blockade suggests that the sympathetic nervous system appears upregulated in response to hyperosmotic stimulus. I also presented evidence of adrenal dysfunction as markers for CA synthesis were blunted in PBDE animals. Further evidence of stress axis disruption was presented in the form of altered heart rate variability and increased plasma corticosterone responses in PBDE-exposed animals. In combination, these results suggest that developmental exposure to environmental toxicant PBDEs alters adrenal function and stress axis responses contributing to elevated blood pressure following hyperosmotic stimulation. Importantly, we presented novel findings that attenuation of HSA activity via ganglionic blockade increases plasma corticosterone. This finding supports the concept of inter-dependency of adrenal cortical and medullary function. Furthermore, disruption of the genes associated with adrenal medullary function in PBDE animals was associated with increased plasma CORT in the absence of changes in expression of HPA-axis relevant genes. PBDEs could, therefore, be increasing plasma corticosterone by disrupting CA synthesis. Exploring how PBDEs alter stress responses has therefore provided tantalizing clues for

understanding paracrine effects within the adrenal gland that arise from exposure to environmental stressors.

The findings in chapter two were expanded upon in chapter three. Ganglionic blockade only partially attenuated pressor responses in PBDE-dosed animals to hyperosmotic simulation; therefore we explored the hypothesis that PBDE exposure disrupts the renin-angiotensin system. We stimulated PBDE-dosed animals with hypertonic saline in the presence of captopril, an ACE inhibitor. We found that the exaggerated pressor response to hyperosmotic stress in PBDE-exposed animals is abolished by captopril. The effect on blood pressure occurred even though captopril did not significantly alter plasma osmolality. Furthermore, we found evidence of RAS disruption in PBDE-dosed animals. Plasma AngII responses to hyperosmotic stimulation in PBDE-dosed animals was blunted and adrenal Agtr1 expression was also blunted in PBDE-exposed animals. In combination the results presented in chapter three provides evidence that the renin-angoiotensin system is adversely affected by developmental PBDE exposure. Furthermore, this evidence demonstrates that adrenal disruption by PBDEs can have profound impacts on other homeostatic functions that are intimately tied to stress axis activity. The precise nature of this disruption and the mechanisms underlying the effectiveness of captopril in reducing blood pressure following hyperosmotic stimulation are in need of further examination. While these effects could very well be centrally

driven, it is exciting to consider that paracrine actions within the adrenal gland may be partially responsible for PBDE toxicity of a wide variety of homeostatic systems.

In chapter four I focused on a different sort of chronic stress, daily sound stress. The finding that that chronic sound stress increases systolic blood pressure supports the concept that psychological stressors can contribute significantly to elevated blood pressure. Evidence was presented that adrenal PACAP expression is elevated in rats subjected to chronic sound stress even as expression of the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT) were unchanged. We also examined adrenal gene markers relevant to hypothalmic-pituitary adrenal axis activation and found that adrenal MC2R expression is blunted in rats subjected to chronic sound stress. As before, stress We then shifted our focus to the hypothalamus itself because studies have documented changes in NMDA NR2B subunit mRNA expression in stressed animals in both the BNST (Ventura-Silva, Pêgo et al. 2012) and the PVN (Ziegler, Cullinan et al. 2005). We discovered that chronic sound stress increased mRNA expression of the Grin 2B gene which encodes the NR2B subunit of NMDA receptors. In combination, chapter four provides evidence that adrenal PACAP and HPA signaling is impacted by chronic sound stress. That PACAP gene expression increases while MC2R expression is blunted provides possible clues to the relationship between PACAP and HPA-related genes in the adrenal gland following chronic stress. Furthermore, the relatively new concept that glutamatergic signaling in

the PVN is transformed by chronic stress is supported by our findings of altered NMDA receptor subunit expression in the PVN.

Finally, in chapter five, I explored a promising therapeutic option to combat the negative effects of stress. Manual therapies such as manipulation and massage have been associated with significant impacts on blood pressure and autonomic nervous system activity. Because mechanistic research in this field is lacking I saw the need to develop a calibrated method for applying manual therapy to rodents. I used a modified neonatal blood pressure cuff to deliver calibrated manual therapy pressures which reduced systolic and mean arterial pressures in male Long-Evans rats in a dose-dependent manner. Furthermore, the calibrated manual therapy method reduced heart rate variability in Long-Evans rats indicating a shift toward parasympathetic responses. Chapter five was aimed at improving techniques for examining the effects of manual therapies in animal models. It is hoped that a better understanding of the mechanisms underlying manual therapy may have broad socioeconomic impact by highlighting the efficacy of safe, minimally invasive alternatives to popular pharmacological treatments.

I began my journey toward a PhD in Neuroscience because of my clinical experiences as they related to patients suffering from diseases of chronic stress. In nearly seven years of study I have deepened my understanding of the impacts of a variety of stressors. I have discovered some novel roles for PACAP in adrenal function and have broadened our

understanding of how environmental toxicants adversely affect the major stress axes. And, I have developed a new technique for studying potential beneficial effects of manual therapies in animal models. Stress has been implicated in a variety of disease states that have broad socioeconomic impact. The research provided herein should expand our understanding of how stressors negatively impact these biological processes. My work at UCR has already enhanced my clinical understanding and I expect that improved research techniques in manual therapies will lead to a greater acceptance of this promising therapeutic intervention.

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