THE ROLE OF THE AMYGDALA IN AUTONOMIC REGULATION OF A HYPERTENSIVE MODEL DURING STRESS EXPOSURE

By

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To my Aunt Janie

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When exposed to stress, individuals with hypertension demonstrate dramatic increases in blood pressure and heart rate compared to normotensive individuals. Many studies have indicated a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system in both human and animal models of hypertension, yet, the amygdala, the emotional center of the brain, has also been implicated. Although, the amygdala has many connections with brain regions regulating the autonomic nervous system, little information has been provided regarding its role in regulating the hyperreactivity of the cardiovascular response to stress in a hypertensive rat model. The following study was conducted to determine if the cardiovascular response to stress in the spontaneously hypertensive rate (SHR) is due to an alteration in the regulation of the autonomic nervous system by the amygdala

Specific Aim1 was designed to determine if neural activity within the amygdala is increased in the spontaneously hypertensive rat (SHR). Fos-like immunoreactivity (FLI) was increased in all subnuclei of the amygdala during air jet stress (AJS), but no difference occurred between strains. Yet, corticotropin releasing hormone (CRH) positive neurons in the central nucleus of the amygdala (CEA) were increased in the SHR compared to the normotensive control during AJS. Additionally, an increase in FLI was demonstrated in the locus coeruleus in

the SHR during stress. Due to the connection between CRH neurons in the CEA and the locus coeruleus, it is possible the cardiovascular response to stress in the SHR is due to abnormal CRH secretion.

Specific Aim 2 sought to determine if antagonism of NPY 1 and 2 receptors (Y1 and Y2) in the CEA as well as γ-amminobutryic acid (GABA) A receptors would modulate the cardiovascular response to stress in a hypertensive model. The results of the study indicated antagonism of Y1 and GABA A receptors reduced the cardiovascular response to stress in the SHR, while in the Wistar both antagonists caused an increase in HR. The dramatic reduction of the HR in the SHR to a level similar to the Wistar suggests inhibition of GABA A receptors in the CEA may increase parasympathetic activity.

Specific Aim 3 focused on if the attenuation of the cardiovascular response to stress in the SHR by antagonism of the CEA was due to an increase in parasympathetic activity. Analysis of heart rate variability analysis was utilized to determine sympathovagal balance in the SHR. NPY Y1 receptor antagonism within the CEA did not affect parasympathetic acitivity. This lack of response may be due to the availability of other NPY receptors for activation. Nevertheless, GABA A antagonism did demonstrate a modest increase in parasympathetic activity during the initial period of stress as well as an increase in heart rate variability and total power. Additionally, the possible stress-induced increase in ventilatory rate in the SHR was reduced by GABA A receptor antagonism. Disinhibition of the CEA appears to affect autonomic regulation in the SHR leading to a normalization of the cardiovascular response to stress.

CHAPTER 1
THE STRESS RESPONSE AND ITS ROLE IN HYPERTENSION

The Concept of Stress

All living beings require constant maintenance of their internal environment to keep body fluids in balance and tissues healthy. In the mid 1800s, Claude Bernard suggested that "all of the vital mechanisms, however, varied they may be, have always one goal, to maintain the uniformity of the conditions of life in the internal environment (milieu interieur) (24)." By the 1930s, Walter B. Cannon modernized the theory of the "staying power of the body" to the term homeostasis which referred to the body's ability to regulate a stable internal environment in response to various perturbations (40). Although, the notion of a static system seems simple, the body's physiology is in a constant state of fluctuation due to exposure to various internal and external disturbances. Depending on the intensity and duration of these stimuli, it has become accepted that these stimuli can cause damage to tissues as well as change the body's ability to adapt.

During the same time period, Han Selye's interest in the commonalities between disease states led to the hypothesis that the disturbances of homeostasis, especially during pathological conditions, required the activation of similar physiological mechanisms. His theory of a general adaptation syndrome introduced the idea of stress being a nonspecific response of the body to any demand it faces. Selye's nonspecific response included three concepts: the alarm reaction, the stage of resistance, and the stage of exhaustion. The alarm reaction is the body's initial response to a stress, requiring the recruitment of the neuroendocrine and autonomic nervous system to stimulate behavioral and physiological changes. The resistance stage occurs when continued exposure to a stress leads to a temporary adaptation of the system to maintain homeostasis which includes peripheral and central changes. Lastly, the exhaustion phase

describes the inability to adapt due to extended exposure to stress (336, 337). Approximately 20 years later, George Chrousos modified Selye's hypothesis and proposed that each stressor generates a specific physiological adaptation, for example, increases in temperature will require sweating and dilation of superficial vessels to release heat. Yet, similar to Selye, Chrousos agreed that the severity of the stressor can lead to a loss of adaptation (50).

The concept of homeostasis pertains to the maintenance of physiological parameters around a desired set-point. In 1988, Sterling and Eyer introduced the term allostasis to encompass the dynamic fluctuations in the cardiovascular system, immune system, and central nervous system due to diurnal changes as well as internal and external stimuli (100). In accordance with Selye and Chrousos, allostatic load is the wear and tear on the body and brain leading to chronic hyperactivity or hypoactivity of the physiological systems that are normally involved in acute adaptations to stress. Several other factors contribute to allostatic load. For instance, anticipation can lead to pre-stimuli activation of the neuroendocrine hormones that produce early adaptive responses. An individual's lifestyle also can add to allostatic load, for example overeating to cope with stress can eventually lead to diabetes or cardiovascular problems. In fact, three types of physiological responses generate allostatic load: frequent stress, failed shut down of the stress systems, or inadequate response to the stimuli (234). Furthermore, Burchfield adds a twist to how we respond to allostatic load, by suggesting that our predisposition to adapt to stressors and our ability to perceive the stimuli can determine how we will respond or minimize our arousal to the stressful stimuli. Simple conditioning also may also play a role in how we adapt to further stressful challenges (35).

Stressful stimuli are generally classified as either physical or psychological stressors. Unfortunately, each stressor will have a specific physiological and behavioral response which

complicates the use of categorization. Englemann stated that systemic stressors, such as pain, hemorrhage, and inflammation, generally rely on activation of the hypothalamus and are genuine challenges to the system (91). Predictive or processive stressors, which include novel environments and fear, can lead to hypothalamic and adrenal activation without physiological challenge. In fact, the psychological response to stress has proven to be an important component whether the system challenged is treated as a physical or psychological stressor. Awareness or anticipation of the stimuli causes changes in both glucocorticoids and cardiovascular responses in order to prepare the body for future challenges. The influence of the conscious mind on adaptation can lead to difficulty in separating specific stimuli as either purely physical or purely psychological stressors, suggesting that both peripheral and central responses must be considered when studying the responses to stress.

Physiological Systems Involved in Stress

Stressful challenges call for a recruitment of several main systems within the brain to orchestrate an efficient physiological and behavioral response. The hypothalamic-pituitary-adrenal axis (HPA axis) is the main contributer to the neuroendocrine response to stress. Secondly, the autonomic nervous system, which includes the parasympathetic (PNS) and sympathetic nervous system (SNS), modulates the cardiovascular and other visceral systems during stress. The level of arousal or attention to stimuli is controlled by the locus coeruleus (A6, LC), a dense cluster of norepinephrine (NE) containing neurons in the rostral brainstem otherwise termed the LC-NE system (396). Finally, the limbic system, a group of brain nuclei, controls emotional and behavioral reactions, as well as modulation of the peripheral responses to stressful stimuli. If any of these systems become out of sync, a dysregulation of the stress response may occur.

HPA Axis

The HPA axis coordinates both peripheral and central neuroendocrine secretions during stress. When activated, cells of the parvocellular region of the paraventricular nucleus of the hypothalamus (PVN) secrete corticotropin releasing hormone (CRH) into median eminence where the portal system transports it to the anterior pituitary to stimulate corticotrophs to release adrenocorticotropin hormone (ACTH) into the circulation (Fig1-1). During chronic stress, arginine vasopressin (AVP) is co-released with CRH to increase secretion of ACTH (3). When ACTH reaches the adrenal glands, cortical release of cortisol is stimulated and circulating levels of cortisol rise. Cortisol then induces catabolism of various nutrients in order to prepare the body for stress. To prevent sustained degradation of the metabolic stores, cortisol operates through a negative feedback pathway to the HPA axis and inhibits sustained ACTH and CRH release at the level of the pituitary and PVN as represented in Fig. 1-1 (183). Throughout the body, cortisol binds to either mineralacorticoid (MR) or glucocorticoid (GR) receptors, allowing for its ability to modify sodium and water balance as well as catabolism during stress. Aside from peripheral actions, cortisol can act centrally in brain regions with dense GR receptor populations, such as the amygdala and hippocampus, to promote learning, memory, and adaptation (245). While cortisol may mediate a negative feedback at the pituitary, there is evidence that this neurohormone also potentiates the secretion of extra-hypothalamic CRH to modulate behavior and peripheral responses (56, 192, 237). The actions of cortisol are important to the adaptation of stress, however, alterations in the HPA axis can generate differential levels of cortisol release, which can then lead to various pathologies.

The level of activation of the HPA axis is determined by various cortical and brainstem inputs integrated within the PVN. The majority of catecholaminergic innervations into the PVN ascend from brainstem regions such as the nucleus of the tractus solitarious (NTS) and rostral

ventrolateral medulla (RVLM) (62, 330). Catecholaminergic release stimulates the secretion of ACTH via CRH (376). Moreover, transection of medullary catecholamingeric pathways attenuated HPA axis reactivity (330). Similarly, serotonergic pathways acting through the dorsal and medial raphe stimulate HPA activity and lesions of these regions also diminish HPA axis activity (171). The parabrachial nucleus (PBN) and periaquaductal gray (PAG) also relay cardiovascular and pain information to the HPA axis (101, 195). Furthermore, circumventricular organs sensitive to circulating hormones send efferent projections to the PVN and modulate HPA reactivity. For example, angiotensin II sensitive neurons in the subfornical organ directly stimulate CRH secretion (4, 214, 286). Aside from other brain regions, intrahypothalamic subnuclei also can modulate the PVN. For example, the NE stimulation of the magnocellular PVN neurons is mediated by glutaminergic interneurons (68). Further modification of HPA Axis activity during stress stems from the limbic system. The bed nucleus of the stria terminalis (BNST) sends efferent inhibitory information to the hypothalamus, although, it has been noted that CRH neurons from the BNST stimulate CRH cells in the PVN (61, 104, 160). Meanwhile, the amygdala signals the BNST, NTS, or PBN to indirectly modulate the HPA axis (242, 291, 334, 400). Thus, when exposed to stressful stimuli, extensive anatomical evidence has included a wide range of brain regions that have the capacity to adjust the activity of the HPA axis.

Sympathetic Nervous System.

The traditional 'fight or flight' theory of stress is a reaction to threatening stimuli associated with the induction of the SNS. Norepinephrine (NE) and epinephrine (EPI) are secreted from the adrenal medulla and act on target effector organs to prepare the body for homeostatic challenges. The consequential responses include increases in blood pressure (BP), heart rate (HR), reduction in digestion and reproduction, and dilation of the pupils. Catecholamines released both into the circulation and locally by SNS postganglionic nerve fibers

act at various adrenergic receptors to cause vasoconstriction and vasodilation of blood vessels to provide nutrients and oxygen to the necessary tissues. Aside from its peripheral actions, neurons in the brainstem that produce catecholamines can modulate the HPA axis response as well as cortical-generated behaviors. For instance, catecholaminergic neurons in the NTS (A2/C2 cell groups) and RVLM (C1) integrates peripheral sensory information to change sympathetic nerve activity (SNA), resulting in changes in vascular contraction and cardiovascular function (134). Additionally, the NTS can be modulated by descending projections from the dorsal medial hypothalamus (DMH), PVN, and amygdala to adjust the cardiovascular responses to stressful stimuli (34, 70, 103, 334).

LC-NE System

The LC, a region important in arousal, is one of the major sources of norepinephrine release throughout the brain during stress (29). Figure 1-2 illustrates the various cortical regions the LC projects to as well as the reciprocal connections between the amygdala and cortex with the LC. Lesions of LC result in a reduction in stress-induced secretion of ACTH and stimulation of the LC can lead to augmented ACTH secretion (208, 406). Although, no direct connection from LC to the PVN has been identified, it is accepted that the LC must act through polysynaptic pathways involving the amygdala, hippocampus, and prefrontal cortex to modulate the HPA axis (62). Conversely, it also has been noted that the amygdala, BNST, NTS, PAG, and Barrington's nucleus act on specific rostral-caudal subdivisions to modulate LC output (85, 191, 371, 397). Glucocorticoids receptors have been located on CRH neurons in the amygdala and BNST suggesting cortisol may indirectly influence the LC's activity (206). Similar to the HPA axis and SNS, the LC-NE system can be modified by central inputs leading to a refined physiological response to specific stress stimuli.

Limbic System

Integration of sensory and emotional information during stress occurs in the limbic system which includes the lateral septum, the BNST, and the amygdala. As the main organizer of emotional aspects of the stress response, the amygdala is a conglomerate of subnuclei that have different functions and modulate different brain regions (Fig 1-3). The lateral amygdala (LA) is considered the "gate" of the amygdala, in that all efferent information from the cortex and thalamus is passed through it. From the LA, the information is distributed to the other subnuclei: the basolateral amygdala (BLA), the central amygdala (CEA), and medial amygdala (MEA). The BLA is generally considered to be important in memory formation by its extensive connections to the hippocampus. Additionally, the BLA is the main regulator of the behavioral responses to stress. The MEA is a small output center implicated in modifying the cardiovascular system and HPA axis. The main output of the amygdala, the CEA, modulates cardiovascular nuclei, visceral functions, the HPA axis, and cortical regions (207). Finally, the BNST has also been considered part of the extended amygdala due it having similar projections as the CEA.

The primary role of the CEA is to coordinate sensory information to influence the visceral responses to incoming stimuli. Electrical and chemical stimulation of the CEA in conscious rats generates increases in both BP and HR, however, while under anesthesia electrical stimulation of the CEA has been reported to elicit a depressor and bradycardic response (109, 167). Other studies involving anesthesia, also demonstrated depressor responses to both electrical and chemical stimulation of the CEA (112, 176). It is apparent that activation of the CEA is important in regulating cardiovascular function and changes in autonomic drive elicited from this region are profoundly influenced by anesthesia. Since anesthesia primarily acts via potentiating

inhibitory synaptic inputs, these observations also suggest that the role of the amygdala in autonomic regulation is easily altered by changes in inhibitory drive.

The amygdala harbors a dense population of γ-aminobutyric acid (GABA) containing neurons that maintain tonic inhibition over this limbic region. Intrinsic GABAergic activity modifies incoming signals through the lateral amygdala and BLA leading to highly selective information departing through the CEA (368). In fact, the BLA itself is modulated by local glutamate and GABA neurons through either a fast GABA A receptor mediated or a slow GABA B receptor mediated inhibitory component (297, 356). Likewise, a population of GABAergic interneurons in the intercalated cells located between the BLA and CEA has been indicated as another inhibitory passage to alter CEA activity (207, 308). In fact, GABA has been implicated in the BLA's role in the modulation of cardiovascular responses during stress. For example, blockade of GABA A receptors in the BLA with the selective receptor antagonist bicuculline elicits increases in BP, HR, and anxiety which are dependent on simultaneous activation of excitatory amino acids (318, 324, 359). Similarly, in the MEA, microinjection of bicuculline causes increases in HR and BP and this response is inhibited by a GABA A agonist (427). Alternatively, microinjection of GABA A agonists into the CEA has been reported to produce anxiolytic behaviors during a social interaction test in rodents (326). In chloralose anesthetized rats, glutamate stimulation of the CEA evokes decreases in MAP and HR, which are reduced when the CEA is pre-treated with GABA. Interestingly, in the same study, the glutamate elicited depressor responses were also reported to be attenuated in the presence of bicuculline, suggesting a potentially complex role for GABA in the CEA (51). Consequently, the selective excitation or inhibition of GABA interneurons within the different subnuclei of the amygdala must be coordinated appropriately to guide the emotional and cardiovascular responses to stress.

As stated above, the CEA is considered one the primary outputs of the amygdala for ascending and descending modulation of the autonomic centers of the brain. The CEA modulates cardiovascular function via anatomical interconnections with several important autonomic centers in the brainstem. For example, rostral projections to the BNST were identified when electrical and chemical lesion of BNST was shown to attenuate the depressor response caused by stimulation of the CEA (304). The CEA also sends direct descending projections to the NTS, dorsal motor nucleus of the vagus (DMV), and RVLM and reciprocally the NTS, VLM, and PBN send autonomic information to the CEA (44, 111, 304, 314). Specifically, the CEA may influence the arterial baroreflex function, an important reflex involved in short-term regulation of blood pressure, due to neuronal connections with the NTS and PBN (134, 164). Additionally, CEA stimulation activates both inhibitory and excitatory neurons in the PAG, an important output in coordinating cardiorespiratory and motor responses to threatening stimuli (19, 57, 67, 311). Thus, the CEA may act through various pathways to modulate autonomic responses to stress.

Aside from the cardiovascular regulatory regions, the limbic system also can interact with the HPA axis and the LC-NE system. The CEA interacts with the HPA axis via direct modulation of the PVN or indirectly via GABAergic neurons in the BNST (125, 368). Catecholamines secreted into the amygdala also can affect the activity of the HPA axis. For example, both alpha and beta adrenergic receptor antagonists injected into the MEA have been reported to attenuate ACTH secretion during stress (218). Catecholamine innervation from the LC to BLA, CEA, and intercalated cells implicates the LC-NE system in modulating amygdalar activity (95). Also, NE diminishes depressor responses elicited by microinjection of glutamine into the CEA and NE mediated inhibition of inhibitory synaptic activity in the BLA has been

shown to be altered by prior exposure to chronic stress (30, 305). Thus, the amygdala plays a primary role in coordinating the HPA axis, LC-NE activity, and limbic system during stress, while intrinsic GABAergic interneurons within the amygdala ensures the resulting response is appropriate.

Hypertension and the Stress Response

High blood pressure or hypertension affects millions of individuals worldwide and is the most common form of cardiovascular disease. Hypertension contributes to many disease states including chronic heart failure, renal failure, and Alzheimer's disease (184, 335). Although, typically thought of as a vascular pathology, it has been hypothesized that chronic mental stress can lead to hypertension. Alternatively, many studies have demonstrated hypertensive patients are hyperresponsive to psychological stressful stimuli, suggesting hypertension can lead to a sensitization of stress regulatory centers in the central nervous system. Both borderline hypertensive and hypertensive patients exhibit increases in BP and cortisol levels during various stressors, such as a mental arithmetic test, compared to normotensive individuals (7, 254). Additionally, hypertensive patients demonstrate increased baseline cortisol levels and reduced frontal lobe volume suggesting impairment of the glucocorticoid negative feedback system described above may be involved in altering brain structures (118). Indeed, in hypertensive patients that died of acute heart failure, both number of CRH neurons and CRH mRNA expression in the magnocellular and parvocellular PVN was found to be increased relative to normotensive individuals that had died from traumatic injury (120). Aside, from an abnormally regulated HPA axis, fMRI studies of individuals with exaggerated BP reactivity to stress have identified that the amygdala may also be dysregulated. For example, there was evidence of reduced gray matter volume, greater amygdala activation, and stronger connectivity between the amygdala and the cortex and pons (114). Finally, basal muscle SNA and plasma epinephrine

levels have been reported to be higher in borderline hypertensive individuals compared to their normotensive counterparts. Moreover, following cold pressor and mental arithmetic tests, SNA and plasma NE levels remains elevated, indicating that exaggerated stress responses also may be found in pre-hypertensive individuals (229). This observation suggests that enhanced sensitivity to stress may contribute to the development and maintenance of hypertension. Aberrant responses to stress in humans with hypertension appear to be related to a dysregulation of the HPA axis, sympathetic nervous system, and the limbic system.

Various hypertensive rat models have been established to study how various risk factors induce the development of hypertension. Genetic models of hypertension are represented by the spontaneously hypertensive rat (SHR) and Dahl salt-sensitive rat. The SHR was developed by Okamoto and Aoki in the 1960s through inbreeding of Wistar rats with high BP (215, 257). The Dahl salt-sensitive rat develops high BP when fed a high salt diet (299). Alternatively, attempts to create chronic stress-induced models of hypertension through the use of prolonged combinations of lights, noises, and shock have generally been unsuccessful; however, chronic exposure to cold has been noted to produce prolonged hypertension (105, 213, 358, 370). Administration of deoxycorticosterone acetate (DOCA) paired with saline drinking fluid and a nephrectomy will form severe pharmacology-induced hypertension in the DOCA-salt model (285). Finally, renal-induced hypertension was created by Goldblatt in 1934 by constricting one renal artery and leaving the contralateral kidney intact or removing it (119). Each of these models are used in order to study the various ways hypertension can develop, however, the SHR has been the primary hypertensive rat model described in regards to exaggerated physiological and behavioral responses to stress stimuli.

Distinctive traits of the SHR include increased peripheral resistance, decreased baroreflex sensitivity, and hyperactivity. Increased neurogenic tone, increased vascular wall thickness, and altered calcium regulation in the vasculature are a few of the routes that can lead to increased peripheral resistance and possibly altering central regulation of blood pressure (11, 172, 217). Removal of the pituitary or adrenal glands before and after the development of hypertension in the SHR reduces BP and maintains normotension suggesting these organs play a role in the development of hypertension (15). Similar to human hypertensive patients, SHR rats exposed to stress demonstrate augmented plasma catecholamines, HR, and BP compared to its genetically similar normotensive control, the Wistar Kyoto (WKY). An alteration in the HPA axis has emerged as a possible reason for the exaggerated behavioral and physiological responses to stress in the SHR. Basal levels of circulating corticosterone are increased while ACTH and CRH levels are low in the SHR (142, 165). During restraint, however, CRH mRNA levels increase in the SHR compared to the WKY (165). Following exposure to acute stressors, the SHR exhibited increases in ACTH, but no change in corticosterone levels compared to the Wistar (83). Alternatively, plasma ACTH levels were reported to decrease while corticosterone levels increased during hemorrhage and ether exposure in the SHR compared to the WKY (142). Thus, stress-specific secretion of glucocorticoids in the SHR appears to deviate from normal, supporting the hypothesis of dysregulation of the HPA axis both at rest and during stress exists in this model of essential hypertension.

SNA is increased in the SHR and appears to be related to the development of hypertension. Increases in MAP and renal SNA (RSNA) begin at 4-6 weeks and increase significantly with age. This progressive rise in baseline MAP appears to be due to resetting of central sympathetic centers, including the baroreflex set point, and not an alteration in

baroreceptor function (172). Central changes in autonomic control centers in the SHR have been linked to the catecholaminergic system since it does not appear to be normal in the SHR. β-adrenergic blockade reduced both basal BP and HR in young and old SHRs, however, it did not attenuate the increases in BP and HR to vibratory stress alluding to a dysfunction in the stress-induced activity of catecholamines in the SHR (137). Reduced α2 adrenergic receptor density in the brainstem, cortex, and cerebellum suggests a down regulation of receptors (259). Additionally, NE levels in the medulla in SHR have been reported to be lower compared to the WKY (413, 419). Furthermore, α2 receptor blockade in the NTS demonstrated an attenuation of the baroreflex-induced bradycardia in the SHR compared to the WKY (148). Alterations in the central regulation of the SNS are evident in the SHR and may be due to hypersensitivity of α2 receptors.

Aside from modulation of the cardiovascular centers, there also is evidence that catecholaminergic neurons in the LC exhibit lower than normal tonic discharge in both the SHR compared to normotensive control (181, 260). Additionally, a higher threshold current is required by the LC of an SHR in order to induce increases in BP compared to the WKY suggesting the LC may only be active during a high intensity stimulus in the SHR (180). The hypoactivity of the LC at rest infers that it may play a major role in regulating arousal during homeostatic challenges in the SHR. For example, basal GABA and glutamate levels are slightly higher in the SHR, but not significantly different from the WKY. Yet, during intravenous administration of NE to increase BP, the secretion of GABA in the LC was significantly decreased in the SHR compared to the WKY. On the other hand, the LC secreted more GABA during tail pinch in the SHR compared to the WKY (174). Furthermore, tail pinch in the conscious SHR also produced a prolonged secretion of NE from the LC compared to the WKY

(175). Thus, the alteration in neuronal activity and release of neurotransmitters in the LC of the SHR may possibly lead to the abnormal regulation of the ANS during stress exposure in the SHR.

The development of hypertension and the exaggerated cardiovascular response to stress also has been hypothesized to be mediated in part by dysfunction of the amygdala in the SHR. This hypothesis was based on both the known role of the amygdala in coordinating emotional and autonomic responses to stress and the outcome of several early studies in the 1980s which demonstrated that electrolytic lesion of the CEA attenuated increases in BP during environmental and noise stress, in addition to blunting the development of hypertension in young SHRs (102, 110, 324). Since electrolytic lesions destroy both neurons and fibers of passage, chemical lesion studies also were performed to confirm previous findings. Following chemical lesion of the CEA with ibotenic acid, attenuation of both basal BP and stress-induced increases in BP were found. However, this result was confounded by parallel reductions in weight in the lesioned SHR which also may have contributed to the slightly lower blood pressure (338). In a more recent study, ablation of the MEA with ibotenic acid was performed and lesion of this region of the amygdala was also reported to modestly attenuate the development of hypertension in the SHR (107). Thus, amygdalar lesions slightly affect basal BP, nevertheless, based on the known function of the limbic system it remains possible the amygdala may play a more important role when the SHR is exposed to a challenge. Indeed, there is some evidence that the density of GABA A receptor binding sites is lowered in the amygdala and hypothalamus of the SHR versus normotensive controls (200). Additionally, while glutamate and GABA rates in the amygdala have been reported to be similar between the SHR and WKY at rest, noise stress was reported to induce a significant increase in glutamate release selectively in the amygdala of the SHR, but not

the WKY (349). It appears that the intrinsic regulation of inhibition of the amygdala is altered in the SHR and may play a role in the exaggerated cardiovascular responses to stress typically observed in the SHR. Nevertheless, a more thorough exploration of the role of the amygdala in the exaggerated cardiovascular response to stress in the SHR is needed to further establish the dysfunction of the limbic system in this hypertensive model.

Neurotransmitters and Neuromodulators Involved in Mediating Physiological Changes to Stress

The stress response incorporates the communication of several neurotransmitters and neuropeptides to generate the numerous physiological changes needed for the system to prepare, adapt, and recover from the stimuli. Earlier, CRH, AVP, ACTH, and cortisol were mentioned as the major players in the activation of the HPA axis. Secondly, the catecholamines, NE and EPI, which integrate SNS and NE-LC signals, were discussed. Interestingly, other neurotransmitters, such as dopamine and serotonin also have been found to be increased in the amygdala following fear conditioning to a tone (426). Additionally, neuropeptide Y (NPY), a neuromodulator often co-released with catecholamines, may be involved in regulating the peripheral as well as central-behavioral responses to stress (153, 331). Intriguingly, endogenous opioids inhibit the actions of CRH in the LC suggesting opiates may be important in the adaptation to stress (395). Similarly, GABA and glutamate exert their inhibitory and excitatory impact, respectively, on all regions of the brain for further fine tuning of the stress response. Due to the brevity of the project, it was necessary to focus on two of the molecules that have an interesting antagonistic relationship in the stress response: CRH and NPY.

CRH

A member of the urocortin family, CRH is a 41 amino acid polypeptide hailed as one of the pivotal neurohormones involved in the stress response. CRH acts through a Gs protein

coupled receptor to activate the adenosine 3',5'-cyclic monophosphate (cAMP)-dependant protein kinase A (PKA) pathway (204). Activation of this pathway leads to a calcium influx into the cell via L-type and P-type calcium channels (201). Consequently, CRH triggers membrane depolarization and an increase in action potential frequency. Additionally, CRH can inhibit potassium currents which aid in maintaining negative resting membrane potentials, thereby increasing the excitability of a neuron (202). Unfortunately, beyond this not much is known about CRH signaling within the cell; many other types of G-protein systems may be a part of the CRH-mediated activation of neurons.

CRH receptors are widely distributed throughout the brain with the densest populations in the pituitary, brainstem, and cerebellum to moderate levels in the BLA, hypothalamus, and PBN to the least in the CEA, BNST, and thalamus (78). CRH binds to either CRH receptor 1 (CRH1) or two subtypes of CRH receptor 2 (CRH2a or CRH2b). Although both receptors can be located in similar brain regions, CRH1 can be mainly found in localized regions of the cortex, BLA, DMH, pons, and pituitary gland while CRH2 are in the lateral septum, MEA, hippocampus, and PVN (47). CRH receptors appear to be important in regulating the HPA axis and behavioral responses generated by the amygdala during various stress stimuli (18, 58, 386, 393).

CRH neuronal populations are located in very specific brain regions, reflecting their functional role during stress. These brain regions include the PVN, amygdala, BNST, and a small number of brainstem regions (238, 239, 267, 372). In the discussion above, CRH neurons in the PVN were mentioned for their role in the HPA axis, yet extrahypothalamic CRH neurons perform an important role in regulating stress with their ability to modulate the HPA axis and SNS reactivity through integration of behavioral and emotional information. For example, CRH efferents projecting from the CEA relay cortical information to modulate both the cardiovascular

and behavioral responses to stress in the following regions: the PVN, LC, NTS, BNST, PBN, and PAG (66, 125, 312, 321, 397, 401). Interconnections between CRH neurons in the CEA and regulation of the PVN have been found to be both direct and indirect involving various neurotransmitters such as GABA and glutamate (161). Alternatively, CRH employs modifications of neuronal activation in other brain regions regulating autonomic responses to stress. Evidence has demonstrated changes in ACTH and AVP when CRH is microinjected into the LC and PBN (43). CRH also generates increases in the frequency of neuronal firing in the LC and increases secretion of NE in the LC which may be due to the direct innervation of noradrenergic dendrites by CRH-positive neurons in the rostral peri-LC (333, 394, 397). Finally, endogenous CRH release within the amygdala allows for further filtering of incoming information. For example, the BLA's regulation of anxious behaviors may be modified by local CRH neurons from the CEA and CRH efferents from the BNST (340). The extent of CRH's influence throughout the brain is vast and may be highly selective for any provided stimulus.

Accordingly, central administration of CRH causes changes in both behavior and physiological responses to stress. In general, intercerebroventricular (ICV) infusion of CRH increases grooming and burying behavior, as well increases in BP, HR, plasma corticosterone, and circulating catecholamines (193). ICV administration of a CRH antagonist attenuates increases in BP, HR, and body temperature generated by both the stress of cage switching or injection of IL-β, suggesting CRH is important in modulating several physiological responses to stress (251). In the amygdala, CRH administration also generates changes in autonomic and behavioral responses basally and during stress. Microinfusion of CRH into the CEA increases HR, but does not appear to affect plasma catecholamines suggesting that CRH neurons in the CEA may be pertinent in regulating PNS output via brainstem regions (410). Additionally,

microinjection of a CRH antagonist, α-helical CRH9-41, into the CEA reduced emotional behavior in socially defeated rats and attenuated freezing behavior (375). Similarly, injecting a CRH antagonist into the amygdala reduced the affective behavior and glucocorticoid release during social conflict (157). Aside from the CEA, the BLA demonstrated an induction of anxiety and panic during social interaction when injected with a CRH agonist, urocortin (316). In fact, endogenous CRH secreted into the BLA aids in regulating emotional behavior and memory consolidation during stress (306). Thus, significant evidence implicates the actions of extrahypothalamic CRH within the amygdala complex is an important regulator of the stress response.

CRH activity in the PVN and the amygdala may be related to duration and type of stress. Both the PVN and amygdala demonstrated increases in CRH mRNA, CRH protein, and c-fos immunoreactivity, an early marker of neuronal activation, during restraint. Nevertheless, the activated CRH-positive cells increased in number during longer bouts of restraint in the PVN and MEA, but were reduced in CEA (59) (163). Alternatively, specific stress stimuli can induce differences in CRH activity between the CEA and the PVN. Audiogenic stress also activated c-fos in CRH neurons in the PVN, but not in the CRH neurons in the CEA (158). Similarly, morphine withdrawal also increases CRH neuronal activation in the PVN, but only non-CRH neurons were activated in the CEA and BNST (138). Finally, immobilization stress increased the number of activated CRH cells in the PVN, but not in the CEA, while amphetamine administration increased the number of activated CRH cells in the both the CEA and the PVN (309). The above studies reflect the discriminatory activation of CRH neurons in both the PVN and CEA allowing for selectivity in the generation of physiological and psychological responses to distinct stress stimuli.

NPY

Neuropeptide tyrosine (NPY) is a versatile neuropeptide involved in regulation of feeding, vasoconstriction, anxiety, and cognition (69, 153, 209, 301). A member of the family that includes pancreatic polypeptide Y (PPY) and peptide YY (PYY), NPY acts via six different receptors, including NPY receptor 1 (Y1) through NPY receptor 6 (Y6) (179). The primary receptors studied to date are Y1 and Y2 which are activated by different peptide regions of NPY; Y1 requires the entire peptide for activation while Y2 needs to bind just the C-terminus to be activated (403, 412). Transcription of NPY starts with cleavage of the Pre-pro-NPY to pro-NPY, with further cleavage to form NPY1-36 which activates Y1 receptors. Further enzymatic processing fragments the peptide to produce NPY3-36 and NPY2-36, which are agonists for Y2 and Y5, respectively (179). Consequently, the actions produced by NPY are dependent on the receptor subtypes and the intracellular signals they generate when activated.

The Y1 receptor is a purtussis sensitive Gi protein-coupled receptor that when activated results in inhibition of intracellular cAMP production which activates both potassium and calcium channels. In contrast, the Y2 receptor is a G protein coupled receptor that inhibits adenylyl cyclase leading to inhibition of high voltage calcium channels (28, 295). In general, Y1 receptors are located primarily post-synaptically, enabling decreases in synaptic transmission. Interestingly, there is some evidence to suggest that Y1 receptors are rapidly internalized following stimulation. Indeed, in a recent in vitro study internalization of the Y1 receptor was shown to occur in the presence of either high levels of a Y1 agonist or Y1 antagonist, suggesting that Y1 receptor activation may be rapid, but short-lived (283). On the other hand, Y2 receptors are mainly found pre-synaptically and modulate secretion of various neurotransmitters, including NPY itself. Moreover, like Y1 receptors, it appears that Y2 receptors do internalize with

prolonged exposure to Y2 agonists, suggesting the action of Y2 receptor activation may be slower and more persistent (277, 278). Although Y1 and Y2 receptors are structurally distinct, both have been found to decrease secretion of GABA and glutamate, establishing NPY's role as a modulator of neurotransmission in the brain (293, 348).

Located throughout the brain, dense populations of NPY neurons have been discovered in the hypothalamus, BNST, NTS, hippocampus, cortex, LC, and the amygdala (49, 127). More importantly, NPY innervations are strategically positioned to modify various homeostatic processes. For example, NPY projections from the CEA, PVN, and BNST to the dorsal vagal complex (DVC) implicating the role of this neuropeptide in modulating viscero-autonomic function (128). The arcuate nucleus of the hypothalamus (ARC) is one of the regions with the densest population of NPY neurons. Neurons in the ARC project to other hypothalamic regions, the amygdala, pons, and brainstem, supporting NPY's ability to modulate a wide range of homoestatic processes (127). NPY has been found to be co-localized in neurons with a variety of neurotransmitters such as somatostatin, GABA, and catecholamines suggesting that its actions may be extremely complex (232, 331). Lastly, NPY receptors are ubiquitous in the brain, but can be found densely populated in different brain regions. For example, both Y1 and Y2 receptors are mainly located in the hippocampus, hypothalamus, cortex, amygdala, and brainstem, but differ in their relative abundances (32, 99, 276, 362). Undoubtedly, the anatomical placement of NPY and its receptors within the brain emphasizes the relevance of this neuropeptide in regulating many neuroprocesses.

Peripherally, NPY acts as a vasoconstrictor residing in noradrenergic innervations of the vasculature, adrenal glands, and the heart (9, 131, 216, 432). Evidence suggests that NPY is co-released from sympathetic nerve terminals during periods of high sympathetic nerve activity

when large dense core vesicles tend to be released. This suggests that release of plasma NPY is highly dependent on the intensity and duration of stress (430). Furthermore, NPY is released during stress to potentiate the actions of catecholamines by intensifying vasoconstrictive actions. Alternatively, there is evidence that catecholamines in turn enhance NPY's action by sensitizing the vasculature to NPY vessels to NPY (429, 431). NPY's activity in the periphery appears to rely on high levels of SNA implicating its role as a regulator of catecholamines during stress and in conditions with high SNA, such as hypertension.

Aside from affecting BP via peripheral vasoconstriction, NPY release can also modulate BP and HR via interaction with the cardiovascular regulating centers in the brainstem (130). Central administration of NPY generally causes hypotension and bradycardia and these autonomic changes have been attributed to activation of the Y1 receptor. In contrast, selective stimulation of Y2 receptors via ICV administration has been shown to increase both BP and HR basally and during social stress and fear conditioning (188, 387). Likewise, NPY 13-36, a Y2 agonist into the autonomic control region, the NTS, produces a pressor response and can counteract the depressor actions of NPY and Y1 antagonists microinjected into the same region (5, 422). This observation is intriguing for several reasons. First, it suggests that in some instances the autonomic effects of Y2 receptor activation may exhibit dominance over Y1. Secondly, the balance of Y1:Y2 receptor expression in autonomic regions of the brain appears to be adjustable and if altered, may lead to aberrant conditions such as hypertension (5, 422). Moreover, there is some evidence that interaction between NPY and α2 receptors may mediate the reduction in BP and HR produced by central NPY administration. For example, NPY has been found to reduce α2 binding sites in the NTS. In the same study, both Y1 and Y2 agonists reduce the affinity of α2 high affinity receptors and antagonized clonidine-induced bradycardia

and hypotension (421). Thus, cardiovascular regulation by NPY relies on specific receptor activation and appears to be related to inhibition of adrenergic receptors.

Many investigations regarding NPY have focused on the role of this neuropeptide in modulating anxiety-type behaviors, as well as adaptation to stressful stimuli. NPY has generally been accepted as an anxiolytic neuropeptide, since when it is administered ICV there is an overall reduction in stress-induced behaviors (156). The extent of NPY's influence during stress is specified by the NPY receptor subtype activated. Plenty of evidence has established Y1 as the receptor generating the anxiolytic responses of NPY. For example, exposure to an elevated plus maze (EPM) test which measures general anxiety, demonstrated that injection of antisense oligonucleotides targeting the Y1 receptors significantly increased anxiety compared to control rats (152, 404). Contrary to NPY's reputation as an anxiolytic, a Y2 agonist administered ICV increased anxiety during EPM (250). Finally, Y2 knock-out (KO) mice demonstrate less anxiety on the elevated plus maze and less immobility during the swim test, suggesting better adaptive skills which may be due to increased secretion of NPY and GABA.(390). Congruently, Y1 activation in the amygdala generates anxiolytic-like effects during the EPM test and social interaction (320). Yet, a Y2 agonist microinjected into the BLA increased anxiety-type behaviors during social interaction in rats (152, 317). Additionally, acute and repeated restraint decreased and increased NPY mRNA levels in the amygdala respectfully, suggesting NPY may be involved in both short-term and long-term adaptations to stress. Overall, NPY is an important regulator of anxiety-induced behaviors with the specificity of its actions determined by activation of its receptors.

CRH and NPY's Relationship During Stress

As outlined above, there is substantial evidence that independently, both CRH and NPY influence the physiological response to stress through their ability to extensively modify both the

peripheral and central nervous system. Although, located in similar brain regions, such as the hypothalamus, amygdala, and LC, there is still little information regarding how CRH and NPY may communicate with each other anatomically. For example, CRH receptors have been found to colocalize on NPY and dopaminergic neurons in the ARC suggesting a direct modulation of these neurons (38). Also, anterograde tracers placed in the ARC have demonstrated that NPY fibers surround CRH neurons in the PVN suggesting a direct input. Yet, Y1 were not found to be co-localized on CRH neurons, but were on other fibers that made contacts with CRH neurons (211). Nevertheless, in another study, Y1 receptors were found to be co-labeled with CRH neurons in the PVN (82). Despite the evidence of an anatomical relationship between NPY and CRH in the PVN, more studies are warranted to characterize how CRH neurons, NPY neurons, and their receptors communicate in other brain regions.

Complementing the anatomical relationship, the interaction between NPY and CRH proves to be cooperative in the PVN. Although, acute and chronic ICV infusion of NPY has been demonstrated to reduce PVN and pituitary CRH levels, most studies demonstrate the reverse response (27). Central administration of NPY increased CRH immunoreactivity and ACTH and corticosterone secretion as well as increased CRH mRNA expression in the hypothalamus (136, 367, 405). In contrast, CRH microinjected into the PVN increased NPY release (246). NPY also stimulated increases in CRH overflow and reduced NE release from the PVN, however, this response was blocked by a Y1 antagonist suggesting Y1 modulates both CRH and NE in the PVN (143). NPY and CRH appear to share coordination of the HPA axis, however, their relationship in regulating emotionality in the amygdala is quite unique.

Compelling evidence has established a fascinating antagonistic relationship between NPY and CRH in regards to their modulation of affective behaviors during stress. As mentioned

before CRH functions are considered anxiogenic while NPY functions are generally anxiolytic. Heilig developed a theory of regulation of emotion in the amygdala highlighting the opposing actions of NPY and CRH as illustrated in Figure 1-4. He proposed that an initial stimulus initiates secretion of CRH into the amygdala driving emotional behaviors to that stimulus. Subsequently, NPY is secreted into the amygdala combatting affective behaviors generated by CRH and attempting to return the system to a balanced emotional state. (Fig 1-4) (154). Several anxiety studies demonstrate this opposing interaction between NPY and CRH in the amygdala. For example, microinjection of NPY into either the CEA or BLA has been shown to induce anxiolytic responses to the EPM and a conflict test, while CRH produced the opposite effects (31). Furthermore, injection of urocortin, a CRH agonist, into the BLA decreased the amount of time spent on the conditioned floor in the two floor choice test suggesting increased levels of anxiety, while pretreatment with NPY prior to urocortin prevented this response (315). CRH and NPY also exhibit rival actions in the secretion of neurotransmitters. For instance, bath application of NPY was shown to inhibit GABA release in a BNST slice preparation, while CRF application primarily mediated its effects via facilitation of GABAergic postsynaptic effects (178). At the cellular level, recent work has demonstrated in immortalized amygdalar cells (AR-5 cell line) that treatment with CRH elicits an increase in intracellular levels of cAMP, while NPY application reduces cAMP levels via inhibition of adenylyl cyclase (343). This suggests a possible antagonism within intracellular signal transduction pathways between the two systems. The antagonistic relationship between CRH and NPY in the amygdala maintains the integrity of the emotional response to stress and if altered, could lead to a dysfunction of the stress response.

NPY and Hypertension

As described above, NPY's role in modulating cardiovascular regulation and SNA has been well established in addition to the role of NPY in modulating sensitivity to stress. As a

consequence, it has been hypothesized that abnormalities in the function of the NPY system, both centrally and/or peripherally, may be an important contributor to the development of hypertension and possibly the trait of elevated reactivity to stress often observed in pre-hypertensive and hypertensive individuals (130). In the brain, NPY immunoreactivity has been reported to be lower in the cortex, midbrain, and medulla in the SHR compared to WKY (219). In contrast, in the ARC, increased basal levels of NPY mRNA have been documented in the SHR, but these levels were decreased during restraint compared to a normotensive control (196, 374). Similarly, NPY's interaction with catecholamines appears to be abnormal in the SHR. For example, clonidine, an alpha-adrenergic agonist, displayed a reduced inhibitory effect on the release of NE in the medulla in the SHR and was shown to require a greater concentration of NPY during coapplication to enhance the clonidine-mediated inhibition compared to the WKY, suggesting a diminished ability for NPY and α2 receptors to affect NE secretion in this model of essential hypertension (225). Additionally, the SHR demonstrated increased NE levels in the brain following NPY microinjection into the PVN compared to NPY-elicited decreases in NE levels in the WKY (415). It is apparent that the reduced NPY levels and an abnormality in NPY modulation of NE secretion may affect SNA in the SHR.

NPY receptors have also demonstrated alterations in function in the hypertensive individual. The aortic-coarcted hypertensive model generated increases in Y2 expression in the NTS while Y1 expression was increased in the petrosal ganglion suggesting both receptors are important in the establishment of hypertension in this model (52). Additionally, the SHR demonstrates increased NPY content and altered Y1 and Y2 activation in the vasculature which contributes to exaggerated neurogenic contraction in the mesenteric arteries compared to normotensive controls (123). The increased potency of NPY receptors has also been

demonstrated in the cardiovascular centers of the SHR. NPY antagonism of α2 mediated through Y1 in the NTS was enhanced in the SHR compared to the WKY (420). Furthermore, central administration of an Y2 caused vasopressor actions in both the SHR and WKY, yet, this response lasted longer in the SHR due to increased binding of NPY to Y2 in the NTS (6). Dysfunction among regulation of NPY and its receptors extends from the vasculature to neuronendocrine and cardiac regulation centers in the brain confirming this neuropeptide as an important target for exploring cardiac hyperreactivity to stress in the SHR.

Summary

From the above discussion, it is evident that individuals with hypertension display an abnormal cardiovascular response to stress. This dysfunction appears to lie within integration of the HPA axis, SNS, and the limbic system. Specifically, the amygdala's ability to regulate autonomic and behavioral responses may be one of the sources contributing to the augmented responses to stress in the SHR. Furthermore, an alteration in the NPY system has been previously documented in the adult SHR which may possibly contribute to the high level of SNA and vascular dysfunction observed in this model of hypertension. Due to an antagonist relationship with CRH, it is possible NPY actions in the amygdala may be a part of the cardiovascular hyper-reactivity to the stress observed in SHR. To further advance our knowledge of brain mechanisms underlying autonomic responses to stress in hypertension, the following studies were undertaken to evaluate the role of the amygdala in the exaggerated cardiovascular response to stress in the SHR, with a specific focus on the roles of CRH and NPY. Moreover, because many of the actions of CRH and NPY are mediated through central modulation of GABA release, we also explored the role of GABA receptor activation of the amygdala in the cardiovascular response to stress in the SHR versus a normotensive control. Three specific aims were undertaken.

Specific Aim 1: Identify how an acute stress stimulus impacts the cardiovascular response and the pattern of c-Fos activation in the amygdala of an animal model of hypertension, the SHR, versus a normotensive control, the Wistar rat. Hypothesis: Acute exposure to air jet stress in the SHR would elicit a greater change in BP and HR compared to the normotensive control and this exaggerated autonomic response in the SHR would be associated with a significant increase in c-fos activation in the PVN and amygdala and a change in CRH expression in the amygdala.

Specific Aim 2: Determine if blockade of Y1, Y2, and GABA A receptors in the CEA will affect the cardiovascular response to stress in the SHR. Hypothesis: Antagonism of both Y1 and GABA A receptors in the CEA will increase the cardiovascular response to air jet stress in the SHR. Antagonism of Y2 receptors in the CEA will decrease cardiovascular responses to air jet stress in the SHR.

Specific Aim 3: Determine if blockade of Y1 and GABA A receptors in the CEA affects the heart rate variability in a hypertensive model compared to a normotensive control during acute stress. Hypothesis: Antagonism of Y1 and GABA A receptors in the CEA will reduce the parasympathetic activation in the SHR during air jet stress.

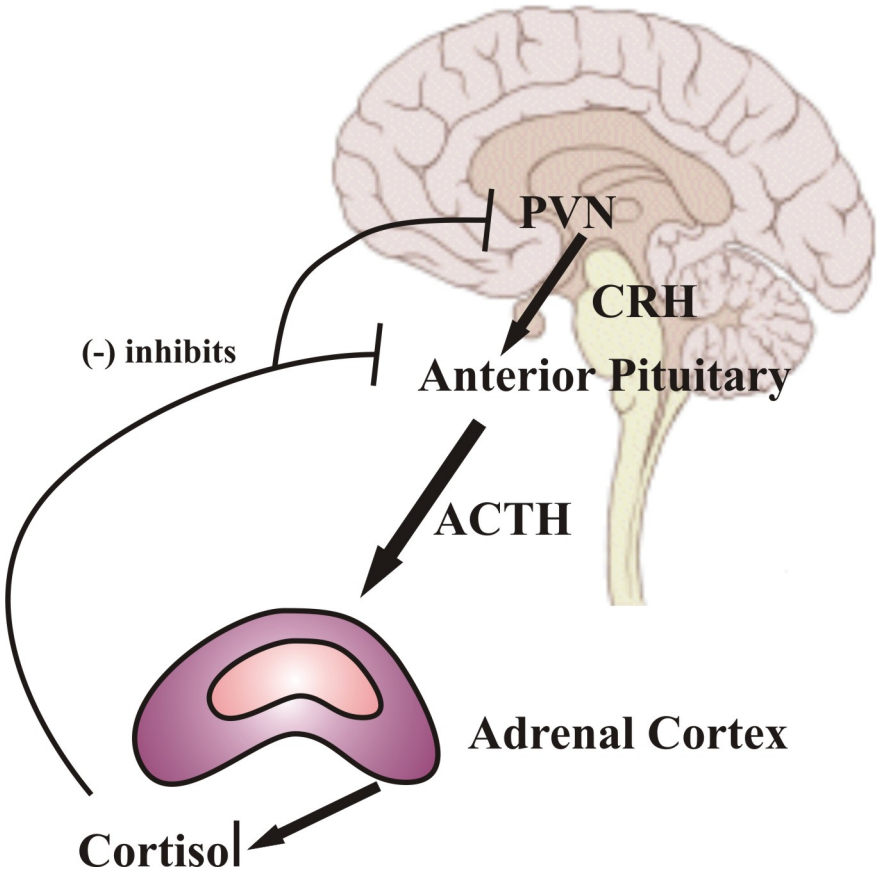

Figure 1-1. Diagram of HPA axis. See text for description. PVN, paraventricular nucleus of the hypothalamus; CRH, corticotropin releasing hormone; ACTH; adrenocorticotropin hormone.

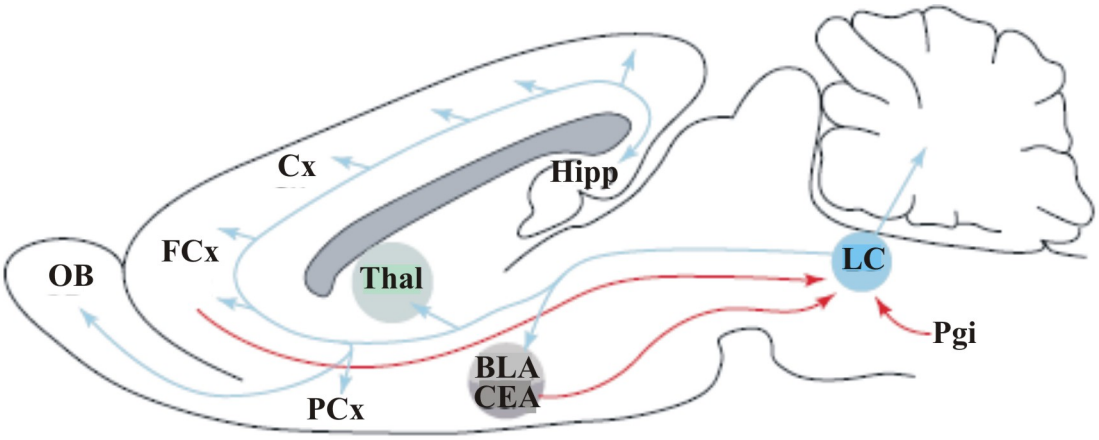

Figure 1-2. Diagram of efferent and afferent connections with the locus coeruleus. Blue lines represent efferent projections from LC. Red lines represent afferent connections to LC. LC, locus coeruleus; PGi, paragigantocellularis nucleus; BLA, basloateral nucleus of the amygdala; CEA, central nucleus of the amygdala; PCx, piriform cortex; OB, olfactory bulb; FCx, frontal cortex; Cx, the neocortex; Thal, thalamus; Hipp, hippocampus. *Adapted and reprinted from Trends in Neuroscience, 28:11, S. Bouret and S.J. Sara, Network reset: a simplified overarching theory of locus coeruleus noradrenaline function, pg.575, 2005, with permission from Elselvier.*

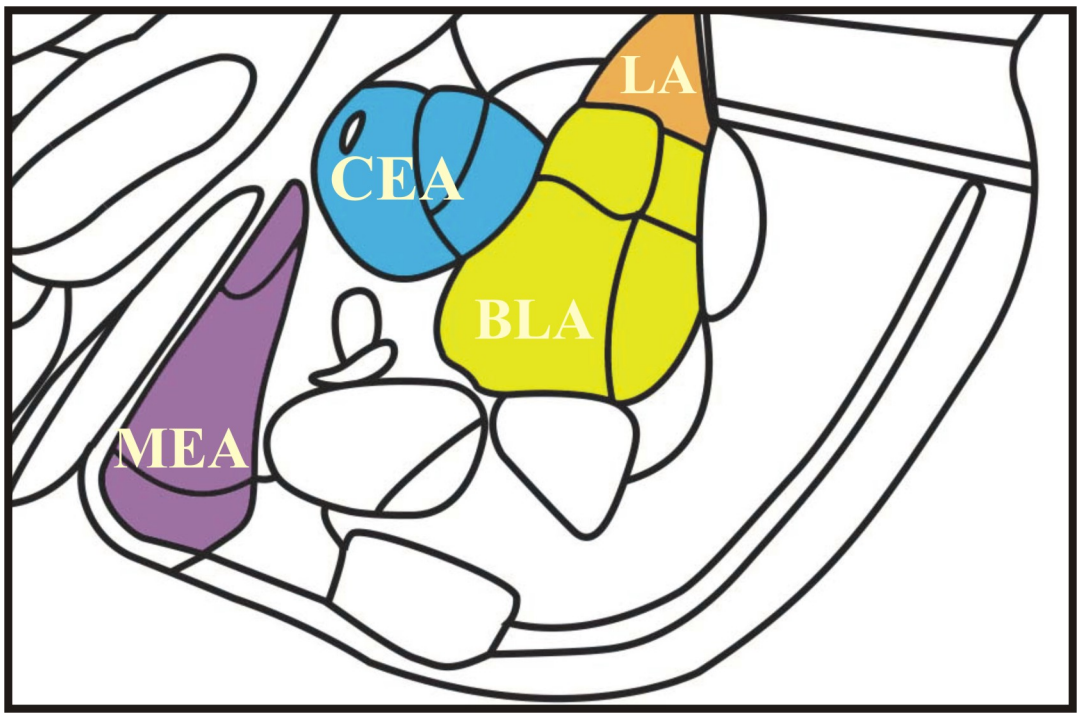

Figure 1-3. Anatomical representation of the subnuclei within the amygdala. CEA, central nucleus of the amygdala; MEA, middle nucleus of the amygdala; BLA, basolateral nucleus of the amygdala, LA, lateral amygdala.

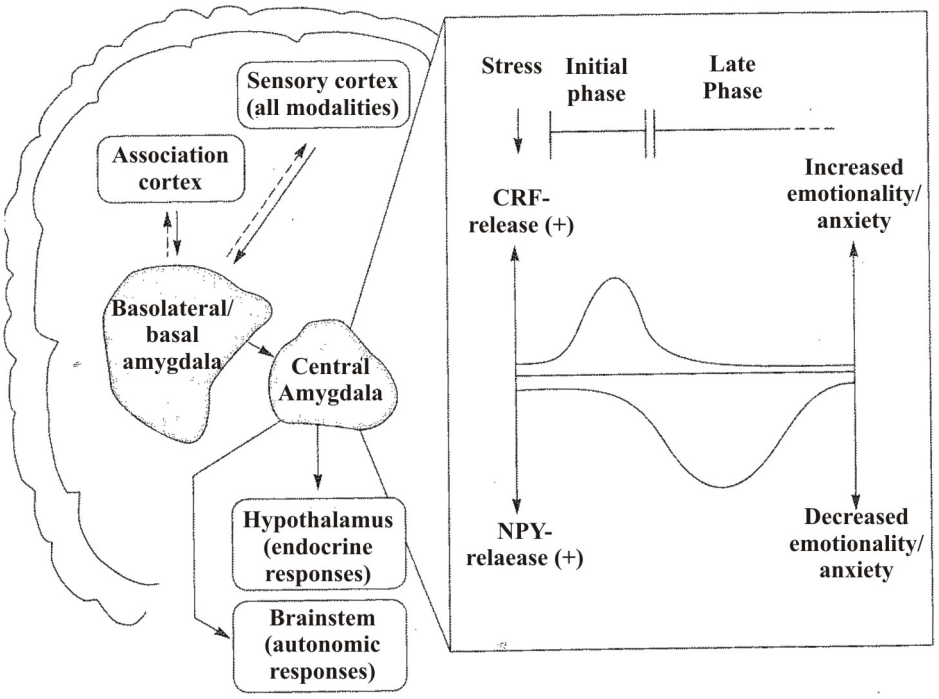

Figure 1-4. Heilig's proposed theory of the opposing regulation of emotionality by CRH and NPY in the amygdala. See text for description. CRH, corticotropin releasing hormone; NPY, neuropeptide Y. *Adapted and reprinted from Trends in Neuroscience, 17:2, M. Heilig et al., Corticotropin-releasing factor and neuropeptide Y: role in emotional integration, pg.83, 1994, with permission from Elsevier.*

CHAPTER 2
CHANGES IN C-FOS AND CORTICOTROPIN RELEASING HORMONE IMMUNOREACTIVITY IN THE AMYGDALA FOLLOWING AIR JET STRESS IN THE SPONTANEOUSLY HYPERTENSIVE RAT

Introduction

The amygdala is a complex structure mediating autonomic, neuroendocrine, and behavioral responses to different types of stress, including internal stressors, such as hypotension or inflammation, and external stressors, like physical restraint or fearful social contexts (63, 73, 74, 316, 339). In humans, feelings of anxiety or fear have been associated with activation of the amygdala and, if lesioned, the amygdala can dampen autonomic and emotional responses to stressful situations (12, 364, 428). Moreover, individuals with exaggerated cardiovascular responses to stressful stimuli show evidence of increased neuronal excitation in the amygdala which is correlated with a predisposition for high blood pressure (114). Thus, changes in the amygdala's integral role in coordinating autonomic and endocrine responses may lead to exaggerated behavioral and physiological responses to stress.

Three main subnuclei encompass the amygdala: the lateral and basolateral complex (BLA), the central nucleus (CEA) and the medial nucleus (MEA) of the amygdala (207). In general, the BLA complex is thought to function as an integrator of various sensory inputs and acts through a gating mechanism to modulate the output of the CEA (207). The CEA, in turn, sends projections to many important autonomic sites throughout the brain, including the hypothalamus, locus coeruleus, parabrachial nucleus, and central gray to modulate autonomic output (67, 125, 312, 397, 401). Additionally, the MEA has been implicated in modifying the hypothalamic-pituitary-adrenal (HPA) axis (218).

The amygdala contains a high concentration of inhibitory neurons; thus it has been proposed that activation of the BLA by fearful or stressful stimuli may functionally inhibit CEA

and MEA projection neurons thereby disinhibiting distal target sites (271, 274). Yet, the exact conditions under which the CEA and MEA projection neurons are excited or inhibited remain to be fully elucidated. Furthermore, many of the neurotransmitters, such as corticotropin releasing hormone (CRH), which can be found in high concentrations within the amygdala, are known to have powerful impacts on neuronal processing during stress (316, 375).

Historically, researchers have agreed that individuals with hypertension often elicit exaggerated cardiovascular, hormonal, and behavioral responses to stress (7, 137). Furthermore, it has been hypothesized that the augmented sympathetic response to environmental stimuli was one of the mechanisms underlying the development of hypertension in an animal model of essential hypertension, the spontaneous hypertensive rat (SHR) (189). Persuading evidence from lesion studies has exposed a role for the CEA in mediating an augmented response to stressful stimuli and the development of high blood pressure in the SHR (102, 110). Likewise, a more recent lesion study has also implicated the MEA in contributing modestly to the development of hypertension in the SHR (107). The MEA may also contribute to an exaggerated abnormal endocrine response to stressful stimuli typically observed in the SHR, possibly through abnormal regulation of the hypothalamic-pituitary-adrenal (HPA) axis (137, 141, 142).

Although evidence suggests that the amygdala contributes to the exaggerated stress response in the SHR, to our knowledge no previous studies have examined the pattern of neuronal activation within the amygdala of the SHR following stress. Prior studies have examined changes in c-Fos, an early marker of neuronal activation, in the brainstem and hypothalamus after air jet stress in the SHR versus normotensive rats (165, 269). In those regions, some significant differences between strains have been identified. Furthermore, since CRH release from the CEA may modulate the response to stress, it is surprising that changes in

activation of CRH neurons within the amygdala in response to stress have also not been previously examined in the SHR (63, 222, 316). Evidence indicating an alteration in the regulation of CRH in other brain regions in the SHR raises the possibility that dysregulation of CRH neurons within the amygdala could also contribute to exaggerated responses to fearful stimuli in this strain (141, 144, 197).

Due to the limited information describing how the different subnuclei of the amygdala respond to acute stress in the SHR, the following investigation was undertaken to test the hypothesis that both the CEA and the MEA of the SHR would demonstrate a greater number of neurons containing c-Fos and CRH staining compared to a normotensive rat in response to acute exposure to stress. Preliminary results from this study have been presented (290).

Methods

General Preparation

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee and followed the NIH guidelines. Male SHR and normotensive Wistar (W) rats (10-12 weeks old; Charles River Laboratories, Wilmington, MA) were randomly assigned to one of two groups: air jet stress (AJS; n=14/strain) and noise control (NC; n=10/strain). Animals were then given a subcutaneous injection of Rimadyl (0.1 mg/kg, Pfizer Animal Health, Exton, MD) and buprenorphine (0.1 mg/kg, Rickett Benckiser Pharmaceuticals, Inc., Richmond, VA) 20 min. prior to the induction of anesthesia. Animals were anesthetized to a surgical level (2-4% isoflurane+oxygen) and an incision was made on the ventral surface of the hind limb. Fat, nerves, and connective tissue was gently dissected from the femoral vasculature. Catheters (Braintree Scientific, Braintree, MA) were placed into both the femoral artery and vein toward the abdominal aorta. Catheters were filled with heparinized saline (100 IU/ml) and plugged with obturators. The catheters were tunneled subcutaneously to the

nape of the neck, exited between the scapulae, and were secured in place with sutures. All incisions were treated with triple antibiotic ointment (Alpharma USPD, Inc., Baltimore, MD) and the animals received a subcutaneous injection of saline (2-3 ml) for fluid replacement. The rat was placed in 100% oxygen for 5 minutes then allowed to recover on a heating pad. An additional injection of bupronorphine (0.1 mg/kg) was given before returning the animal to its home cage.

Animals were housed individually following surgery and allowed 48 hours to recover prior to experimentation. On the first day following surgery, the animals were brought to the lab to be weighed, handled, and acclimated to the experimental container. Acclimation for the AJS and NC groups also included a 50-60 min. period of exposure to air noise (~75-80 decibels) while resting in the experimental chamber.

Experimental Protocol

All experiments took place between 7:00 a.m. and 12 p.m. On the day of the experiment, the exteriorized catheters were connected to PE 50 tubing containing heparinized saline (100 IU/ml). The animals were placed into the experimental chamber where the catheters were attached to a swivel and tethering system (Instech Laboratories, Inc., Plymouth, MA) which allowed for unrestrained movement within the chamber. The arterial catheter was connected to a pressure transducer (Stoelting Inc., Wood Dale, IL) for continuous measurement of pulsatile and mean arterial pressure (AP). The experimental chamber consisted of a circular container (15 cm radius) with three tubes (~0.5 cm diameter) inserted into the wall of the chamber, one tube every ~30 cm (equidistance around the circumference) at a height of approximately ~5 cm from the bottom of the chamber. The tubes were connected to a three-way valve manifold for regulation of the air stream through each tube. During the AJS protocol, pressurized room air flowed

continuously at 20 Psi through one of the three tubes. AJS and NC treated animals were recorded on separate days.

At the beginning of the experiment, a 90 min. baseline of AP was recorded. During this baseline period, both AJS and NC groups were exposed to air noise associated with 20 Psi room air released from tubing outside of the chamber. For the AJS group, after 90 min. of air noise, the pressurized air was switched from being directed away from the chamber to being directed into the chamber through one of three inflow tubes. The AJS protocol was separated into two distinct periods: an initial 5 min. period of random AJS (continuous stream of air entering the chamber from a random direction and at random durations) immediately followed by a 15 min. period of controlled AJS (the direction of the air flow was changed every minute). After the 20 min. of AJS, rats were allowed to recover for 90 min. without continued noise. Following the baseline period, the NC group experienced the noise component of the AJS without the flow of air into the apparatus for 20 min. followed by the 90 min. of quiet recovery. At the termination of the experiment, all animals were deeply anesthetized with sodium pentobarbital (80-100 mg/kg) and immediately transcardially perfused with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.2). Brains were removed and post fixed in 4% paraformaldehyde overnight followed by submersion in a 30% sucrose solution for 48-72 hours. Prior to processing, a small cut was made along the rostral-caudal extent of the right ventral surface of the brain to distinguish left and right sides. Next, the tissue was frozen and sectioned into 40 μm coronal slices.

Immunohistochemistry

Sliced tissue was soaked in phosphate buffered saline (PBS) for approximately 24 hours and then washed in 3% donkey serum and 0.4% Triton X in PBS (3%DS-T-PBS) for one hour. The tissue was incubated overnight in rabbit anti-Fos antibody (1:2000 dilution, Santa Cruz

Biotechnology, Santa Cruz, CA) diluted with 1% DS-T-PBS. The next day the tissue was washed in 1% DS-T-PBS for one hour, incubated in donkey-anti-rabbit biotinylated immunoglobulin G (Ig) antibody (1:500; Jackson Immuno Research, West Grove, PA) for two hours, and then re-rinsed for 1 hour. Next, the tissue was incubated for 30 min in an avidin biotinylated-peroxidase tertiary molecule solution (ABC Vectastain Kit, Vector Labs, Burlingame, CA). Following a final wash in 1% DS-T-PBS, the tissue was stained with Vector SC (Vector Labs) revealing the Fos-like Immunoreactivity (FLI) as gray/blue stain in the nucleus of the cell. The brain tissue was then re-incubated in 3% DS-T-PBS for one hour, followed by incubation in guinea pig anti-CRH (1:10,000; Peninsula Laboratories, Inc., San Carlos, CA) for 12-24 hours. The tissue was rinsed in 1% DS-T-PBS for one hour, incubated with donkey anti-guinea pig biotinylated Ig antibody (1:500; Jackson ImmunoResearch) for two hours, re-rinsed for 1 hour, and then incubated for 30 min in ABC solution, followed by a 1% DS-T-PBS rinse. Finally, the tissue was stained with 0.05% diaminobensidine hydrochloride (DAB, Vector Labs)) diluted in 2.5% ammonium sulfate; 0.00% hydrogen peroxide in 0.05 M Tris-HCL. Tissue was rinsed three times in PBS and mounted on slides and dried. Slides were dehydrated and coverslipped.

Data Analysis

For the cardiovascular data, heart rate (HR) was derived from the interval between each successive systolic peak of pulsatile AP and then averaged along with mean arterial pressure (MAP). Three time periods were analyzed for average MAP and HR, including baseline, the treatment period (AJS or noise), and recovery. The baseline time point was defined as a 2 min. average approximately 20 min. prior to the treatment period when AP and HR remained steady for 5 min. During the treatment period, MAP and HR were averaged over successive two min.

intervals and two recovery time points, at 30 and 60 min. from the onset of the treatment period were also measured. The change from baseline in MAP and HR was calculated for each animal

For immunohistochemical analysis, two slices per animal were imaged for each of the following brain regions: three rostral-caudle extents of the amygdala, two rostral-caudal extents of the LC, the arcuate nucleus (ARC), PVN, and dorsal medial hypothalamus (DMH). Brain regions were identified by anatomical landmarks, such as fiber tracks and surface indentations, defined by the Rat Stereotaxic Atlas (281). For the amygdala, the rostral extent was -1.6-2.1 mm, the middle extent was -2.2-2.6 mm, and the caudal extent was -2.7-3.2 mm caudal to bregma. Specifically, FLI in the CEA, BLA, and the MEA was quantified. The rostral and caudal regions of the LC were defined as -9.3-9.7 mm and -9.8-10.2 mm caudal to bregma, respectively. The ARC was identified between -3.1-3.3 mm caudal to bregma. The magnocellular (mPVN) and parvocellular (pPVN) subnuclei of the PVN were identified between -1.7-1.9 mm caudal to bregma. Finally, the DMH was located between -2.8-3.1 mm caudal to bregma. The criteria for Fos-like immunoreactivity (FLI) was based on the presence of gray/blue stain corresponding to the cell's nucleus. CRH-positive cells were identified by a brown color filling the cytosol and axonal and dendritic processes of the neuron. Each brain region was imaged at 10x and counted manually in Adobe Photoshop 7.0. All cell counts were made from the left side of the brain. Cell counts from the two slices per each brain region were averaged. Preliminary analysis identified that the rCEA did not contain CRH-positive cells and FLI was relatively low in all treatment groups, thus quantification of CRH and FLI labeling was limited to the caudal and middle amygdalar regions. Additionally, no cells co-labeled for FLI and CRH were evident in the CEA and thus co-labeling could not be quantified.

Statistical Analysis

Cardiovascular data were analyzed using a three-way analysis of the variance (ANOVA) with repeated measures comparing treatment groups and strains against the designated time points (STATVIEW). The treatment and recovery periods were analyzed. A three-way ANOVA was also used for comparing strain, anatomical extent, and treatment effects for the immunohistochemical data for the amygdala and locus coeruleus between strains. In the amygdala, initial analysis identified no caudal-middle location effect for FLI or CRH positive cells, thus, the number of FLI or CRH-positive cells from the middle and caudal regions of each subnucleus of amygdala were summed. Further analysis of the effect of strain and treatment was then done using a two-way ANOVA. The hypothalamic subuclei were also analyzed via a two way ANOVA between strain and treatment. For all analyses, when a significant interaction occurred, three different statistical comparisons were made: 1) strain differences between control groups; 2) strain differences between AJS groups; and 3) differences within strain between treatment groups. The comparisons were made using an unpaired t-test in which the significance level was adjusted by the Bonferroni test for multiple comparisons. Statistical significance was set at $P<0.05$.

Results

Cardiovascular Response to Air Jet Stress

In the present study, conscious adult male SHR and Wistar (W) rats were placed unrestrained in an experimental chamber and exposed to one of the following conditions: 90 min. of air noise followed immediately by 20 min. of AJS (n=14) or exposed to 110 min of air noise (NC, n=9). Figure 2-1 illustrates the typical cardiovascular response before, during, and immediately following AJS for each rat strain. At the onset of AJS, arterial pressure increased in both strains, however, the increase was greater in the SHR compared to the Wistar. HR also

increased rapidly at the onset of AJS and the initial rise from baseline also appeared greater in the SHR. Furthermore, as AJS continued, HR in the Wistar rat (Fig. 2-1A) gradually began to fall, followed by a slight rise at the end of AJS. In contrast, during sustained AJS, HR in the SHR remained steady with only a slight decrease near the end of the stress period (Fig. 2-1B). At the offset of AJS, MAP and HR gradually returned to baseline in both strains.

Figure 2-2 demonstrates the average change from baseline for MAP and HR during the 20 min. of AJS, the corresponding NC period, and during the recovery. Prior to the onset of the treatment, during the 90 min. air noise baseline period, MAP of the SHRs was significantly higher compared to the Wistars (162±3 vs. 115±2 mmHg; respectively) and baseline HR of the SHRs was significantly lower compared to the Wistars (350±7 vs. 385±13 beats/min; respectively). During the treatment period (AJS or NC), statistical analysis of the changes in MAP using a three-way ANOVA only identified a significant interaction between strain and treatment, the effect associated with individual time points was not significant ($P>0.5065$). Subsequent analysis using a two-way ANOVA identified that the average increase in MAP with all time points combined in the SHR during AJS was significantly greater than the increase in MAP elicited in the W-AJS and both the NC groups ($P<0.0001$). The increase in MAP during AJS elicited by the W-AJS group was also significantly different from the SHR-NC group ($P=0.0056$). Comparison between the NC groups did not demonstrate any difference during the treatment period. Recovery points for all groups were not significantly different from each other.

Statistical analysis of the change in HR during the treatment period using a three-way ANOVA did not identify a significant interaction with time, however the strain and treatment effect was significant ($P=0.0224$). As a consequence, all time points were combined and a two-way ANOVA (strain and treatment) identified that the increase in HR during AJS in the SHR

was significantly greater than that observed in the W-AJS group and both NC groups ($P<0.0001$). The change in HR for the W-AJS group was also significantly greater that the change in HR demonstrated in both NCs ($P<0.0011$). The NC groups did not exhibit any noticeable fluctuations in HR during the experiment and there was no significant difference between the strains. During the recovery period, there was no significant difference in HR between all four groups.

Changes in FLI and CRH Staining in the Amygdala Following AJS

To identify whether the exaggerated cardiovascular response to AJS in the SHR was associated with increased neuronal activation in a specific region of the amygdala, FLI in three main subnuclei of the amygdala was quantified. Visual representation of three different subnuclei of the amygdala evaluated is shown in Figure 2-3A. Contrary to our original hypothesis, statistical analysis within each subnucleus did not identify any strain effect. Within each subnucleus, however, there was a significant treatment effect. In the BLA, for example, there was a significant increase in FLI in the AJS compared to the NC groups ($P<0.0001$; Fig. 2-3B). In both the CEA and MEA, FLI in the AJS groups also increased significantly above the NC (Fig. 2-3C&D; $P=0.0024$ and $P<0.0001$, respectively).

In Fig. 2-4A, typical examples of CRH staining observed in the CEA of a W-AJS and a SHR-AJS animal are illustrated. A three-way statistical ANOVA did not demonstrate an interaction between strain, treatment, and the caudal-middle extent of the CEA ($P=0.6144$), but there was a significant effect of treatment ($P=0.0056$) and strain ($P=0.0002$). Further analysis with the CRH cell counts from the middle and caudal regions summed also identified a significant effect of strain ($P=0.0321$) and treatment ($p=0.0038$), but the interaction between groups was not significant ($P=0.3570$). Despite the significant effect of strain, the subsequent post hoc analysis of the strain effect did not result in a significant increase ($P=0.0618$) as

compared to the treatment effect (P=0.0074). Thus, AJS was identified to significantly increase CRH labeling in both strains by approximately two fold and independent of treatment, CRH-positive cells were more numerous in the SHR compared to the Wistar.

Changes in FLI in LC and the Hypothalamus Following AJS

Figure 2-5A depicts the number of FLI cells in the rostral LC of a SHR and Wistar exposed to AJS. A three-way ANOVA between the caudal (cLC) and rostral LC (rLC) demonstrated a significant effect of strain, treatment, and the caudal-rostral location (P=0.0385). In the rLC, there was a significant increase in FLI in the SHR-AJS group compared to the W-AJS group and both NC groups (P<0.0011). The W-AJS group also demonstrated a significant increase in FLI levels in the rLC compared to both NC groups (P<.0.0002). Conversely, in the cLC, both SHR and Wistar AJS groups demonstrated significant increases in FLI compared to the NC groups (P=.0.0002 and P<0.0013, respectively), but no significant difference between the SHR-AJS and W-AJS groups was identified. No differences between the NC groups in either the cLC or rLC were detected.

Figure 2-6 illustrates the effect of AJS on FLI levels throughout the hypothalamus. All hypothalamic regions evaluated demonstrated an effect of treatment on FLI, but no strain differences were identified. For example, in both the magnocellular and parvocellular regions of the PVN, AJS induced a significant increase in FLI compared to NC groups (P=0.0009 and P<0.0001, respectively; Fig. 2-6A). In the dorsomedial nucleus of the hypothalamus (DMH) and the arcuate nucleus of the hypothalamus (ARC) there was also a significant increase in FLI in the AJS groups compared to the NC groups (Fig. 2-6B, P<0.0001 and P<0.0001, respectively).

Discussion

The putative contribution of select subnuclei in the amygdala to the exaggerated cardiovascular response to acute stress observed in hypertensive individuals was evaluated in the

current study. In both nomotensive and hypertensive conscious rats, the unrestrained AJS model utilized in the present study generated a robust increase in both MAP and HR that was significantly greater in the hypertensive group (SHR) compared to the normotensive controls. Exposure to AJS induced a significant increase in FLI within all subnuclei of the amygdala evaluated, but contrary to our original hypothesis, no strain effect was identified. The only significant strain effect identified in the amygdala was associated with the number of CRH-positive neurons observed in the CEA. In response to AJS, the number of CRH-positive neurons increased in both strains compared to the NC groups and the number of CRH neurons was greater in the SHR groups compared to the Wistar groups despite the lack of a significant interaction between strain and treatment. Interestingly, we did identify that the exaggerated cardiovascular response to AJS in the SHR was associated with significantly higher levels of FLI in the rostral LC compared to all other treatment groups. This observation is intriguing since there is a well characterized anatomical connection between CRH neurons in the CEA and the rostral pole of the LC (396, 397). Thus, both the CRH levels in the CEA and FLI in the rostral LC in the SHR are elevated in response to AJS compared to a normotensive control providing new evidence that dysregulation of the these two regions may contribute significantly to the exaggerated cardiovascular response to acute stress observed in certain forms of hypertension (55, 174).

Cardiovascular response during AJS exposure in the SHR. In the present study, we developed a novel model of AJS without restraint to study the pattern of brain activation involved in stress. This model was developed on recent evidence suggesting that patterns of brain activation, particularly in the amygdala, are dependent on the type of stressor applied, either psychological or physical (73, 110). Most AJS models previously used to test for stress

response differences in the SHR compared to normotensive strains have utilized some method of restraint while exposing the animals to AJS. Since restraint is a form of stress itself, those methods may have potentially contaminated the results by testing multiple stress stimuli simultaneously (102, 110, 233). We also conditioned the rats to the air noise component of AJS prior to testing to prevent any added effects induced by audiogenic stress (233, 268). With the former precautions, the SHR responded to the AJS with a marked increase in MAP and HR compared to the normotensive Wistar. Other stressors applied to the SHR such as restraint, noise, and alerting stimuli have also been shown to elicit cardiovascular responses comparable to those generated by our AJS model (147, 273). Our results indicate that, in response to a processive stressor, the sympathetic nervous system of the SHR is innately hyperactive.

Amygdalar activation during stress in the SHR. AJS exposure revealed an increased activation of FLI in several subnuclei of the amygdala independent of strain. The increase in FLI detected in the MEA was considerably greater than those observed in the BLA or CEA in the SHR, an observation that is consistent with reports from normotensive rats (74, 75). We had hypothesized that AJS would induce a greater rise in FLI in the MEA of the SHR compared to the Wistar, based on a recent observation that chemical lesion of the MEA in the SHR reduces the level of hypertension developed in the SHR (107). Furthermore, activation of the MEA in response to stress has been linked to the release of hormones and there is evidence that the HPA axis may be dysregulated in the SHR (75, 76, 142). Despite evidence of the MEA's possible role in stress, the results of the present study do not implicate the MEA in playing a unique role in the exaggerated cardiovascular response to stress typically observed in the SHR.

Contrary to our hypothesis that the CEA of the SHR would also demonstrate increased numbers in FLI-positive neurons in response to AJS compared to the Wistar, our study

demonstrated only a treatment effect regarding the CEA. Multiple studies have demonstrated a reduced level of FLI in the CEA compared to control in respect to processive stessors, like restraint, while physiological stressors such as hypotension generate the opposite response (74, 158, 309). Indeed, when baseline levels of FLI are elevated by amphetamine administration, the addition of an external stressor has been reported to reduce FLI in the CEA (73). Furthermore, amphetamine exposure did not generate an increased number of CRH-positive neurons, suggesting the interconnections within the CEA may be actively inhibited by certain stimuli (72).

In the present study, we did observe an increase in CRH-positive neurons in the CEA in response to AJS, with a noticeable increase in the SHR compared to the Wistar. The only prior evidence regarding dysregulation of CRH levels in the SHR refers to CRH mRNA expression in the PVN. Previous investigators have identified that basal CRH mRNA levels in the PVN of the SHR is either lower or not different compared to a normotensive strain (141, 144). During restraint, however, CRH mRNA expression in the PVN increases more in the SHR (197). Increased expression of CRH in the PVN in response to acute stress has been hypothesized to be related to the altered regulation of HPA axis implicated in the SHR (142). The present study is the first to evaluate CRH protein levels in the amygdala of the SHR and the results suggest that comparable to the PVN, CRH expression appears to be increased in the CEA of the SHR in response to stress.

The third region of the amygdala to be evaluated in the present study was the BLA. The BLA mainly modulates behavioral output generated during times of both stress and non-stress. Acting through γ-aminobutyric acid (GABA) intercalating projection interneurons, the BLA can exert tonic inhibition over the CEA (207). The increased FLI in the BLA observed following AJS in the present study suggests the activation of the BLA may lead to increased inhibition of

the CEA during stress. Since the only strain related difference we observed in the CEA in response to AJS was identified in the CRH-positive neurons, it is possible that the exaggerated response to AJS in the SHR is mediated by inadequate inhibition from the BLA to the CEA's CRH-positive neurons. Alternatively, the exaggerated cardiovascular response may be regulated through another pathway. The prefrontal cortex (PfC) also acts on the GABAergic intercalated neurons to tonically inhibit the amygdala to prevent activation of cardioacceleratory areas. During stress, the PfC withdraws control allowing for disinhibition of the CEA, resulting in subsequent disinhibition in brain regions downstream and an increase in sympathetic drive (312, 384). It is possible that a competitive inhibition also acts within the intercalated cells exciting specific populations of neurons in the CEA generating a unique response to specific stress stimuli. Notwithstanding, the limitations of the c-fos technology only allows us to measure neuronal excitation while measuring neuronal inhibition could prove to be beneficial in dissecting the amygdala's role in regulating the cardiovascular response to stress.

FLI in the locus coeruleus following AJS. Unlike the pattern of FLI in the amygdala, the rostral LC demonstrated a substantial increase in FLI in the SHR-AJS group that was significantly greater than that induced in the W-AJS group. Only small increases in FLI in the LC in response to air puff stress have been previously reported in the SHR compared to another normotensive control, the WKY rat. In that study, however, single puffs of air were used to induce stress, compared to the 20 min. of continuous AJS used in the present study (269). The difference in the intensity of the stress may have led to the robust activation of the rLC exhibited in the present study in the SHR. Acknowledged alterations in both the anatomy and activity of the LC in the SHR include reduced basal neuronal discharge, decreased α-2 adrenergic receptor sensitivity, and increased dendritic branching (90, 98, 260). During activation of the baroreflex,

the LC is generally inhibited by GABAergic input, however, in the SHR, there has not been a noticeable change in GABA secretion following intravenous infusion of norepinephrine (174, 350). Alternatively, basal secretion of GABA in the LC is higher in the SHR compared to the normotensive rat, suggesting the large pool of GABAergic input in the LC in the SHR prevents appropriate reflex modulation of pressor responses. On the other hand, when exposed to a moderate stressor such as tail pinch, GABA is released in higher levels in the SHR giving further evidence a dysfunction in the LC's role in modulating the stress response (174). The enhanced sensitivity to sensory stimuli may have led to the increased activation in LC of the SHR during this study and may be more related to behavioral responses rather than cardiovascular activity during stress (399).

The LC core has a dense population of noradrenergic neurons that either project rostrally to the forebrain or caudally to the spinal cord (228, 294). Lesion and microinjection studies have demonstrated that the caudal portion of the LC is active during hypotension while the rostral component responds to pressor responses (13, 42). The increased FLI in the rLC of SHR demonstrates a possible hyperarousal of the LC leading to increased noradrenergic release into the limbic system and the HPA axis to modulate stress-like behaviors (33, 266). CRH efferents from the CEA and bed nucleus stria terminalis, target the rostrolateral peri-LC, a dendritic region rich in noradrenergic nerve terminals located at the rostral pole of the LC (397). More specifically, there is evidence that descending input from CRH neurons in the CEA mediates stress related excitation of LC in normotensive animals (63, 64, 397). CRH injected into the LC leads to increased neuronal discharge and cortical norepinephrine release (353, 394). Injections of CRH in the LC of the SHR demonstrated smaller increases in neuronal activation compared to normotensive controls furthering the theory that the LC is abnormal in the SHR (55).

Additionally, afferents from the nucleus tractus solitarius (NTS), a medullary cardiovascular center, targets the rostro-medial peri-LC for autonomic influences on LC activity (398). The integration of limbic and autonomic-related information centralizes in the peri-LC and drives release of norepinephrine throughout the brain during stress. Both the increases in CRH neurons in the CEA and FLI in the rLC in our study suggest that the relationship between these two brain regions may be involved in the exaggerated cardiovascular reactivity in the SHR during stress.

FLI in hypothalamic structures following AJS. In addition to the amygdala and LC, we examined the pattern of FLI in three hypothalamic regions known to be involved in stress-related responses: the DMH, ARC, and PVN (391). Interestingly, evaluation of the DMH, ARC, and PVN demonstrated similar increases in FLI in both the Wistar and SHR following AJS. Since all three hypothalamic subnuclei have been implicated in mediating autonomic responses to stress, and the response to AJS was exaggerated in the SHR, it was unexpected that parallel rises in FLI would be exhibited in both the Wistar and the SHR (103, 269, 365, 366). Moreover, several previous studies have demonstrated significant increases in FLI in the hypothalamus and the brainstem in response to AJS and restraint in the SHR when compared to the WKY rat (165, 197, 269). Yet, the results of the present study suggest that the capacity for neuronal activation in the hypothalamus during AJS in the absence of physical restraint may be relatively normal in the SHR when compared to a different normotensive strain.

To determine if the noise accompanying the AJS protocol contributed to the FLI levels demonstrated in these animals, we ran a control group exposed only to the noise component. In all brain regions analyzed, noise did not induce high levels of FLI in either strain. Previous studies have demonstrated increases in c-fos mRNA levels in the forebrain, hypothalamus, pons, and brainstem with increasing noise intensity (36). The noise range in our study was near 75-80

decibels which, as described in the literature, only induces small increases in FLI levels in the hypothalamus and LC. Moreover, in the amygdala, previous studies have demonstrated that increasing noise intensities induce increased levels of c-fos mRNA in the MEA and BLA while the CEA demonstrates reduced level of c-fos mRNA. In contrast, in the current study relatively low levels of FLI were exhibited in all subnuclei of the amygdala in our NC group (73, 74). It is possible that the lack of FLI observed in the NC group was a function of time, since the exposure to the noise was 110 min. followed by a 90 min recovery. By the end of the experiment, any c-fos generated by the noise would have subsided since c-fos protein levels are thought to peak ~90-120 min. following exposure to a stimulus (249). Chronic audiogenic stress also displayed low activation of c-fos mRNA expression supporting the low levels exhibited in our noise control (39) . Overall, analysis of the noise control confirmed that increases in FLI levels with regard to the AJS groups are unique to that stimulus.

Methodological Considerations. Three methodological considerations should be taken into account in the interpretation of the present results. First, in the majority of previous studies investigating the impact of stress on regional brain activation in the SHR have utilized the WKY as a normotensive control (60, 73, 389). In the present study, we chose to use the Wistar as a control over the traditional WKY based on evidence that the WKY strain may be hypo-responsive to stressful stimuli compared to other normotensive strains (26, 63). Thus, some of the differences in our results and previous studies may be related to the normotensive strain used for comparison. Secondly, we chose to use c-fos as the immediate early gene marker of neuronal activation. Previous studies have shown that noise and restraint can reduce the level of c-fos activity induced by amphetamine in the amygdala, suggesting that stress may be associated with inhibition of the CEA in the normotensive animal, however, c-fos is only a marker of excitation

(63). Furthermore, another immediate early gene marker, phosphoylated cAMP response-element binding protein (PCREB), which is responsible for various signaling cascades within the cell, has also been shown to be increased during stress in a distinctive pattern compared to c-fos (72). Future studies evaluating both PCREB and c-fos labeling are likely to provide a more complete picture of activation of the amygdala in the SHR exposed to stress, particularly in regard to changes in CRH transcription which appears to be dependent on CREB (166, 194). Finally, it is possible the amount of time spent habituating the animal to the AJS apparatus may not have been sufficient as demonstrated by the high basal HR in the Wistar groups. Additionally, the tether may be considered a confounding factor due to it being a partial restraint. Both novel environments and handling can induce neuronal activation, therefore it is important to eliminate all possible factors that can affect the response to a solitary stress stimulus.

Conclusion. The results of the current study provide new information regarding activation of the amygdala in the SHR following exposure to unrestrained AJS. In all brain regions examined, there was a lack of a strain effect except for an elevation of CRH labeling in the SHR and an elevation of FLI in the rLC in the SHR compared to the Wistar after AJS. These observations suggest that the SHR is hypersensitive to arousal stimuli and the exaggerated neuronal activation of neurons in the rLC may be related to CRH efferents from the CEA. Overall, it appears the exaggerated stress response in the SHR may be associated with an abnormal regulation of the sympathetic system possibly driven by altered inputs to the LC. These observations provide a possible target for future investigations into the treatment for hypertension and exaggerated responses to stress.

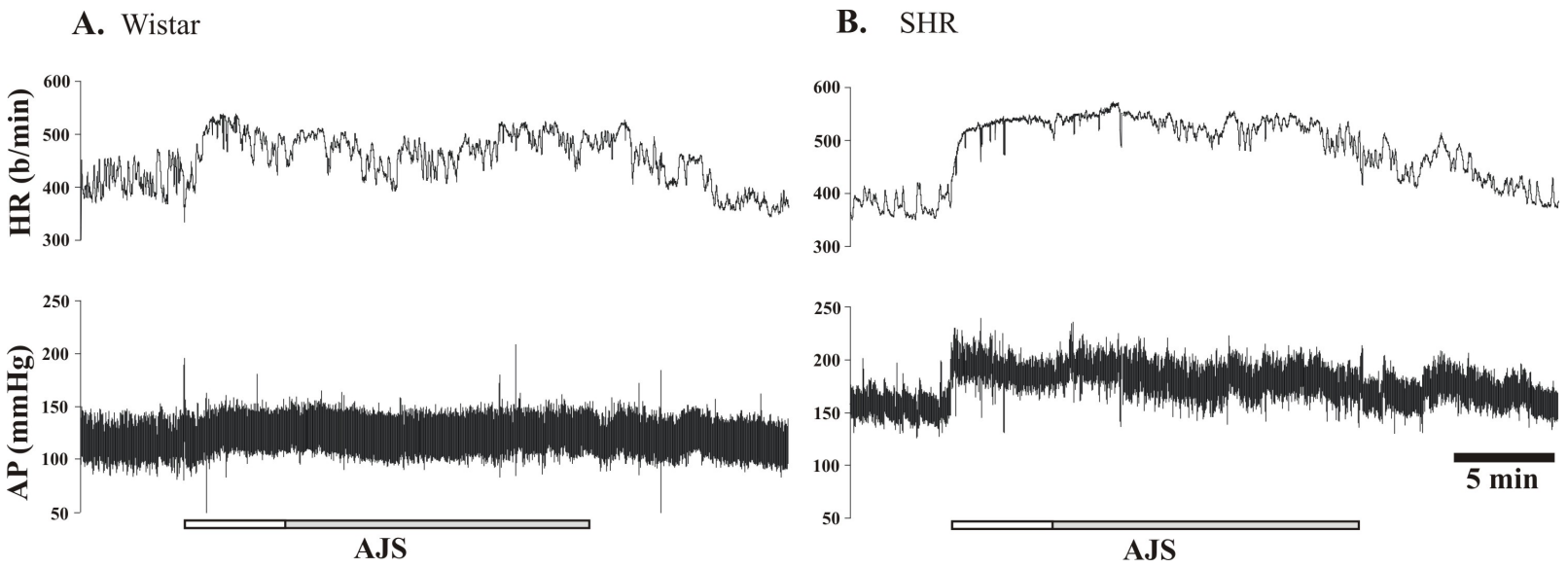

Figure 2-1. Raw cardiovascular response to air jet stress. Arterial pressure (AP) and heart rate (HR) from a single unrestrained male Wistar (A) and SHR (B) rat before, during, and immediately following exposure to unrestrained AJS. Gray and white horizontal bar represents the 20 min. duration of air jet stress (AJS), including an initial 5 min. of randomly directed AJS (white) and 15 min. of sustained AJS in which the direction of air changes in 1 min. intervals (gray). The total recording represents 35 min.

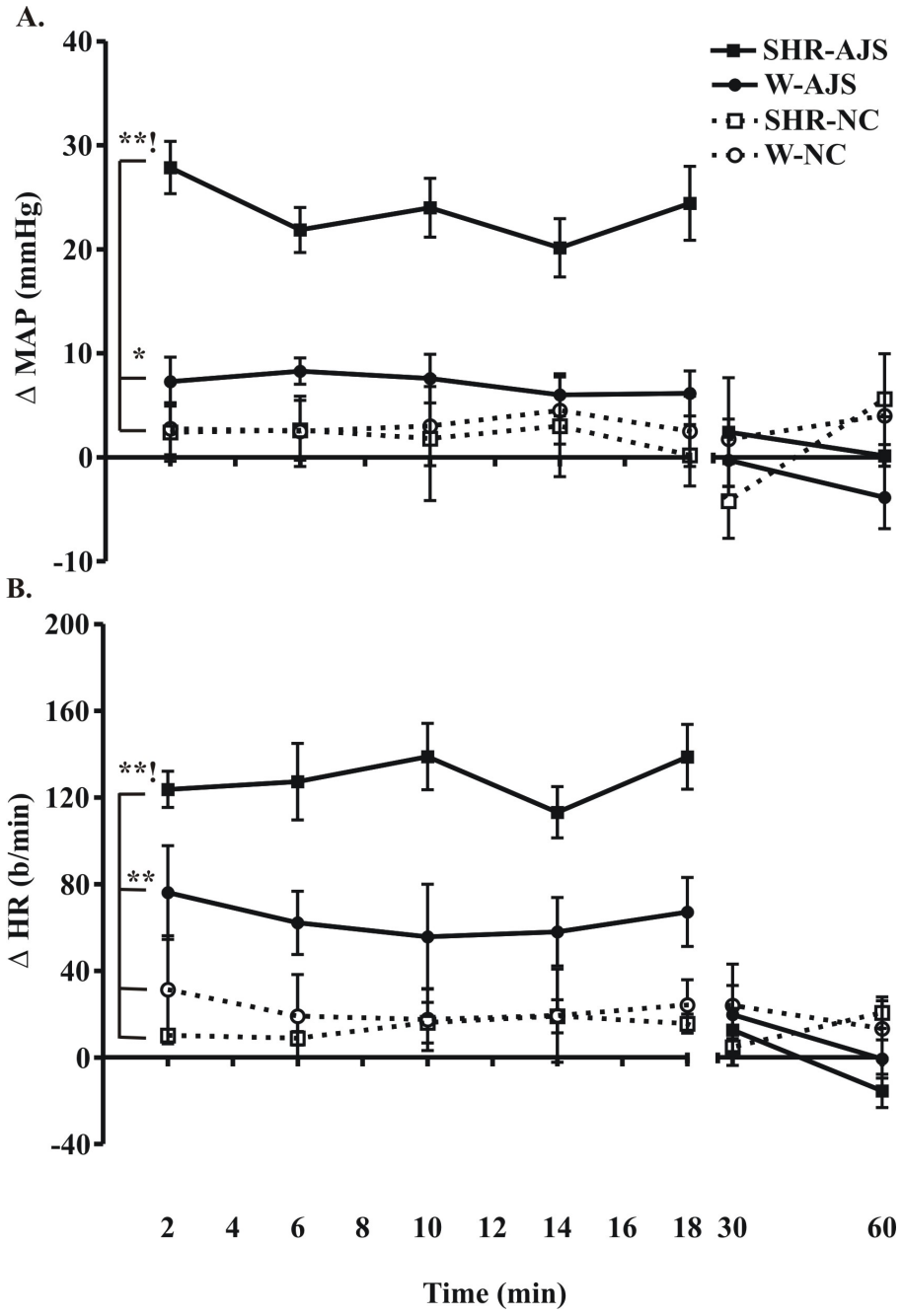

Figure 2-2. Delta cardiovascular response to air jet stress. Change from baseline for mean arterial pressure (A) and heart rate (B) in response to air jet stress (AJS) or noise control (NC) between SHR and Wistar rats. Numbers on x-axis represent the final time point from the averaged 2 min. intervals. W-NC (n=4), W-AJS (n=7), SHR-NC (n=5), and SHR-AJS (n=7). *P<0.0083 indicates significance between AJS and opposing strain's NC. **P<0.0083 indicates significance between AJS and both NC groups. !P<0.0083 indicates significance between AJS groups. W, Wistar; SHR, Spontaneously Hypertensive Rat; MAP, mean arterial pressure; HR, heart rate.

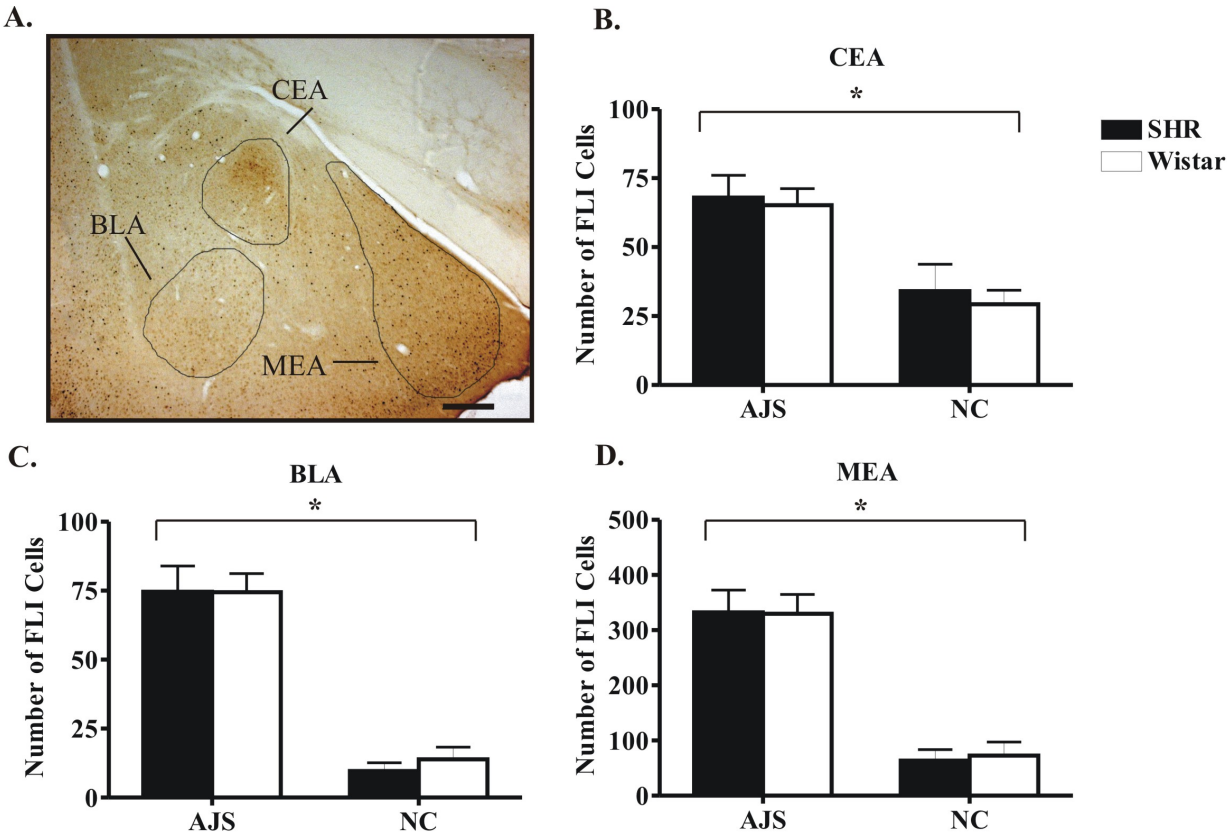

Figure 2-3. FLI cell counts in the central, basolateral, and medial nucleus of the amygdala. A) Visual representation of the caudal portion of the subnuclei of the amygdala from a single rat. Black bar represents 50 µm. B) Number of FLI-positive neurons in the CEA. C) Number of FLI-positive cells in the BLA. D) Number of FLI-positive neurons in MEA. *P<0.05 indicates significant difference between AJS and NC groups. W-NC (n=5), W-AJS (n=7), SHR-NC (n=5), and SHR-AJS (n=7). AJS, air jet stress; NC, noise control; FLI, fos-like immunoreactivity; CEA, central nucleus of the amygdala; BLA, basolateral nucleus of the amygdala; MEA, medial nucleus of the amygdala. Group definitions are in Fig 2.

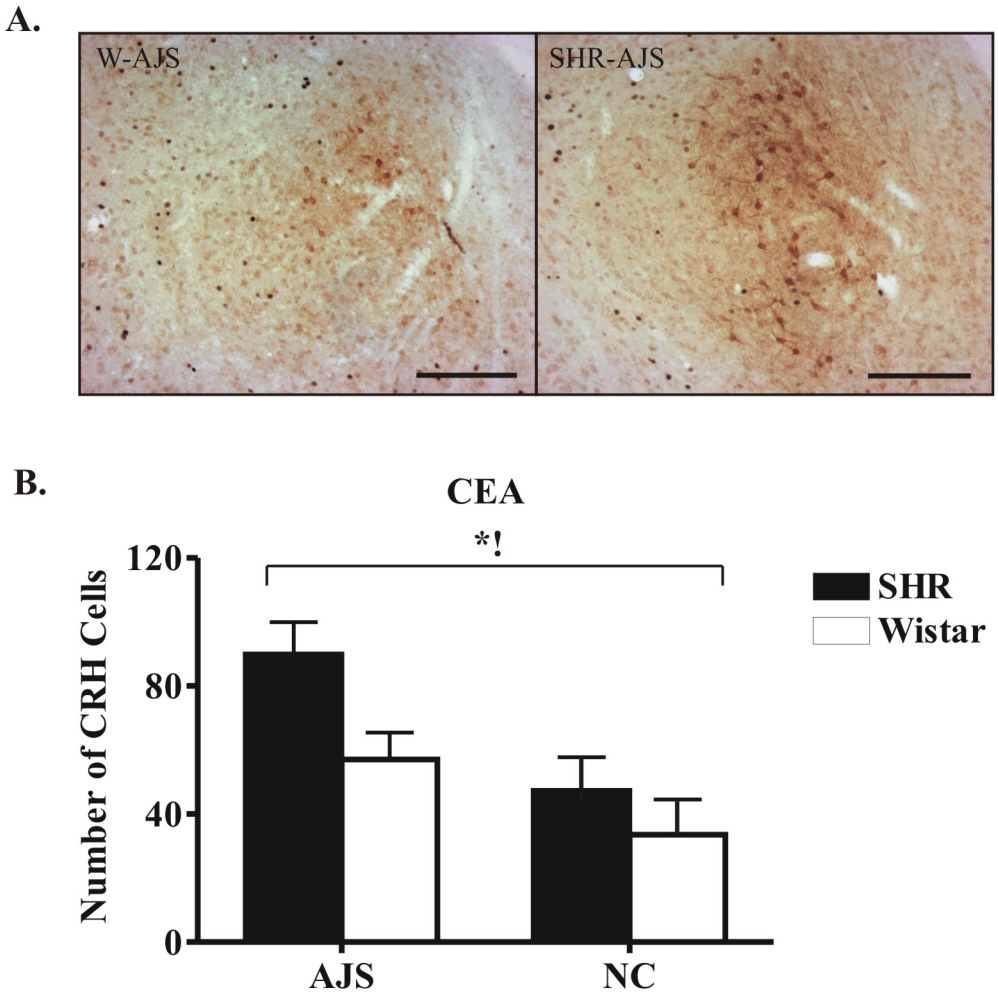

Figure 2-4. CRH-positive cells in the central nucleus of the amygdala. A) CRH staining in the CEA representing both the W-AJS and SHR-AJS groups. Black bar represents 100 μm. B) Number of CRH-positive cells in the CEA. *P<0.05 indicates significant difference between AJS and NC groups. !P<0.0618 indicates strain difference between SHR and Wistar groups. W-NC (n=5), W-AJS (n=6), SHR-NC (n=5), and SHR-AJS (n=6). CRH, corticotropin releasing hormone; cCEA, caudal central nucleus of the amygdala; mCEA, middle central nucleus of the amygdala. Group definitions are in Fig 2.

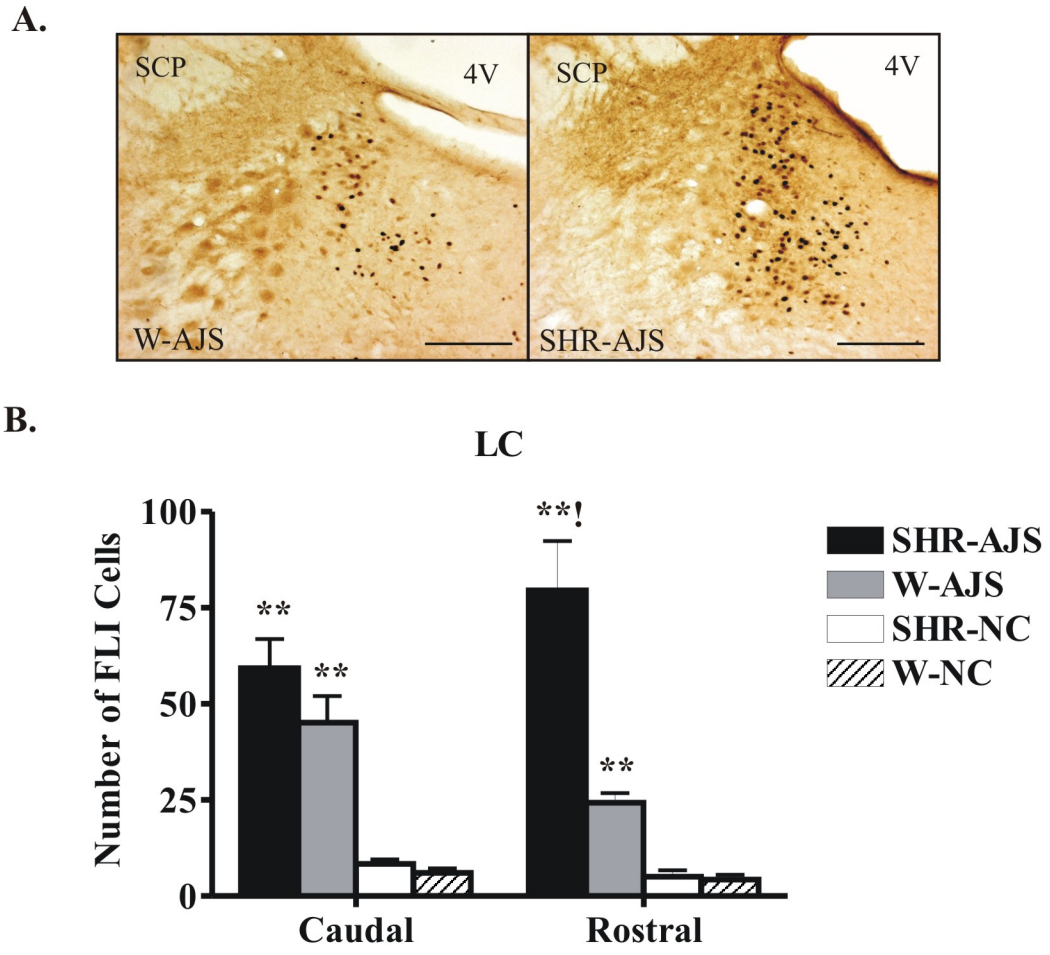

Figure 2-5. Pattern of FLI in the locus coeruleus. A) Images of rLC from a single animal within both AJS groups. Black bar represents 100 μm. B) Number of FLI in the caudal (cLC) and rostral (rLC) regions. *P<0.0083 indicates significance between AJS and opposing strain's NC. **P<0.0083 indicates significance between AJS and both NC groups. !P<0.0083 indicates significance between AJS groups. W-NC (n=5), W-AJS (n=7), SHR-NC (n=5), and SHR-AJS (n=7). SCP, superior cerebellar peduncle; 4V, fourth ventricle; cLC, caudal locus coeruleus; rLC, rostral locus coeruleus. Group definitions provided in Figure 3.

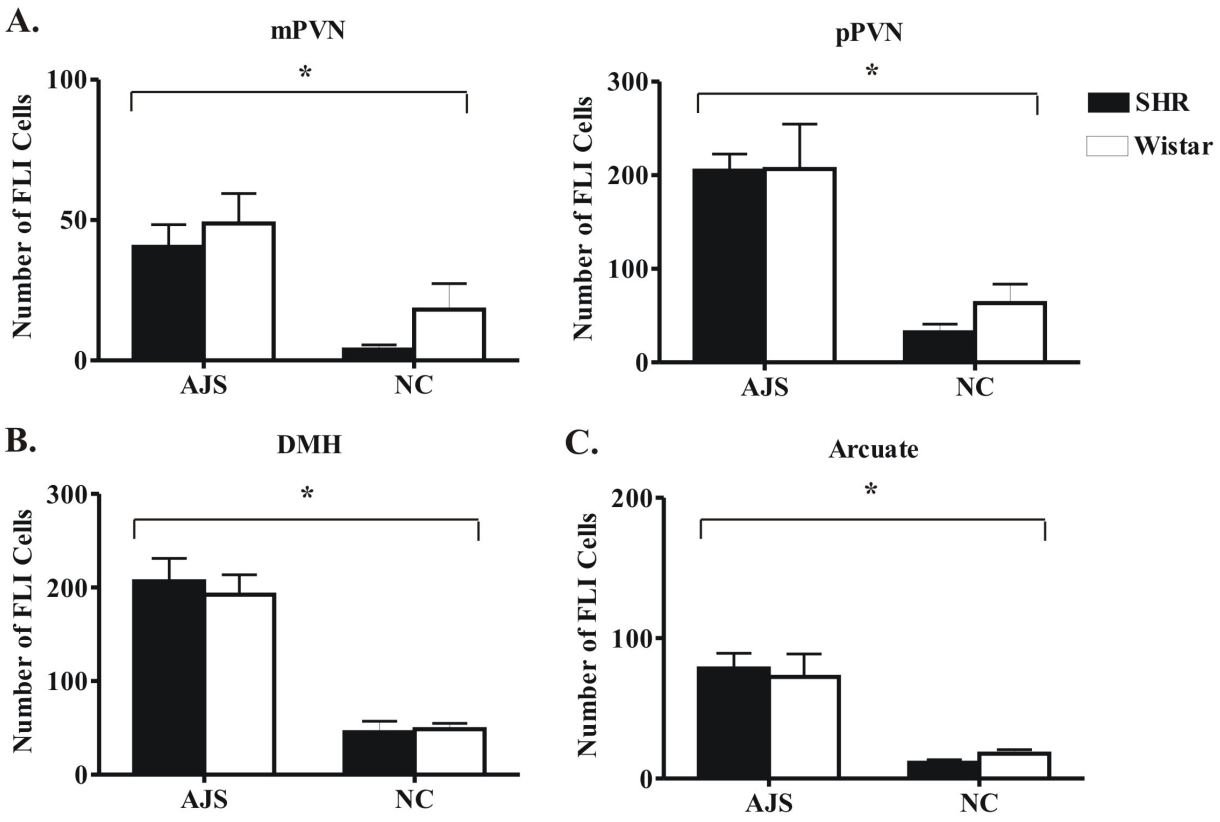

Figure 2-6. Pattern of FLI in the hypothalamus. A.) Number of FLI in magnocellular PVN (mPVN) and parvocellular PVN (pPVN). B.) Number of FLI in the DMH. C.) Number of FLI in the ARC. *P<0.05 indicates significance between AJS and NC groups. W-NC (n=5), W-AJS (n=7), SHR-NC (n=5), and SHR-AJS (n=7). PVN, paraventricular nucleus; DMH, dorsomedial hypothalamus; ARC, arcuate nucleus. Group definitions provided in Figure 3.

CHAPTER 3
DISINHIBITION OF CEA MODULATES THE CARDIOVASCULAR RESPONSE TO STRESS IN THE SHR

Introduction

Hypertension is a risk factor for a variety of pathologies such as heart disease, stroke, and Alzheimer's disease (264, 335). When exposed to mental stressors such as public speaking or mathematic tests, individuals diagnosed with hypertension exhibit increased blood pressure (BP), tachycardia, and high levels of plasma glucocorticoids and catecholamines compared to their normotensive counterparts (7, 88, 229, 254). Similarly, rat models of hypertension demonstrate augmented cardiovascular and hormonal responses to various stressors such as restraint, noise, air jet stress, and ethanol treatment (21, 137, 142, 165, 189). Typically, the hypersensitivity to stress has been attributed to an alteration in the hypothalamic-pituitary-adrenal (HPA) axis due to high basal glucococorticoid levels in both human and the spontaneously hypertensive rat (SHR) (118, 142, 165). Additionally, high sympathetic nerve activity in hypertensive individuals has also been suspected for augmenting the cardiovascular response to acute stressors (172). Although, hypertension leads to various organ and tissue damage in the periphery, it is evident that changes within the central nervous system may be instrumental in generating the exaggerated responses to stressful stimuli in hypertensive individuals.

Many brain regions contribute to the various behavioral and physiological responses elicited by a perceived stress. Specifically, the amygdala, the emotion processing center of the brain, coordinates the physiological responses to stress with efferent information sent from the cortex. In a normotensive individual, the amygdala has been shown to modify sympathetic activity through direct projections to cardiovascular centers in the brainstem, such as the nucleus of the tractus solitarious (NTS) and the rostral ventrolateral medulla (RVLM) (111, 312, 314). Also, descending inhibition of parasympathetic activity by the amygdala facilitates stress-related

tachycardic responses (44, 111, 304, 314). Aside from modulating the autonomic system, the amygdala also interacts with the HPA axis to modulate glucococorticoid secretion (125, 218). Thus, the amygdala is poised to be a potentially important target site for mediating the abnormal autonomic responses typically observed in hypertensive patients when exposed to stress.

Interestingly, little is known regarding the role of the amygdala in the cardiovascular response to stress in a hypertensive individual. To date only a small number of studies have identified potential factors which may contribute to dysregulation of the amygdala in hypertension. For example, functional magnetic resonance imaging studies of humans diagnosed with stress-induced BP reactivity have identified evidence of hyperactivity in the amygdala during stress, a reduction in gray matter volume within the amygdala, and a stronger connectivity between the amygdala and cortical and brainstem regions (114). Additionally, histological evaluation of the brains of hypertensive patients who succumbed to heart disease demonstrated reduced number of corticotropin releasing hormone (CRH) neurons in the amygdala. These amygdaloid neurons represent one of the largest efferent populations of CRH-positive neurons outside of the hypothalamus and act to modify brainstem, pontine, and cortical neuronal function (124, 321, 372). In the SHR, lesioning of the central nucleus of the amygdala (CEA), the main output of the amygdala, has been shown to attenuate both the development of hypertension and the cardiovascular response to stress in the SHR (102, 110, 324). More recently, lesion of the medial nucleus of the amygdala (MEA) has also been shown to attenuate the development of hypertension in the SHR (107). Alternatively, direct stimulation of the amygdala does not generate any blood pressure or heart rate differences between the SHR and the normotensive Wistar-Kyoto rat (WKY), raising the possibility that the amygdala may be more important in regulating cardiovascular responses during challenging situations rather than during a basal state

(109). Accordingly, secretion of glutamate within the amygdala does not appear to differ between the SHR and WKY at rest or during baroreflex challenges, but glutamate levels were reported to be markedly elevated in the SHR during an acute stress compared to the WKY (349). Thus, there is some evidence to support the hypothesis that dysregulation of the amygdala in the SHR plays an important role in eliciting an exaggerated cardiovascular response to stress, however, the neurotransmitters involved in mediating this augmented response have yet to be elucidated.

Neuropeptide Y (NPY) is a novel neuropeptide that functions to regulate food intake, peripheral vasoconstriction, catecholamine secretion, and anxiety (69, 209, 404). Generally co-released with catecholamines, NPY has been shown to modulate several brain regions including the hypothalamus, hippocampus, amygdala and brainstem (49, 153, 216, 276, 331). The various actions of NPY are dependent upon the six receptor subtypes it can bind to, however in both behavioral and cardiovascular regulation during stress, most studies have focused on the function of the NPY receptor 1 (Y1) and the NPY receptor 2 (Y2). In the brain selective activation of Y1 receptors has been found to be the mediator of NPY-generated bradycardia and hypotension, while Y2 receptor activation elicits hypertension and tachycardia (5). An opposing action of these two NPY receptors on behavioral responses to stress has also been reported.

Originally hypothesized by Heilig et al., extrahypothalamic NPY is thought to be released during the latter phase of stress to oppose the actions of stress-induced CRH release in the amygdala (154, 319). The delayed secretion of NPY is thought to be a compensatory measure to reduce anxiety and emotionality during sustained stress. In fact, numerous studies have the discovered that not only does Y1 mediate NPY's antagonistic actions on CRH, but Y1 receptors appear to mediate NPY's general anxiolytic effect observed when administered during

behavioral stressors (31, 152, 155, 320, 404). On the other hand, Y2 receptors produce anxiogenic responses to stress by suppressing endogenous NPY release, thereby counteracting Y1's inherent anxiolytic nature (53, 186, 250, 317, 390). Overall, the cardiovascular and behavioral responses to stress may be highly dependent on the balance of Y1 and Y2 receptors activated by NPY. Furthermore, there is some evidence of dysregulation of NPY and its receptors in the SHR. For example, it has been reported that NPY mRNA expression in the arcuate nucleus of the hypothalamus is greater in the SHR compared to the WKY at rest, but is markedly reduced following restraint stress (196, 235, 374). Moreover, increased binding affinity and increased density of Y2 receptors in the dorsal medulla (NTS) of the SHR compared to the WKY has been identified, suggesting that autonomic dysregulation in the SHR may be due to Y2 domination in cardiovascular centers (6). Unfortunately, alterations in NPY function and the relative contribution of the different receptor subtypes to the exaggerated autonomic response to stress has not been previously explored in the amygdala of the SHR.

In addition to modulating CRH release, NPY has also been shown to modulate the stress response through its relationship with the γ-aminobutyric acid (GABA) neurons. For example, chronic GABA A receptor activation with diazapam has been shown to increase the Y1 mRNA expression in mice overexpressing Y1 (93, 255). Alternatively, Y2 activation suppresses GABA release by inhibiting voltage-gated calcium channels (369). Although, the interaction between GABA and NPY has not been investigated in the SHR, an alteration in the GABAergic system has been reported in several brain regions of the SHR. For example, neuronal firing following microinjection of a GABA A receptor antagonist in the hypothalamus is decreased in the SHR compared to the WKY, while GABA B receptor-mediated neuronal firing is increased (212). Additionally, GABA A receptors are reduced in the PVN in the SHR compared to the WKY

(200). Also, GABA secretion in the locus coeruleus (LC) of the SHR is reduced during a hypertensive challenge in the SHR compared to the WKY (174). Finally, GABA A receptors have been shown to be down regulated in the amygdala of the SHR compared to the WKY (200, 232). Thus, if NPY does not function appropriately in the amygdala of the SHR, it may be related to its relationship with GABA.

Current evidence has demonstrated that both NPY and GABA systems are dysregulated in SHR, however, their role in amygdala and descending regulation of cardiovascular responses to stress in the SHR has not been previously examined. Thus, the present study was undertaken to test the hypothesis that Y1 and GABA A receptor blockade in the CEA will modulate the cardiovascular response to acute stress in the SHR differently compared to a normotensive control, the Wistar. More specifically, based on our current knowledge of the functional interaction between the two systems it was hypothesized that modulation of GABA A and Y1 receptors would be less effective in modulating mean arterial pressure (MAP) and heart rate (HR) responses to acute exposure to air jet stress in the SHR compared to the Wistar. Additionally, we hypothesized that Y2 antagonism would induce the opposite effect generated by Y1 during acute stress. Preliminary data from this study has been presented (289).

Methods

Animals and Surgical Preparation

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee and followed the NIH guidelines. Male SHR and Wistar rats (10-12 weeks old; Charles River Laboratories, Wilmington, MA) were randomly assigned to one of two groups, vehicle control or drug-treatment, prior to instrumentation. Animals were given a subcutaneous injection of Rimadyl (0.1 mg/kg, Pfizer Animal Health, Exton, MD) and buprenorphine (0.1 mg/kg, Rickett Benckiser Pharmaceuticals, Inc., Richmond, VA) 20 min.

prior to the induction of anesthesia. Animals were then anesthetized to a surgical level (2-4% isoflurane+oxygen), secured in a stereotaxic headholder, and an incision was made along the midline of the skull. Stereotaxic coordinates used for cannula placement in the CEA were -2.4-2.6 mm distal from bregma and 4.5-4.6 mm lateral from midline according to Paxinos and Watson (281). Distal measurements for the SHR were adjusted according to the following equation ((lambda (mm) - bregma (mm))/9.0 mm) x 2.4mm=distance from bregma. Bilateral holes were drilled and guide cannulas (7.0-7.5 mm, Plastics One, Inc., Roanoke, Virginia) were lowered into the brain 0.5 mm above the CEA. Two additional holes were drilled in the skull for two stainless steel mounting screws (3/16 mm) and secured to the skull with dental acrylic (Lang Dental Manufacturing Co., Inc., Wheeling, IL). Dummy caps were placed into the cannulas to ensure patency and all incisions were closed and covered with topical antibiotic ointment (Pharmaderm, Melville, NY). The animals were on 100% oxygen for 5 minutes then allowed to recover on a heating pad. During the recovery period the animals were given a subcutaneous injection of saline (2-3 ml) for fluid replacement and an additional injection of buprinorphine (0.1 mg/kg) prior to returning their home cage.

Five to seven days following the first surgery, the animals were re-anesthetized and instrumented with arterial and venous catheters. Pre and post surgical procedures were the same as for the central brain cannulation. Briefly, following the induction of anesthesia to a surgical plane, an incision was made on the ventral surface of the hind limb, and the femoral vasculature was gently dissected from the surrounding connective tissue. Catheters (Braintree Scientific, Braintree, MA) filled with heparinized saline (100 IU/ml) were then placed into the femoral artery and vein directed toward the abdominal aorta. Catheters were plugged, tunneled subcutaneously exiting between the scapulae at the nape of the neck, and secured with sutures.

Animals were allowed 48 hours to recover prior to experimentation. During the day following catheterization, animals were brought to the lab to be weighed, handled, and acclimated to the experimental container. Acclimation included a 50-60 min. period of exposure to air noise (~75-80 decibels) while resting in the experimental chamber.

Experimental Protocol

On the day of the experiment, the exteriorized catheters were connected to PE 50 tubing containing heparinized saline (10-50 IU/ml) and the dummy cannula were removed from the guide cannula. The animals were then placed into the experimental container and the arterial catheter was connected to a pressure transducer (Stoelting Inc., Wood Dale, IL) for continuous measurement of pulsatile and mean arterial BP. The experimental chamber consisted of a circular container (15 cm radius) with three tubes (~0.5 cm diameter) inserted into the wall of the chamber, one tube every ~30 cm (equidistance around the circumference) at a height of approximately ~5 cm from the bottom of the chamber. The tubes were connected to a three-way valve manifold for regulation of the air stream through each tube.

Following a 60 min. baseline period the animals were randomly assigned to receive one of 3 possible solutions microinjected bilaterally (300 nl per side) into the CEA, including artificial cerebral spinal fluid (ACSF), 8.5, 17, or 51 nM GR231118, or 173 μM bicuculline methobromide (BIC). ACSF was made by dissolving 122 mM NaCl, 3 mM KCl, 31.74 mM $NaHCO_3$, and 1.3 mM $CaCl_2$ into deionized water (pH=7.4). GR231118, a NPY Y1 receptor antagonist (Tocris Biosciences, Ellisville, MI), was dissolved in ACSF. BIC (Tocris Biosciences, Ellisville, MI), a GABA A receptor antagonist, was also dissolved in ACSF. Concentrations were based on previously published studies (84, 122, 275).

Localized microinjections were made over 10 seconds using a 1 μl Hamilton syringe connected to PE 50 tubing attached to a 33 gauge internal injection cannula placed into the guide

cannula (8.0-8.3mm, Plastics 1, Inc., Roanoake, VA). Injections were made while the animals were left unrestrained in the testing chamber. The rats rested for 5 min. following the central injection of BIC or ACSF or 10 min. following GR231118 microinjection. During the final two min. of the post-injection waiting period, air noise, pressurized air directed away from the testing chamber, was turned on. After the two min. of noise, the pressurized air was switched to being directed into the chamber through one of the three inflow tubes, beginning the air jet stress (AJS) period. AJS included an initial 5 min. period of random AJS (continuous stream of air entering the chamber from a random direction and at random durations) immediately followed by a 15 min. of controlled AJS (the direction of the air flow was changed randomly every minute). Following the offset of AJS, rats were allowed to recover for 60 min.

All animals that had received either an ACSF or GR231118 microinjection during the first AJS period received a second central injection and underwent a second exposure to AJS. First injections of ACSF were followed by microinjection of GR231118 or BIC. Alternatively, first injections of GR231118 were followed by a second injection of 3 μM BIIE0246, a NPY Y2 receptor antagonist, dissolved in 10% DMSO (Tocris Biosciences, Ellisville, MI) (2). Similar to the first injection, 8 min. following the central injection, air noise was turned on for 2 min. and then at 10 min. post central injection the AJS protocol was repeated followed by a 30 min. recovery.

At the termination of the experiment, all animals were deeply anesthetized with an overdose of sodium pentobarbital (100-200 mg/kg; Ovation Pharmaceuticals, Inc., Dearfield, IL). 2% fluorogold (Fluorochrome, LLC., Denver, CO; 300nl) was injected bilaterally into the CEA to mark the injection sites. Brains were removed and soaked in 4% paraformaldehyde overnight. Prior to sectioning, a small cut was made along the rostral-caudal extent of the right

ventral surface of the brain to distinguish left and right sides. Next, the tissue was frozen and sectioned into 40 μm coronal slices.

Injection Site Analysis

Injection sites were imaged by a Zeiss Axioscope 2 (Carl Zeiss Microimaging, Inc., Thornwood, NY) connected to a Cool Snap camera (Photometrics, Tuscon, AZ) using QED Image software (Media Cybernetics, Inc, Bethseda MD). Two images were taken per animal: one fluorescent image of the fluorogold injection and a bright field image for anatomical identification to determine if the injection hit the CEA or missed. The CEA injection was considered a "hit" or accurate when the fluorogold injection was centralized into the CEA. Both bilateral and unilateral injections were pooled based on similar cardiovascular response characteristics. Central injections were considered "misses" when the fluorogold injection was above, lateral, and medial to the coordinates of the CEA.

Data Analysis

Following identification of whether the central injection site "hit" the CEA or missed, the results from the experiments in which the CEA was accurately targeted were grouped and analyzed for drug treatment effect. All responses to Y1 receptor blockade of different doses (8.8-51 nM) were combined based on similarities between responses. In addition, for the ACSF group within each strain, data from five "hits" in the CEA and two "misses' from the surrounding regions were grouped together due to the lack of a difference in response characteristics. Finally, a group of experiments in which the Y1 receptor antagonist missed the CEA was analyzed for each strain to identify the specificity of the drug effect into the CEA versus surrounding regions.

For all experiments identified for further analysis, HR was first derived from the interval between each successive systolic peak of pulsatile BP and then averaged in parallel with MAP

(Spike2 Software, Cambridge Electronic Design Limited, Cambridge, England). Four separate time periods were quantified, including a pre-injection baseline, a post injection baseline, the 20 min. AJS period, and a recovery time period. The pre-injection baseline period included a two min. average of MAP and HR taken 15-20 min. prior to the central injection. The post-injection baseline period was a two min. average taken two min. prior to the onset of air noise. During the 20 min. AJS period and the following recovery period, one min. averages of MAP and HR were used for analysis. The change in MAP (ΔMAP) and HR (ΔHR) relative to baseline was then calculated during and following the offset of AJS. In addition, to further evaluate changes in the response to AJS, the area under the curve (AUC) for ΔMAP and ΔHR for two five minute periods (Graphpad, Software, Inc., La Jolla, CA), including the initial (1-6 min.) and final period (15-20 min. see Figure 3-1) of AJS, were determined.

Statistical Analysis

First, to evaluate the effect of CEA injection on baseline MAP and HR, a two-way ANOVA with repeated measures within each strain was used to identify treatment (drug injected) and time (pre vs. post injection) effects (STATVIEW). In both the SHR and Wistar rats, the pre and post injection baselines did not demonstrate a significant interaction between time and treatment for either MAP ($P=0.9688$, SHR and $P=0.09505$, Wistar, respectively) or HR ($P=0.9179$, SHR, or $P=0.9813$, Wistar). Thus, for all Wistar and SHR groups both baselines were combined to generate a single pre-AJS average baseline. The effect of strain and treatment on the averaged pre-AJS baseline for MAP and HR was then evaluated using a two-way ANOVA.

Next, a two-way ANOVA with repeated measures was used to identify significant differences between strains following ACSF microinjection during AJS. A one-way ANOVA

was used to identify the effect of strain on both the initial and final stress response for both ΔMAP and ΔHR following ACSF microinjection.

The effect of drug treatment on the ΔMAP and ΔHR within strains was evaluated using a two-way ANOVA with repeated measures. Prior to this analysis, data from the first and second injections for the SHR-Y1 (first, n=3 and second, n=3), SHR-BIC (first, n=2 and second, n=2) and Wistar-BIC (first, n=2 and second, n=2) treatment groups were combined. Justification for the consolidation of data from both first and second injections was based on an ANOVA with repeated measures was of the SHR-Y1 data, which contained the largest number of animals/group and identified no significant differences for the ΔMAP ($P=0.9349$). In contrast, for the delta HR, there was a significant interaction between time and injection in the SHR-Y1 group ($P=0.0054$). Further analysis, however, identified that the only time points which were significantly different for HR between the first and second injections were limited to the first three min. of AJS ($P<0.0293$). To identify whether this initial difference in the ΔHR would translate to the AUC measurement, a one-way ANOVA was performed and there was no significant difference in AUC for ΔHR between first and second injections of GR231118 in the SHR during the initial period of stress ($P=0.0822$). As a result, the first and second injections for all groups mentioned above were combined.

The recovery periods for ΔMAP and ΔHR between treatments were analyzed separately (time point at 5 and 10 min. post stress) using either a two way ANOVA. Finally, a two-way ANOVA was used to evaluate the effect of strain and drug treatment on both the initial and the final ΔMAP and ΔHR AUC during AJS stress. Whenever indicated, a one-way ANOVA was used to identify the effect of treatment or strain and a post hoc Scheffe test for multiple

comparisons was employed. All data are represented as mean±SEM and statistical significance was set at $P<0.05$.

Results

The Cardiovascular Response to AJS

The average cardiovascular response to AJS for both the SHR (n=7) and the Wistar rats (n=7) following ACSF microinjection into the amygdala is displayed in Fig3-1A and B. A two-way ANOVA with repeated measures identified a significant interaction between strain and time ($P<0.0001$) for both MAP and HR. Post hoc analysis identified that for all time points, the SHR demonstrated a significantly higher MAP compared to the Wistar ($P<0.0001$). HR also demonstrated a significant interaction between strain and time ($P<0.0001$). Both strains demonstrated similar increases in HR during the onset of AJS, but by the fourth min. of AJS, HR of the Wistar rats began to decline, while HR in the SHRs remained steady. Consequently, HR was significantly greater in the SHR versus the Wistar rats between the fourth and seventh min. of AJS ($P<0.0354$). As AJS continued, HR in the SHR began to decline and HR was not significantly different between strains at any other time point during AJS. At the offset of AJS, HR in the SHR returned to baseline quickly. In contrast, HR in the Wistar rats remained elevated, and as a consequence during the initial part of (min. 1-4) of the recovery period, HR in the Wistar was significantly elevated compared to the SHR ($P<0.0263$).

To determine if AJS generated a significant difference in the absolute rise of MAP and HR from baseline during either the initial or final periods of AJS period between strains, the AUC for the ΔMAP and ΔHR was analyzed during both the initial and final 5 min. of AJS (illustrated by gray bars in Figure 3-1A and B). As shown in Figure 3-1C, during both the initial and the final period of AJS, the AUC for ΔMAP was significantly greater in the SHR compared to the Wistar ($P=0.0004$ and $P=0.0526$, respectively). Similarly, the AUC for the ΔHR was

significantly elevated in the SHR compared to the Wistar rats during the initial AJS period (P=0.0044), however, no significant difference between strains was identified in the AUC for ΔHR during the final period of AJS.

Injection Sites with the CEA

The inset in Figure 3-2B illustrates the location of the CEA relative to the other subnuclei of the amygdala. For determining placement of injection, an example of the spread of a fluorogold microinjection placed into the CEA is exhibited in Figure 3-2C. Accurate and missed injections used for the subsequent cardiovascular analysis are displayed throughout the rostral-caudal extent of the amygdala with injections for the SHR illustrated on the left and for the Wistar, injections pictured on the right, for illustration purposes only. (Fig. 3-2A). For ACSF groups, both accurate and missed injections are displayed in yellow squares (n=7/strain, see methods for description of analysis). Although combined for cardiovascular analysis, first and second injections are displayed separately (orange circles=BIC (n=2/strain), green circles=GR231118 (SHR, n=3; Wistar, 5/strain), and gray squares=GR231118 (SHR, n=3) or BIC second injection (n=2/strain), following ACSF. Finally, missed GR231118 injections are represented by black squares (SHR, n=6; Wistar, n=8).

Average Baseline Comparison between Strains

In Table 3-1, a two-way ANOVA was used to compare strain and treatment for the average MAP and HR baselines. A significant strain effect was found for both MAP and HR ($P<0.0001$ and $P=0.0473$, respectfully), but no significant interaction between treatment and strain was identified. For all treatment groups combined, the SHR exhibited a significantly higher baseline MAP compared to the Wistar ($P<0.0001$). On the other hand, the Wistar demonstrated a higher baseline HR compared to the SHR for all treatment groups ($P=0.0123$).

The Effect of Y1 Receptor Antagonism on the Cardiovascular Response to AJS

Figure 3-3 demonstrates the impact of microinjection of the Y1 receptor antagonist GR231118 into the CEA on the cardiovascular response to AJS in the SHR (n=6) versus Wistar rats (n=5). In the SHR, Y1 receptor blockade in the CEA showed a trend to reduce both the ΔMAP and the ΔHR during AJS compared to ACSF microinjection. A two-way ANOVA with repeated measures identified a significant interaction between time and treatment for both ΔMAP and ΔHR (Fig. 3A, P=0.0255 and P=0.0455, respectively), however, the treatment effect alone was not significant (P=0.2159 and P=0.1299, respectfully). Further analysis of the interaction effect between treatment and time only identified significant differences between ACSF and Y1 receptor blockade at the 4-5 min. time point for the ΔMAP (P=0.0501) and at the 3-4 and 13-14 min. time point for the ΔHR (P=0.0516 and P=0.0321).

Alternatively, microinjection of GR231118 into the CEA of the Wistar had the opposite effect and tended to augment the ΔMAP and ΔHR during AJS. A two-way ANOVA with repeated measures did not identify any significant interaction between time and treatment following GR231118 microinjection for either ΔMAP or ΔHR in the Wistars. There was, however, a significant effect of treatment for ΔHR (P=0.0486). The treatment effect for ΔMAP was not significant (Fig. 3B, P=0.3742).

During the recovery period there was no significant treatment effect or interaction of time and treatment for either the SHR or the Wistar groups.

The Effect of GABA A Receptor Antagonism on the Cardiovascular Response to AJS

As shown in Figure 3-4, administration of BIC into the CEA induced an even greater attenuation of ΔMAP and ΔHR during AJS in the SHR (n=4) relative to Y1 receptor antagonism that was particularly prominent during the initial period of AJS exposure. A two-way ANOVA with repeated measures identified both a significant treatment effect (Fig. 3-4A, P=0.0063) and

interaction for ΔHR (P=0.0029). For the ΔMAP there was only a significant interaction between treatment and time (P=0.0255). Post-hoc analysis demonstrated that BIC significantly attenuated the ΔMAP (P<0.0215) and ΔHR (P<0.0397) response to AJS during the first 8-10 min. of AJS compared to ACSF microinjection.

In contrast, in the Wistar rats, microinjection of BIC into the CEA did not appear to change the ΔMAP response to AJS (n=4), while the ΔHR tended to increase (Fig. 3-4B). A two-way ANOVA with repeated measures demonstrated no significant treatment effect for either ΔMAP (P=0.9379) and ΔHR (P=0.2353) during AJS in the Wistar rats. Moreover, no interaction between time and treatment was identified for either ΔMAP or ΔHR (P=0.9937 and P=0.2075, respectfully).

During the recovery period, the ΔHR in the Wistar rats did demonstrated a significant treatment effect (P=0.0142). Nevertheless, post hoc analysis determined the treatment effect for the Wistar ΔHR only tended to be greater in BIC group (P=0.0824) during recovery compared to ACSF. No significant treatment effects or interaction between treatment and time during the recovery period were identified for ΔMAP in the Wistar (P=0.8911) or for ΔMAP and ΔHR (P>0.0654 and P>0.2893) in the SHR.

The Effect of Y1/Y2 Receptor Antagonism on the Cardiovascular Response to AJS

Finally, the effect of Y1/Y2 receptor blockade was evaluated. As shown in Figure 3-5A, microinjection of the Y2 receptor antagonist BIIE0246 into the CEA one hour following microinjection of the Y1 receptor antagonist in the SHR (n=3) appeared to attenuate the initial ΔHR response to AJS, but did not markedly alter the ΔMAP response compared to ACSF. A two-way ANOVA, identified a significant interaction between time and treatment in the ΔHR of the SHR (P=0.0159). Yet, post hoc analysis only identified a significant attenuation of the ΔHR at the 1-2 min. time point during the initial period of AJS (P=0.0295), an effect that may have

been due to the impact of a second injection (see Data Analysis section). No treatment effect or interaction between GR231118/BIIE0246 treatment and time was identified the ΔMAP (P>0. 2887) during AJS in the SHR.

In contrast, GR231118/BIIE0246 microinjection into the CEA tended to augment the response to AJS in the Wistar rats (Fig. 3-5B, n=4). Indeed, a two-way ANOVA with repeated measures demonstrated a significant increase in ΔMAP during AJS following Y1/Y2 receptor blockade (treatment effect; P=0.0001). There was, however, no significant interaction between treatment and time for the ΔMAP during AJS (P=0.2887). Alternatively, Y1/Y2 receptor blockade increased ΔHR during AJS in the Wistar and a significant interaction between treatment and time (P=0.054) was identified, but the treatment effect was not significant (Fig, 3-5B, P=0.0617). Further analysis of the interaction between treatment and time for ΔHR determined a significant effect at min. 4-6, 8-9, and 13-17 during AJS in the Wistar (P<0.0523).

The recovery period for both the SHR and Wistar did not demonstrate a treatment effect for either ΔMAP or ΔHR (P>0.1208 and P>0.0602, respectfully). Similarly, neither strain demonstrated a significant interaction of time and treatment during the recovery period for either ΔMAP or ΔHR (P>0.6513 and P>0.2715).

Comparison of Missed Drug Injections and ACSF Controls

To confirm the specificity of drug action within the CEA, microinjections of GR231118 that were identified post hoc to be outside of the CEA, or "misses," were analyzed as a group. As shown in Figure 3-6, the missed injections did not elicit a change in the ΔMAP during AJS in the SHR (n= 6; P>0.4666). In contrast, the SHR-Missed Injection group did appear to sustain a greater ΔHR during the final period of stress compared to the ACSF injected animals. A two-way ANOVA with repeated measures identified a significant interaction between time and

treatment (P=0.0138) and further analysis identified that the missed injection of the Y1 receptor antagonist elicited a significantly greater ΔHR during the last 6 min. of AJS (P<0.0531).

Similarly, microinjections made outside of the CEA in Wistar rats did not induce any a significant treatment effect on ΔMAP during AJS (n= 8; Fig. 3-6B, P=0.4844) or a significant interaction between time and treatment (P=0.1166). There was also no significant interaction between time and treatment for the ΔHR (P=0.9039). Yet, a significant effect of the missed injections on the ΔHR during AJS was evident in the Wistar (treatment effect, P<0.0001). During the recovery period, however, there was no significant interaction between time and treatment or treatment effect alone for either strain for ΔMAP or ΔHR (P>0.125 and P>0.66, respectfully).

Effect of Hypertension on the Cardiovascular Response to AJS During GABA A and NPY Y1 Receptor Blockade

To evaluate the impact of the various treatments between strains on the cardiovascular response to AJS, the AUC for ΔMAP and ΔHR was compared between SHR and Wistar rats following microinjection of ACSF, GR231118, or BIC into the amygdala. As shown in Figure 3-7A, a two-way ANOVA of the AUC for ΔMAP and ΔHR during the initial phase of AJS identified a significant interaction between strain and treatment (P=0.0528 and P=0.0472, respectfully). Further analysis identified a significant difference between SHR and Wistar-ACSF groups (P=0.0004) and between Wistar-ASCF and SHR-GR231118 (p=0.0198) groups for AUC ΔMAP. Likewise AUC ΔHR for the SHR-ASCF group was found to be significantly greater during the initial part of stress compared to the Wistar-ACSF group (P=0.0044). In contrast, analysis of the final 5 min of AJS for AUC ΔMAP and AUC ΔHR did not identify any significant interactions between strain and treatment (P=0.5017 and P=0.0836, respectively). Yet, AUC ΔHR did display a significant treatment effect (p=0.0166), which identified that

administration of GR231118 into the CEA tended to increase the AUC ΔHR during the last 5 min of AJS independent of strain.

Finally, Figure 8 illustrates the impact of GABA A receptor blockade on the AUC for ΔMAP and ΔHR. A two-way ANOVA of the AUC for ΔMAP and ΔHR during the initial phase of AJS identified a significant interaction between strain and treatment (P=0.0013 and P=0.0130, respectfully). Similar to the pattern described above, further analysis identified a significant difference between the SHR and Wistar following ACSF microinjection for both AUC ΔMAP and AUC ΔHR ($p<0.0044$). Administration of BIC also significantly reduced both the AUC ΔMAP (P=0.0023) and AUC ΔHR (P=0.0015) in the SHR during the initial phase of stress and eliminated differences in both the AUC ΔMAP and the AUC ΔHR between strains. Analysis of the final period of AJS for AUC ΔMAP did not identify any significant interactions between strain and treatment (P=0.5141). In contrast, analysis of AUC for ΔHR did identify a significant interaction (P=0.0417) between strain and treatment, but further analysis did not identify any significant individual differences (P>0.1162).

Discussion

Extensive evidence has indicated that abnormal regulation of both the NPY and GABAergic systems within the brainstem and hypothalamus of the SHR contributed to autonomic dysregulation in this animal model of essential hypertension. The current study demonstrates for the first time that an alteration of both of these systems extends into the amygdala of the SHR and has a substantial influence on the cardiovascular response to stress. Similar to restraint and other stressors, our model of AJS generated an exaggerated cardiovascular response to stress in the SHR compared to the normotensive Wistar (233). Previous research had demonstrated that central activation of Y1 receptors causes hypotension and bradycardia, so we predicted that the blockade of Y1 would cause the opposite response

during stress in both strains (5). Contrary to our hypothesis, Y1 blockade within the CEA caused a slight attenuation of both MAP and HR during AJS in the SHR. Meanwhile, the Wistar demonstrated a slight increase in MAP and HR implying that Y1 receptors modulate the cardiovascular response to stress differently in a hypertensive state. Administration of a Y2 antagonist also produced a similar response in the HR of SHR, but increased both MAP and HR in the Wistar indicating that all functional regulation of NPY receptors may be altered in the SHR. Similarly, blockade of GABA A receptors generally causes an increase in MAP and HR when injected into autonomic control regions of the brain, however, administration of BIC into the CEA resulted in an impressive attenuation of both MAP and HR in the SHR compared to the augmentation of HR in the Wistar (122). The decreased cardiovascular responses in the SHR following Y1 and GABA A blockade in the CEA provide compelling evidence that both systems are modified by hypertension and contribute to exaggerates autonomic responses to stress.

Effect of Y1 blockade on the cardiovascular response to AJS. Both ICV administration and microinjection into the NTS of Y1 agonist have been shown to induce hypotension, bradycardia, and reduced anxiety responses to stress in the normotensive rat (5, 188, 252, 422). Similarly, a reduction in MAP and HR following administration of Y1 agonist ICV or into the NTS was demonstrated in the SHR, however, it was attenuated compared to the WKY (6, 379). In the present study, administration of a Y1 antagonist in the CEA of the normotensive Wistar rats also induced an increase in both MAP and HR supporting our original hypothesis that blocking Y1 receptors would lead to an increase in the cardiovascular response to stress. In contrast, Y1 receptor blockade in the SHR caused a slight reduction in both MAP and HR during AJS. Although information is limited regarding the role of Y1 in the CEA in the cardiovascular response to stress in the SHR, several possible reasons may account for the

GR23118-mediated attenuation of the cardiovascular response we have observed. First, endogenous NPY levels in the brain are decreased in the brain of the SHR with age compared to the normotensive rat (219, 279). Furthermore, the Y1 receptors demonstrate a reduction in binding in the NTS indicating an inability for NPY to inhibit sympathetic activity in the SHR (6). Thus, due to the altered functionality of the Y1 receptor, the Y1 antagonist may have granted other NPY receptors to become activated during the release of endogenous NPY into the CEA in response to the stress, leading to the reduction in the cardiovascular responses seen in the SHR.

In opposition to Y1 depressor-mediated responses, the Y2 has been regarded as a playing a more major role in the regulation of sympathetic activity in hypertension due to it's increased binding activity, increased mRNA expression in models of hypertension, increased binding affinity to the C-terminus of NPY and NPY fragments, and ability to autoregulate NPY secretion and other neurotransmitters (6, 37, 52, 226, 240, 347, 369). Activation of Y2 receptors in the NTS leads to a pressor response in both normotensive and hypertensive models which overrides any Y1-mediated depressor response (5, 422). Additionally, increased density of Y2 in the dorsal vagal complex and evidence that Y2 blockade prevents NPY-mediated bradycardia suggests Y2 is the dominate modulator of parasympathetic regulation of HR (235, 355). Meanwhile, functional interaction with Y1, Y2 and Y5 has been identified in regulating calcium release in the hippocampus with Y2 taking on the principle role (346). Our study tested whether the Y2 blockade in the CEA following a first injection of GR231118, would generate a reduction in the cardiovascular response during AJS. In the SHR, BIIE0246 caused only a slight attenuation of MAP during the initial period of stress while HR demonstrated a significant reduction. In contrast, the rise in MAP and HR during AJS following BIIE0246 injection is increased in the Wistar. It is possible that by eliminating Y2 suppression of NPY, it allows for

more NPY secretion and a reduction in the cardiovascular response. Nevertheless, if NPY levels are lower in the SHR, this may explain the small reduction in MAP and HR following Y2 blockade (219). Thus, it is possible that the different effects of both Y1 and Y2 blockade on the cardiovascular response in the SHR and Wistar may be due to an alteration in NPY receptors.

Another plausible theory implicates the relationship between NPY and norepinephrine (NE). In both the peripheral and the central nervous system, NPY is co-released with NE to modify its actions (87, 224, 331). In the SHR, NE levels are reduced with aging, however, the release of NE remains the same in the hypothalamus during immobilization stress (383). Similarly, NE release during territiorial stress is increased in both the NTS and amygdala of the SHR (89). In a slice preparation, NPY's ability to reduce NE release following stimulation of the PVN was attenuated suggesting an inadequacy to inhibit sympathetic nerve activity in the SHR (392). Additionally, it has been demonstrated that increased catecholamine levels were not affected by injection of NPY into the PVN of the SHR compared to a reduction in the Wistar (415). Interestingly, an intriguing antagonist relationship between NPY and α2 adrenergic receptors has been implicated in the NTS in which Y1 and Y2 prevent α2 depressor responses in the normotensive rat (421). Yet, in the SHR, the reduced density of α2 receptors in conjunction with low NE in the brainstem may contribute to the development of hypertension (162, 413, 419, 424). Furthermore, ICV administration of an α2 agonist reduced baseline MAP and HR in the SHR, however, it did not reduce MAP, HR, or mesenteric vascular resistance in the SHR during AJS (177). Also, anatomical evidence has demonstrated that α2 receptors are on neurons that project from the CEA to the dorsal vagal complex implicating a possible pathway to regulate HR (116). The above evidence suggests that blockade of NPY receptors in amygdala allows for

activation of α2 receptors causing a decrease in MAP and HR during stress and this response is augmented in the SHR.

The effects of GABA A blockade on the cardiovascular response to AJS. Since many of the effects of NPY are mediated through modulation of GABA, in the present study we also evaluated the effect of GABA receptor blockade in the CEA. In general, central administration of the GABA A receptor antagonist, BIC, into the hypothalamus and brainstem triggers increases in both MAP and HR. The cardiovascular and neuroendocrine response to BIC, specifically in the dorsal medial hypothalamus (DMH), has been described as a similar response to stress in both anesthetized and conscious rat (17, 81, 182). The augmented cardiovascular response produce by BIC has also been previously described in microinjection experiments into the BLA, but not in the CEA (122, 325). There is also evidence that excitatory neurotransmitter, glutamate (GLU), is involved in GABA A inhibition (318, 359). Although, BIC microinjection has been reported to not affect the depressor response caused by glutamate stimulation in the CEA of an anesthetized Wistar, our study demonstrated an attenuation of stress-induced increases in MAP and HR in response to BIC administration into the CEA of the SHR, while the HR response in the Wistar rats was increased (51). Thus, it is apparent that a strain difference exists in the cardiovascular regulation by the amygdala during stress.

Several studies point toward reasons why a reduction in cardiovascular response AJS following GABA A blockade in the CEA was observed in the SHR. First, there is evidence that GABA secretion in the amygdala does not change in response to noise stress, but causes increases in GLU release in the SHR, suggesting an alteration in the regulation of GABA in the SHR compared to the Wistar (349). This notion coupled with evidence demonstrating a lower GABA A receptor binding capacity in the amygdala and hypothalamus of the SHR, suggesting a

reduced inability to inhibit emotional and autonomic responses to stress (200). Additionally, low GABA turnover rates in the hypothalamus and medulla unifies the theory that alterations in the GABAergic system contributes to the increased sympathetic activity in the SHR (329). Finally, a compensatory gain of control by GABA B receptors has been indicated in the SHR. For example, increased GABA B expression has been exhibited in the NTS of the SHR (361). In the PVN, GABA B receptors are also upregulated post synaptically. Furthermore, presynaptic GABA B receptors enhance glutamate and attenuate GABA transmission in the SHR (212). MAP and HR was also found to be reduced following microinjection of a GABA B receptor agonist into the hypothalamus in the SHR (378). Moreover, there is evidence in that activation of GABA B receptors reduces excitatory and inhibitory transmission in the BLA of a normotensive rat, however, there is limited information regarding the role of GABA B receptors in the amygdala of the SHR (418). The increased activity of GABA B receptors in the SHR may have generated the attenuation in the cardiovascular response to stress presented in the current study.

GABA and NPY have also been demonstrated to interact with each other. Anatomical evidence has demonstrated co-labeling with NPY and GABA in neurons within the cortex, amygdala, and NTS (14, 159, 232, 284). Y1 receptors have also been found to have an anatomical interaction with GABA neurons in the amygdala (92, 256). In fact, Y1/LacZ transgenic mice demonstrated increased expression of Y1 during chronic activation of GABA A receptors in the amygdala (255). NPY also was found to reverse GABA A receptor inhibition in the electrically stimulated hypothalamus, however, the effect was not specific to NPY receptor (293). Yet, in the thalamus, Y1 receptor activation reduces excitability of neurons while Y2 downregulates GABA release by inhibiting calcium influx (369). Also, Y2 activation has been

found to depress GABA transmission (178). GABA A agonist, dexamethasone, reduced NPY elicited cardiovascular responses in the NTS and this effect was blocked by BIC (262). To date, the interaction between GABA and NPY in the CEA has not been identified in the SHR, but may explain the attenuation in the cardiovascular response to AJS caused by GABA A or Y1 blockade demonstrated in the current study.

The effects of Y1 and GABA A receptor antagonism on "normalizing" the cardiovascular response to AJS in the SHR. The current results have uncovered compelling evidence that the exaggerated cardiovascular response in the SHR may partly be due to dysfunction of the inhibitory regulation within the CEA. Specifically, both Y1 receptor and GABA A blockade in the CEA attenuated both the ΔMAP and ΔHR AUC during the initial period of AJS in the SHR while the reverse response occurred in the Wistar. In fact, GABA A antagonism magnified the reduction of the cardiovascular response to the point that the SHR resembled the Wistar's response to AJS. The normalization of the cardiovascular response to stress may be related to the connections the amygdala has with brain regions regulating baroreflex information. For example, information from both chemoreceptors and baroreceptors is relayed to the CEA through the PBN (44, 45). In turn, the amygdala sends information to the NTS, RVLM, and dorsal vagal complex to modulate autonomic regulation of the cardiovascular system (128, 314, 401). Furthermore, the amygdala has been implicated in regulating the increased sensitivity to baroreflex suppression in the SHR during arousal which is reversed following lesioning of the amygdala (190). By blocking Y1 or GABA A receptors in the amygdala, it is possible the CEA regulation over baroreflex suppression was enhanced in the SHR returning the cardiovascular response to stress to a normotensive response. Furthermore, the resulting consequences of the blockade may also indicate dysfunction within the GABA

interneurons in the amygdala leading to disinhibition of cardiovascular centers during stress in the SHR.

Methodological Considerations. Three methodological considerations need to be made in regards to the results of the current study. The Y1 antagonist used, GR231118, has also been indicated as an agonist for Y4 receptors (275, 332). Concentration of Y4 receptors is mainly located in the hypothalamus and brainstem, however, low levels of Y4 have been indicated in the amygdala (99, 381, 408). Y4 KO mice also express reduced vasoconstriction and vagal inhibitory activity in regards to NPY in the periphery indicating its role in regulating sympathetic activity (354). Furthermore, Y4 KO mice also demonstrated in reduction in typical increases in motor activity, anxiety, and depression (265, 381). Although, Y4 also has higher affinity for pancreatic peptide y compared to NPY, it is possible that activation of the Y4 agonist may have contributed to the attenuated cardiovascular response demonstrated in the SHR during AJS (388). Secondly, the SHR-Missed Inj group did not generate any larger differences in both MAP and HR compared to the SHR-ACSF group. In contrast, the W-Missed Inj group demonstrated a significant increase in HR. The noted location of the misses tended to be located in the basal ganglia, specifically caudate putamen and globus pallidus. In fact, NPY neurons have been found in sporadic locations in the basal ganglia with Y1 being specifically found in the caudate putamen (48, 357, 414). Additionally, NPY levels are increased in the caudate-putamen during social isolation and the elevated plus maze (385). In general, the basal ganglia deals with motor control, however, parts of the basal ganglia interact with the cortex and limbic system to contribute to emotional behaviors. The spread of the Y1 antagonist in the W-Missed Inj may have modulated NPY activity in the basal ganglia allowing for subsequent disinhibition of downstream brain regions regulating heart rate. Stimulation of the basal ganglia could also

lead to increased motor activity generating a compensatory increase in HR in the Wistar. Finally, the significantly high baseline HR in the W may be due to a strain difference, since baseline HR in the SHR is generally low. It also could be due to the increased baroreceptor reactivity in the Wistar compared to the SHR (20). Overall, the regulation of HR appears to be sensitive in the Wistar during both resting and stressful challenges

Summary. The current study demonstrated a strain difference in the effects of Y1 and GABA A blockade in the CEA during acute stress. The attenuation of the cardiovascular response to stress in the SHR by both GR231118 and BIC was opposite of what we hypothesized. Consequently, our results provide compelling evidence that a dysregulation of both the GABA and NPY receptors in the CEA may lead to further disinhibition in cardiovascular centers resulting in an exaggerated cardiovascular response to stress in the SHR. Further studies are required to determine if the attenuation of the cardiovascular response may be dependent on GABA B activation or due to an alteration in the release of NE, since NPY interacted with both these systems. The mechanisms driving hypersensitivity to acute stressors in hypertensive individuals is complex and appears to be orchestrated by an intermingling of several neurotransmitters and their receptors with the CEA acting as one of the central brain regions involved in coordinating these abnormal responses.

Table 3-1. Average MAP and HR.

Strain	Treatment	MAP*	HR*
SHR	ACSF	170±3	377±12
	GR231118	173±2	359±7
	Bicuculline	176±3	368±8
	GR231118+ BIEE0246	172±6	371±24
	Missed Inj.	170±7	363±13
Wistar	ACSF	119±2	372±9
	GR231118	123±4	377±12
	Bicuculline	119±2	390±17
	GR231118+ BIIE0246	122±5	355±6
	Missed Inj.	123±2	390±6

*P<with respect to strain differences between all groups.

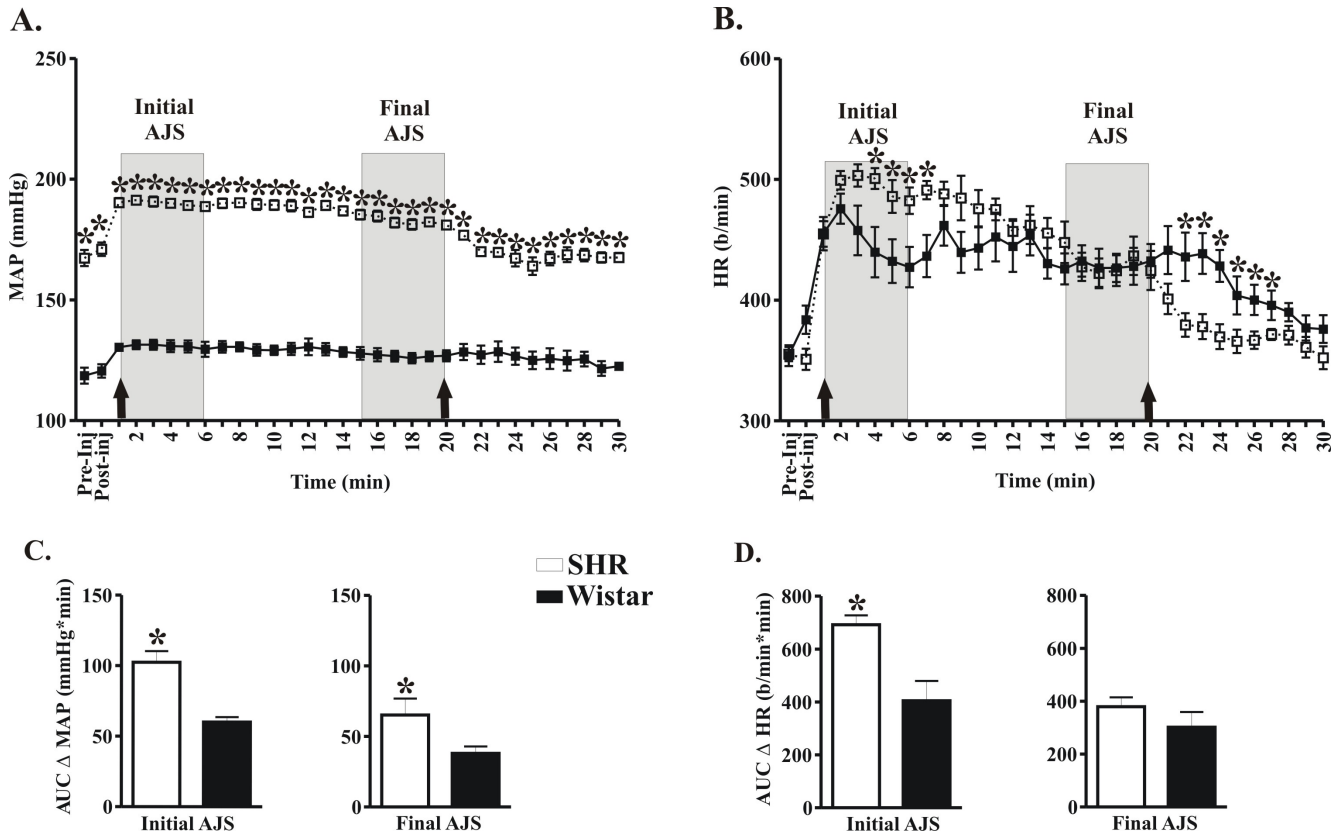

Figure 3-1. Average cardiovascular response to air jet stress (AJS) in the SHR versus the Wistar rat. Average A) MAP and B) HR responses before, during, and after exposure to 20 min. of AJS for both the SHR (n=7) and Wistar rats (n=7). Black arrows represent beginning and end of AJS exposure. Pre injection (Pre-Inj) baseline was taken approximately 15-20 prior to ACSF injection into the amygdala. Post injection baseline (Post-Inj) 4 min. prior to the onset of AJS. Vertical gray bars designate the initial and final period during AJS taken for area under the curve (AUC) analysis. C) Comparison of the change in MAP from baseline AUC during the initial and final stage of AJS. D) Comparison of the change in HR from baseline AUC during the initial and final stage of AJS. *P<0.05 represents significant difference between SHR and Wistar values.

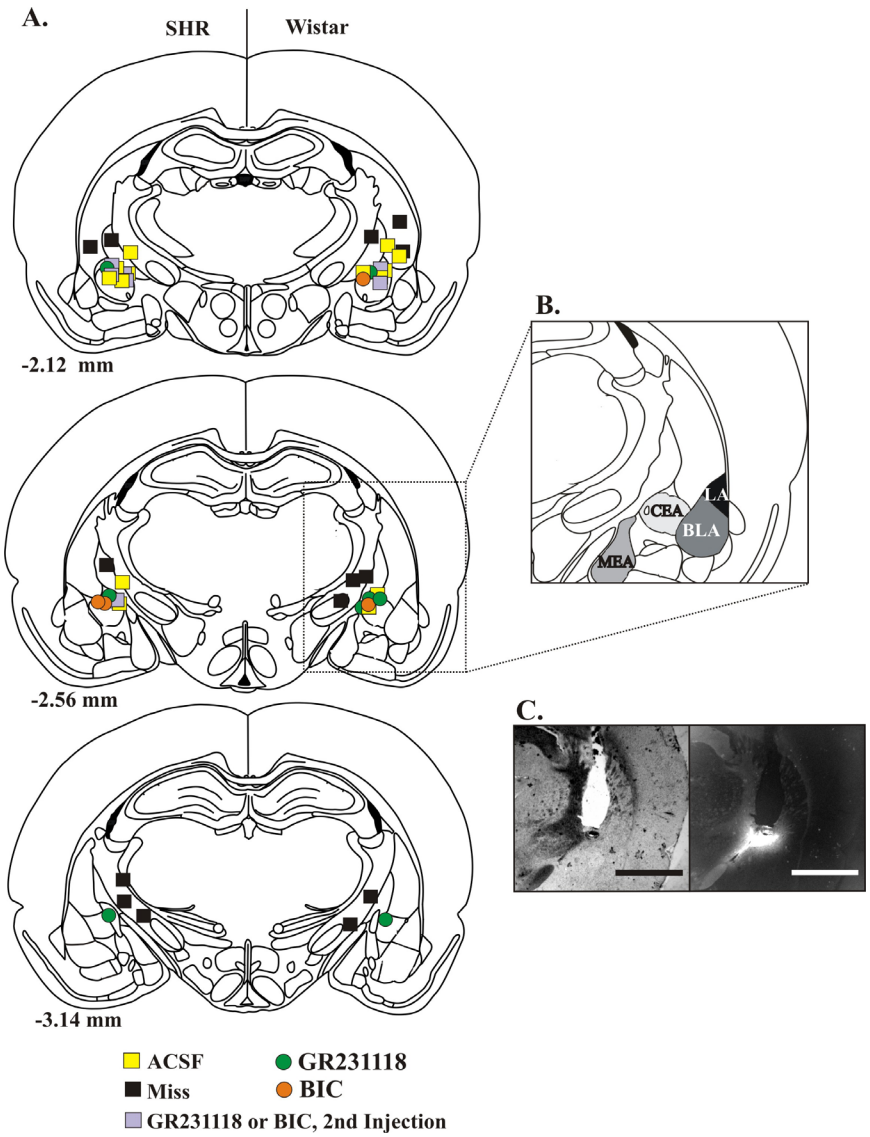

Figure 3-2. Reconstruction of microinjection sites within the amygdala. A) Rostral to caudal extent of recovered injection sites made in the central nucleus of the amygdala (CEA) and surrounding subnuclei of the amygdala. Sites marked on the left represent SHR microinjections locations. Sites marked on the right represent Wistar microinjection locations. Yellow squares represent ACSF injections. Green circles represent first GR231118 injections. Orange circle represents BIC injections. Light gray squares reflect a second injection of GR231118 or BIC following ACSF. Black squares represent missed injections. B) Enlarged illustration of the amygdala complex designating the location of the CEA, MEA, BLA, and LA. C) Representation of an injection into the CEA by both a bright field image for anatomical designation (left panel) and a fluorescent image depicting the spread of fluorogold (right panel). White bar represents 1000 microns. MEA, medial nucleus of the amygdala; LA, lateral nucleus of the amygdala, BLA, basolateral nucleus of the amygdala; BIC, bicuculline; ACSF, artificial cerebral spinal fluid.

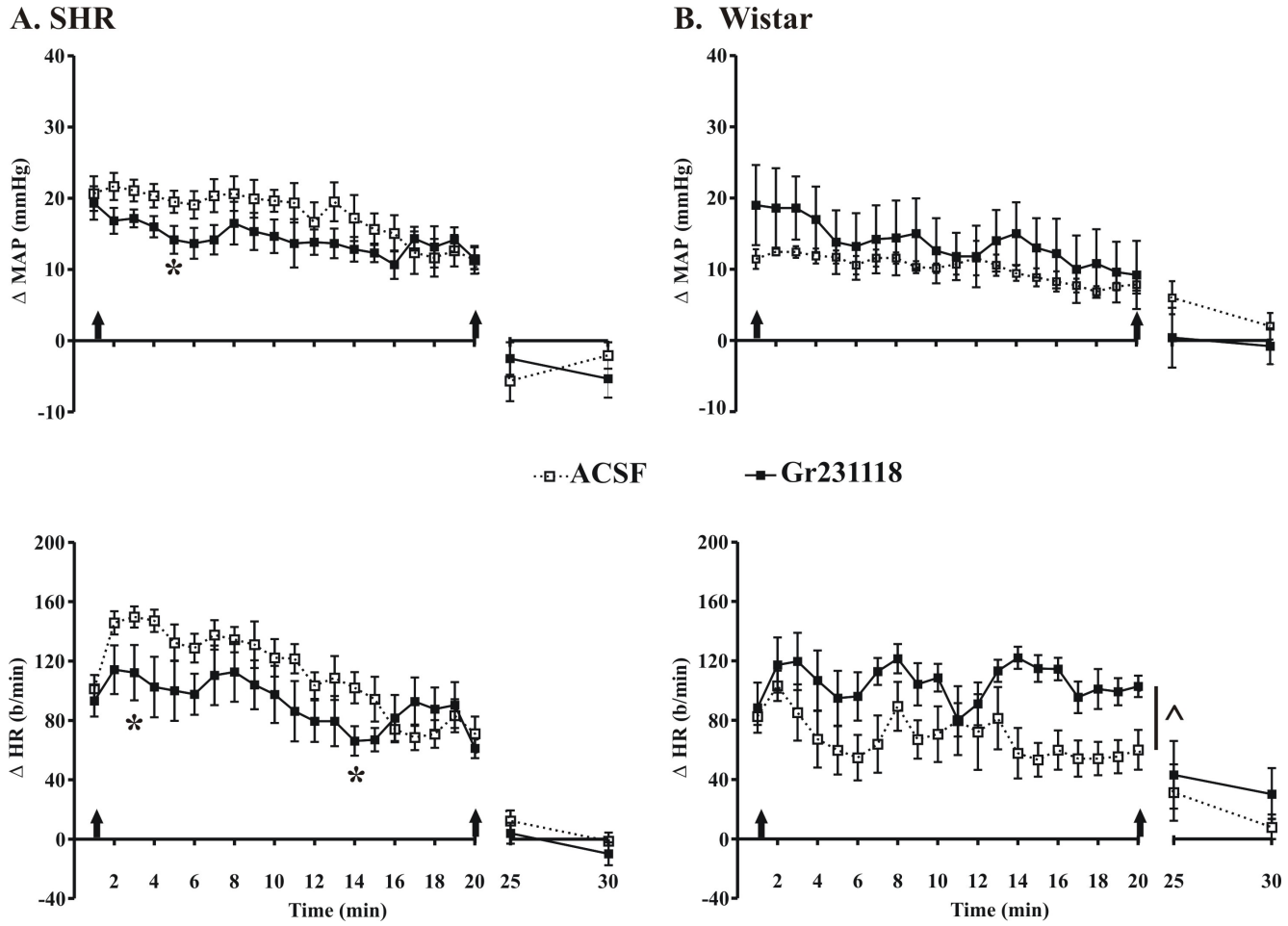

Figure 3-3. Effect of microinjection of GR231118, a Y1 receptor antagonist, into the CEA on the cardiovascular response to AJS. A) ΔMAP and B) ΔHR for the SHR (n=6) and Wistar (n=8) rats administered GR231118 or ACSF (n=7/strain) or GR231118 prior to AJS. Black arrows designate the AJS period. The recovery period is represented by two time points following the offset of AJS (25 and 30 min.). *P<0.05 represents significant difference between treatment groups with a time interaction. ^P<0.05 represents significant treatment effect.

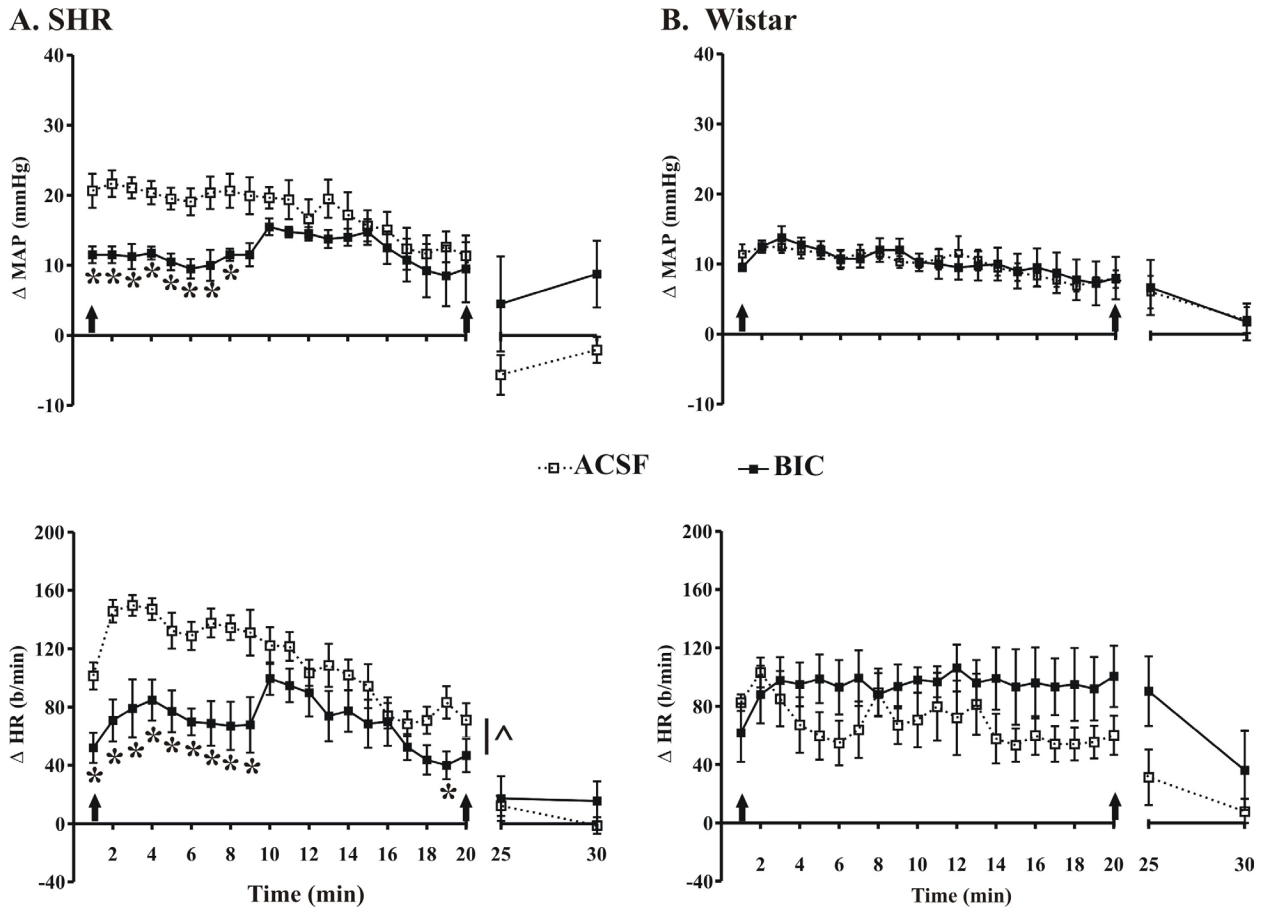

Figure 3-4. Effect of microinjection of bicuculline (BIC) into the CEA on the cardiovascular response to AJS. A) ΔMAP and B) ΔHR for the SHR and Wistar rats administered BIC (n=4/strain) and ACSF (n=7/strain) prior to AJS. Black arrows designate the AJS period. The recovery period is represented by two time points following the offset of AJS (25 and 30 min.). *P<0.05 represents significant difference between treatment groups with a time interaction. ^P<0.05 represents significant treatment effect.

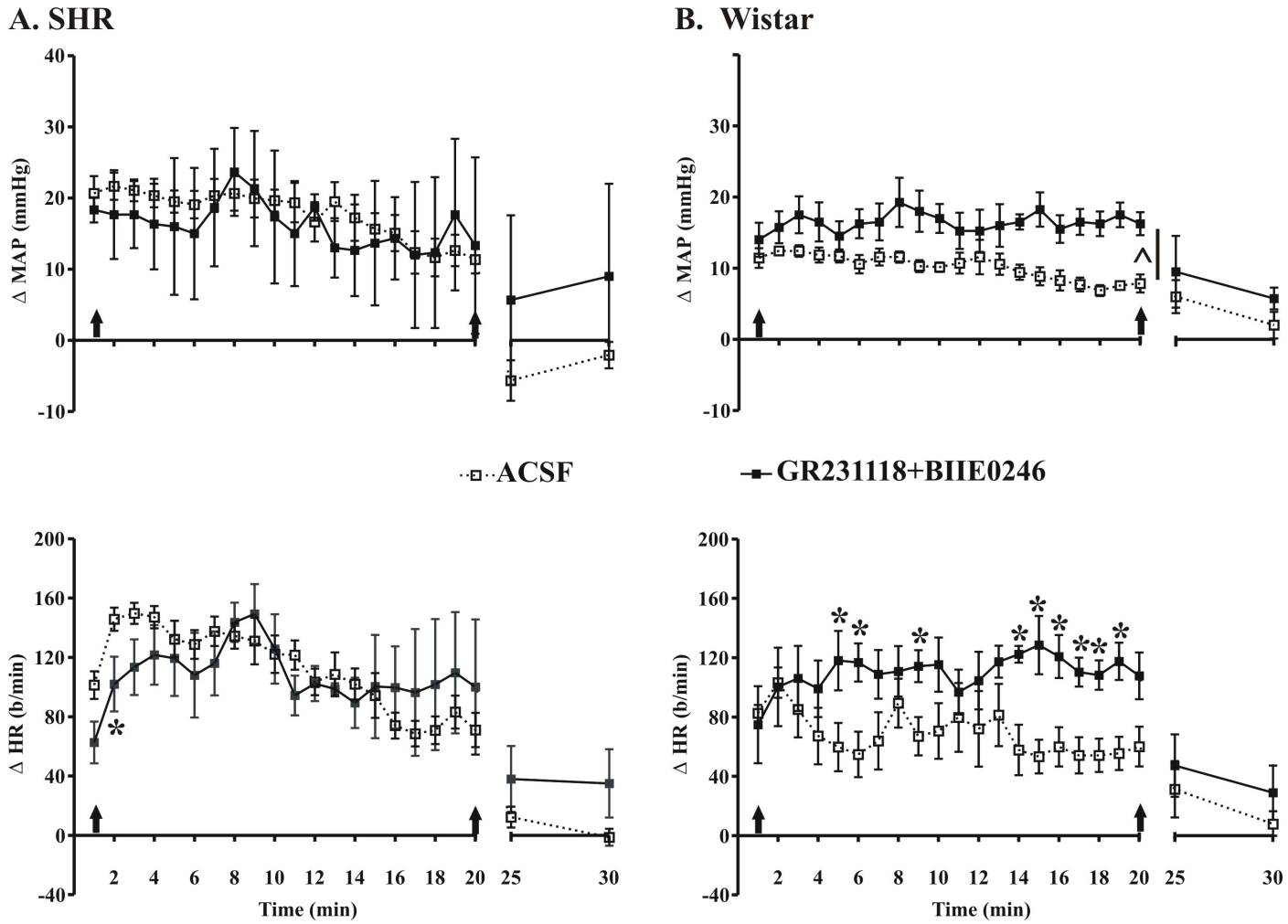

Figure 3-5. Effect of microinjection of BIIE0246, a Y2 receptor antagonist, one hour following GR231118 microinjection into the CEA on the cardiovascular response to AJS. A) ΔMAP and B) ΔHR for the SHR (n=3) and Wistar (n=4) rats administered GR231118/BIIE0246 or ACSF (n=7/strain) or prior to AJS. Black arrows designate the AJS period. The recovery period is represented by two time points following the offset of AJS (25 and 30 min.). *P<0.05 represents significant difference between treatment groups with a time interaction. ^P<0.05 represents significant treatment effect.

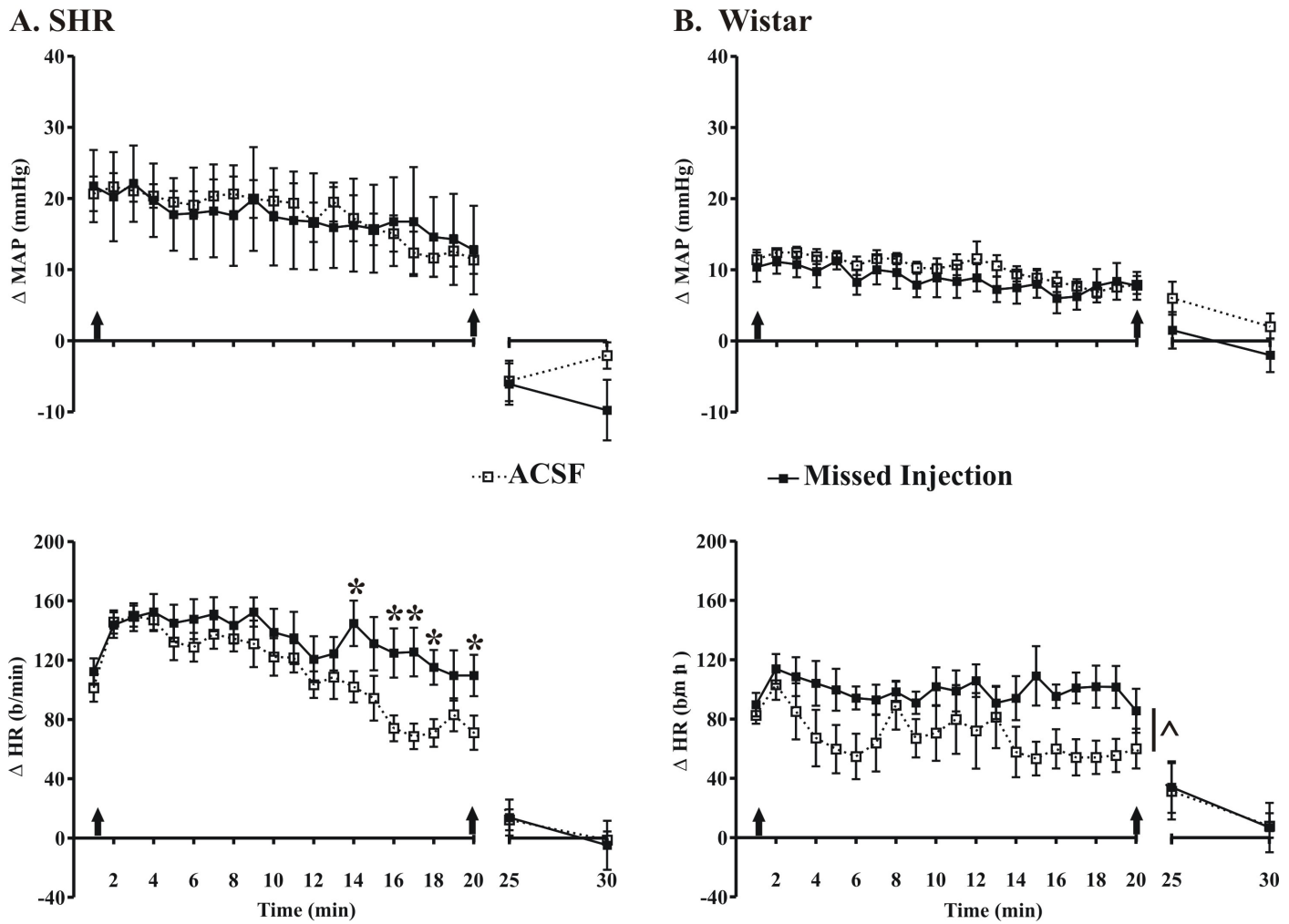

Figure 3-6. Effect of microinjection of GR231118 outside the CEA on the cardiovascular response to AJS. A) ΔMAP and B) ΔHR for the SHR (n=6) and Wistar (n=8) rats administered GR231118 outside of the CEA or ACSF (n=7/group) or prior to AJS. Black arrows designate the AJS period. The recovery period is represented by two time points following the offset of AJS (25 and 30 min.). *P<0.05 represents significant difference between treatment groups with a time interaction. ^P<0.05 represents significant treatment effect.

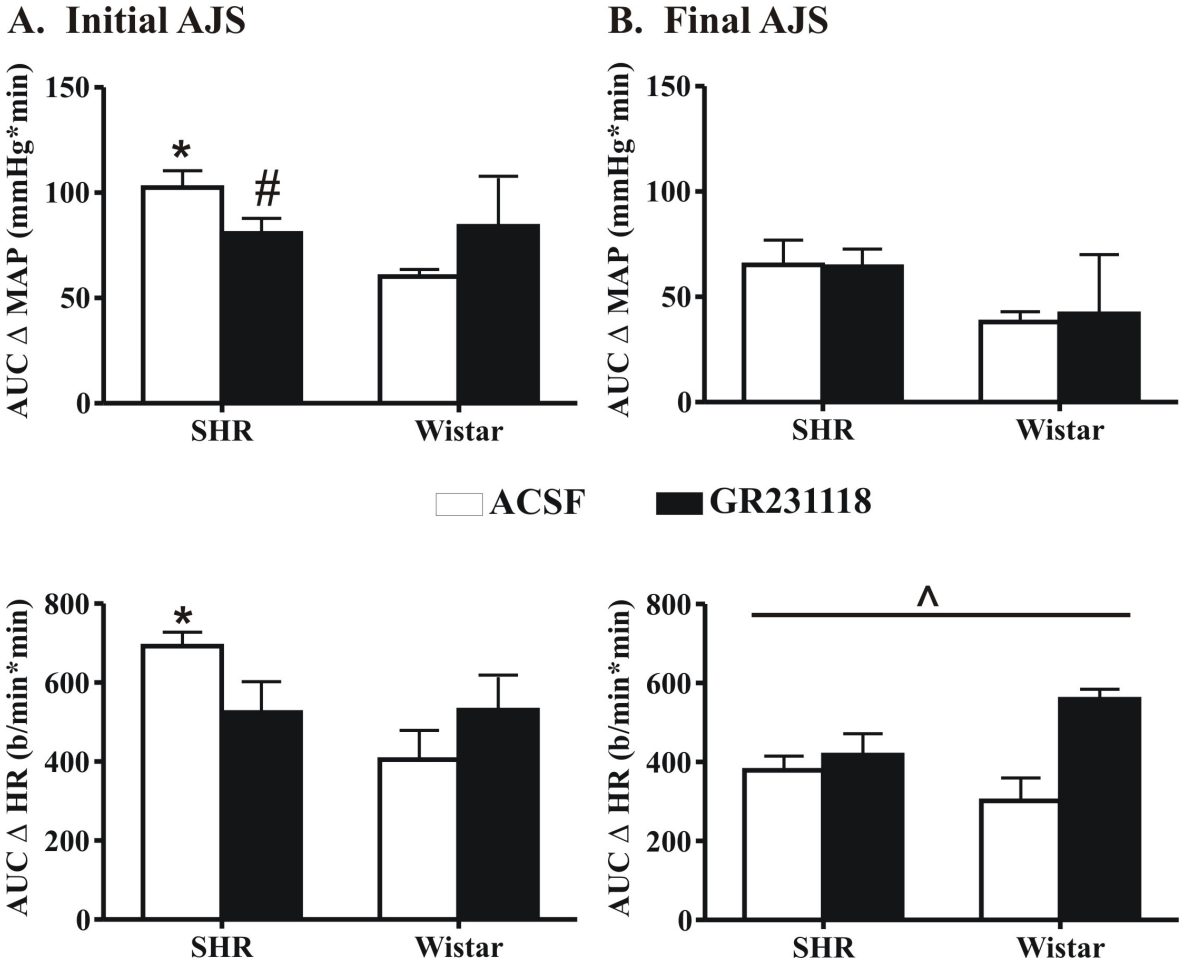

Figure 3-7. Effect of the microinjection of GR231118 into the CEA on the initial and final responses to AJS between strains. A.) Area under the curve (AUC) for ΔMAP and Δ HR during the initial period of AJS following ACSF and GR23118 microinjection in CEA of the SHR versus Wistar rats. B.) AUC for ΔMAP and Δ HR during the final period for AJS following microinjection in the SHR versus Wistar rats. *P<0.05 represents significant difference between ACSF groups. ^P<0.05 represents significant treatment effect.

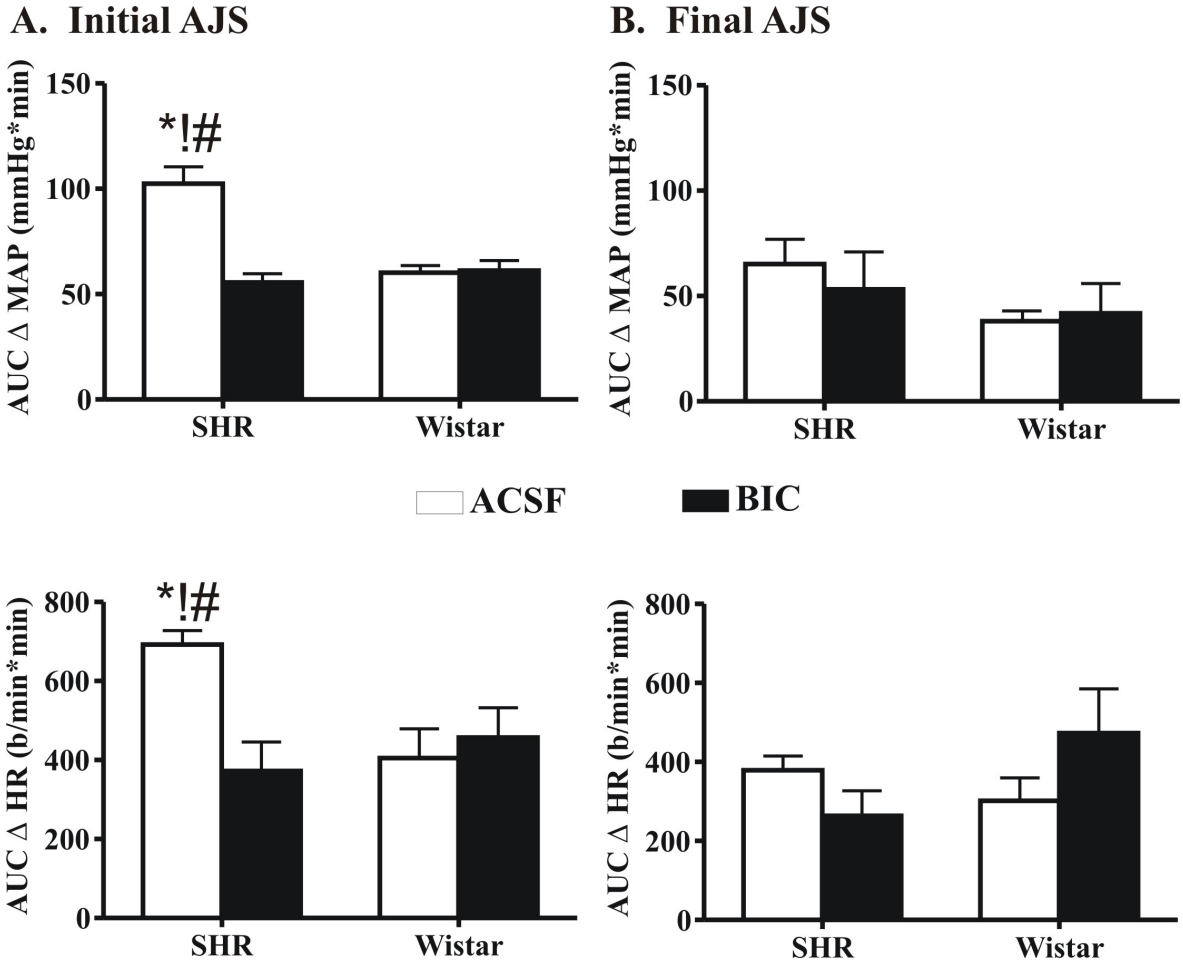

Figure 3-8. Effect of the microinjection of BIC into the CEA on the initial and final responses to AJS between strains. A.) Area under the curve (AUC) for ΔMAP and Δ HR during the initial period of AJS following ACSF and GR23118 microinjection in CEA of the SHR versus Wistar rats. B.) AUC for ΔMAP and Δ HR during the final period for AJS following microinjection in the SHR versus Wistar rats. *P<0.05 represents significant difference between ACSF groups. !P<0.05 represents significant difference of treatment in the SHR. #P<0.05 represents significant difference between SHR-ACSF and W-BIC.

CHAPTER 4
ALTERATIONS IN THE AUTONOMIC RESPONSE TO STRESS FOLLOWING DISINHIBITION OF THE CEA

Introduction

The development of hypertension affects millions of individuals and leads to a multitude of serious conditions including various cardiovascular diseases, stroke, and Alzheimer's disease (184, 335). Most studies have focused on the mechanisms initiating the development of high blood pressure and developing therapeutic and preventative measures to combat hypertension. It is well established that increased sympathetic nerve activity is a main contributor to the development of hypertension, however, the reason for a parallel drop in vagal regulation of heart rate (HR) during increases in blood pressure has not been fully elucidated (149, 150, 172, 173). Although there is a strong correlation between cardiac hypertrophy and reduced vagal function, a change in central regulation of HR needs to be considered (151). Baroreflex sensitivity is reduced in both hypertensive humans and animals suggesting that impairment in the regulation of the autonomic nervous system in the cardiovascular centers in the brainstem contributes to the maintenance of high blood pressure (150, 172, 217). Compelling evidence also suggests several forebrain regions crucial in regulating mean arterial pressure (MAP) and HR while at rest or during stressful challenges are also altered in the hypertensive state (103, 360, 365, 382). Hyperreactivity of the cardiovascular system to stressful stimuli has been identified as a defining characteristic in many hypertensive individuals suggesting an abnormal central regulation of the stress response (7, 137, 233, 254). Analyzing autonomic balance in hypertensive individuals may aid in determining if abnormal regulation by forebrain regions contributes to the exaggerated cardiovascular response to acute stress.

Several methods can be used to measure changes in autonomic regulation, but heart rate variability (HRV) analysis is a versatile, non-invasive tool that can be used to study a variety of

homeostatic challenges, as well as be an indicator of the development of future conditions. HRV analysis determines the number of times that a particular time interval between successive R-R peaks occurs over a given period of analysis. Three distinct interval ranges or frequencies have been identified to contribute to power spectrum analysis of HR: a very low frequency (VLF), low frequency (LF), and high frequency (HF) (223, 272). The VLF has been designated as measure of humoral and temperature influence on HR (363). Once considered an index of sympathetic activity, the LF component is now defined as a combination of both sympathetic and parasympathetic influence (263, 287). Lastly, the HF component has been defined as the main indicator of parasympathetic dominance (263). Additionally, the HF component has been considered an indicator of respiratory sinus arrhythmia or the strength of the synchronization between respiration and heart rate (205, 425). At present, the LF/HF ratio is considered a better measure of sympathetic drive or sympathovagal balance (223). Indeed, HRV analysis can provide insight into the dynamic fluctuation in autonomic regulation during physiological challenges. A correlation between reduced HRV and various cardiovascular and psychological pathologies has been demonstrated in humans and animals with hypertension, diabetes, and panic disorder (97, 135, 231). In contrast, the benefits of various drug therapies, meditation, and exercise have also demonstrated improvements in HRV (71, 282, 417).

Various brain regions are important in modulating the physiological responses to stress, however, the amygdala is unique in that it has the ability to integrate both autonomic regulation and emotional behavior. The ability of the amygdala, specifically the central nucleus (CEA), to modulate the autonomic system lies in its direct and indirect connections to part of the nucleus of the solitary tract (NTS) known as the dorsal vagal complex (DVC), the rostroventrolateral medulla (RVLM), and the hypothalamus (126, 128, 314, 401). In fact, lesioning of the CEA

reduces MAP and HR during noise stress in the spontaneously hypertensive rat (SHR) indicating it is an important regulator of the autonomic nervous system during challenges (110). Alternatively, intraamygdalar injection of the GABA A receptor agonist, muscimol has been shown to significantly attenuate the HR response to restraint stress. Moreover, HRV analysis demonstrated an increase in LF power and a decrease in HF power during restraint and these responses were reversed by muscimol microinjected into the CEA (323). Evidently the amygdala plays a role in modulating the autonomic nervous system during stress, particularly in regulating the heart cycle.

Several neurotransmitters secreted by or within the amygdala have the ability to modulate autonomic control. For example, neuropeptide tyrosine, also referred to as neuropeptide Y (NPY), is a neuromodulator known to be involved in regulating sympathetic activity (69, 209, 404). Peripherally, NPY-immunoreactive nerves are found throughout the heart and vasculature and modulate HR and MAP directly by modifying the actions of norepinephrine (NE, (108, 132)). Furthermore, NPY also acts peripherally to inhibit vagal activity, subsequently affecting the cardiac cycle length and this effect is enhanced by alpha adrenergic receptor blockade (407). Centrally, NPY administered intercerebroventricular (ICV) or into the NTS induces a reduction in MAP, HR, sympathetic nerve activity, and modulates baroreflex gain; these effects which are mediated by the activation of NPY receptor 1 (Y1, (188, 230, 422)). Anatomically, NPY innervations modulate the outputs of the CEA, hypothalamus, and NTS suggesting NPY may act indirectly to modulate brain regions involved in autonomic regulation (108, 126). Interestingly, HRV analysis has demonstrated that ICV administration of NPY attenuates stress-induced decreases in R-R intervals and decreases HR variability associated with fear conditioning (387). Centrally, NPY has also been shown to reduce measures of anxiety when administered into the

amygdala, however, little is known about NPY's influence on the amygdala's role in autonomic regulation.

In the previous chapter, the blockade of both Y1 and GABA A receptors in the CEA led to an attenuation of the exaggerated cardiovascular response during stress in the SHR, an effect that was primarily associated with the initial period of stress. In contrast, central blockade of the same receptor subtypes in a normotensive strain, the Wistar rat, induced the opposite effect on HR. Yet, how blockade of endogenous NPY and GABA receptors in the CEA is specifically involved in modulating autonomic drive to the heart, remains to be determined. Based on our previous findings, the present study was undertaken to evaluate changes in HRV associated with Y1 and GABA A receptor blockade in the CEA during stress as a possible indicator of alterations in autonomic drive in the SHR. It was hypothesized that blockade of Y1 and GABA A receptors in the SHR would induce an increase in the HF component, demonstrating an increase in parasympathetic activity during acute stress. Additionally, it was hypothesized that receptor blockade would induce a reduction in the LF/HF ratio in the SHR during stress due to attenuation in the sympathetic response. The opposite effects were hypothesized to be induced in the Wistar rats following GABA A and Y1 receptor blockade. HRV analysis had been previously used by our lab to evaluate changes in autonomic drive to the heart during a different type of stress, severe hemorrhage (288).

Methods

Animals and Surgical Preparation

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee and followed the NIH guidelines. Data used for analysis in this study were also utilized in the previous chapter therefore the preparation and stress protocols were the same and are only described briefly. Male SHR and the Wistar rats (10-12 weeks old;

Charles River Laboratories, Wilmington, MA) were randomly assigned to one of two groups, vehicle control or drug-treatment, prior to instrumentation. Under anesthesia (2-4% isoflurane+oxygen), bilateral cannula (7.0-7.5 mm, Plastics One, Inc., Roanoke, Virginia) were chronically implanted into the CEA (-2.4-2.6 mm distal from bregma and 4.5-4.6 mm lateral from midline according to Paxinos and Watson (281)). Following surgery, all incisions were treated with topical antibiotic ointment (Pharmaderm, Melville, NY) and the animals received a subcutaneous injection of saline (2-3 ml) and Buprinorphine (0.1 mg/kg) before being returned to their home cage.

Five to seven days post cannulation, the animals were re-anesthetized and instrumented with femoral arterial catheters. Pre and post surgical procedures were the same as for the central brain cannulation. Catheters (Braintree Scientific, Braintree, MA) were placed into both the femoral artery and vein toward the abdominal aorta. Catheters were filled with heparinized saline (100 IU/ml) and plugged with obturators. The catheters were tunneled subcutaneously to the nape of the neck, exited between the scapulae, and were secured with sutures.

Animals were allowed 48 hours to recover following femoral catheterization prior to experimentation. During the day following catheterization, animals were brought to the lab to be weighed, handled, and acclimated to the experimental container. Acclimation included a 50-60 min. period of exposure to air noise (~75-80 decibels) while resting in the experimental chamber.

Experimental Protocol

On the day of the experiment, the exteriorized catheters were connected to PE 50 tubing containing heparinized saline (100 IU/ml). The animals were placed into the experimental container and the arterial catheter was connected to a pressure transducer (Stoelting Inc., Wood Dale, IL) for continuous measurement of pulsatile and MAP.

Following a 60 min. baseline period the animals were randomly assigned to receive one of 3 possible solutions microinjected (300 nl per side) bilaterally into the CEA, including artificial cerebral spinal fluid (ACSF; n=14), 8.5, 17, or 51 nM GR231118 (n=11), or 173 μM bicuculline methobromide (n=8). ACSF was made by dissolving 122 mM NaCl, 3 mM KCl, 31.74 mM $NaHCO_3$, and 1.3 mM $CaCl_2$ into deionized water (pH=7.4). GR231118, a NPY Y1 receptor antagonist, (Tocris Biosciences, Ellisville, MI) was dissolved in ACSF. Bicuculline methobromide (Tocris Biosciences, Ellisville, MI), a GABA A antagonist, was also dissolved in ACSF. Concentrations were based on previously published studies (84, 122, 275).

Localized microinjections were made over 10 seconds using a 1 μl Hamilton syringe connected to PE 50 tubing attached to a 33 gauge internal injection cannula (ventral depth relative to the surface of the skull, 8.0-8.3mm, Plastics 1, Inc., Roanoake, VA). Injections were made while the animals were left unrestrained in the testing chamber. The rats rested for five and ten min. following the central injection of BIC or ACSF and GR231118, respectively. During the final two minutes, the air noise was turned on. After the two minutes of noise, the pressurized air was switched from being directed away from the chamber to being directed into the chamber through one of three inflow tubes. The AJS protocol included an initial 5 min. period of random AJS (continuous stream of air entering the chamber from a random direction and at random durations) immediately followed by a 15 min. of controlled AJS (the direction of the air flow was changed every minute). Following the offset of AJS, rats were allowed to recover for an hour.

Following recovery, all animals that received ACSF microinjection during the first injection received a second microinjection of either GR231118 or BIC. Similar to the first injection, 8 min. following the central injection, air noise was turned on and then after 2 min. of

noise, pressurized air was switched into the chamber and the a second bout of AJS was repeated followed by a 30 min. recovery. No animals received more than two bouts of AJS.

At the termination of the experiment, all animals were deeply anesthetized with a lethal dose of sodium pentobarbital (Ovation Pharmaceuticals, Inc., Dearfield, IL). Fluorogold (Fluorochrome, LLC., Denver, CO; 300nl) was injected into the CEA for marking the injection site. Brains were removed and soaked in 4% paraformaldehyde overnight. Prior to sectioning, a small cut was made along the rostral-caudal extent of the right ventral surface of the brain to distinguish left and right sides. Next, the tissue was frozen and sectioned into 40 μm coronal slices. See previous chapter for the method used to determine appropriate and missed injections.

Heart Rate Variability Analysis

HR was determined offline by detection of the interval between systolic peak pulses in the AP signal (Sampled at 100 Hz; Spike 2 software; Cambridge Electronic Design Limited, Cambridge, Eng.). For HRV analysis, three 5 min. time periods were chosen for analysis: baseline (within 30 min. of microinjection), initial (minute 1-6 during AJS), and final (last 5 min. of AJS). A recovery period (5 min. from offset of AJS) was also used for the linear regression analysis, but not used for HRV analysis due to the lack of difference between treatments during the recovery period identified in the previous chapter. Within each chosen segment, HR was then converted into a tachogram, a record of time between heart beats or RRI. The filtered tachogram was then analyzed in the frequency domain using HRV software (Biosignal Analysis Group; University of Kuopio, Finland (253)). In the software used, the tachogram was interpolated at 10 Hz and detrended via the smoothness priors formulation (alpha=1000; (253, 380)). The autoregressive model was set to the 40th order. The Welch's Periodogram window width was designated to 512 points with an overlap of 256 points in the Hanning window. In the rat, the frequency components of HRV are designated by the following frequency ranges:

0.16-0.6 Hz (LF), and 0.6-3.0 Hz (HF) (168). Frequency domain characteristics analyzed included the power or area under the curve for the HF component, LF component, the ratio of LF/HF power, and the HF peak frequency. Additionally, the HF and LF components were summed to analyze total power.

Statistical Analysis

For comparison of baseline HRV parameters, baseline values from groups were combined for each strain and analyzed by an unpaired t-test. HRV data was analyzed using a two-way analysis of the variance (ANOVA) with repeated measures comparing either strain or treatment against the designated time periods (STATVIEW). When indicated, a one-way ANOVA analysis comparing treatment or strain effects was utilized. A Bonferroni adjusted paired t-test was used to evaluate time effects when indicated.

A linear regression analysis was also utilized to confirm the relationship between HR and R-R interval variability. Average HR was calculated for the baseline, initial AJS, final AJS, and recovery periods and was plotted against the log of the root mean square difference among successive R-R intervals (RMSSD) for each animal in each treatment group per strain. R^2 values were used to identify how well the data fit a trend line and significance was determined by the slope. All data are represented as the mean±SEM. Statistical significance was set at $P<0.05$ for all analyses.

Results

The Effect of Air Jet Stress on HR and HRV

Figure 4-1A demonstrates the typical HR response to AJS following an injection of ACSF in a chronically instrumented conscious SHR versus a Wistar rat. Both strains demonstrated a rapid rise in HR during the first few minutes of AJS. Following this initial response, however, HR in the two strains quickly deviated. HR in the SHR remained elevated then slowly declined

only during the last 5 min. of stress. In contrast, HR in the Wistar fell after the first 2 min. of AJS and then stayed only modestly elevated through the remaining AJS period. Following the offset of AJS, the HR of both strains returned to near baseline levels within 5 min.

In Figure 4-1A, the time periods used for HRV analysis during AJS are represented by the open boxes. In addition, a baseline period was taken 20-30 min. prior to CEA microinjection and AJS. In this example, AJS was applied following ACSF microinjection into the CEA of both animals. Typical resulting power spectrums from each strain at these different time points are represented in Figure 4-1B and 4-1C and were dramatically different. At rest, the HF component was the dominant peak in the SHR. During the initial period of AJS, the power in both LF and HF peaks was substantially reduced relative to baseline and the HF peak shifted to the right. During the final period of AJS, the power in the LF range appeared to return to baseline levels and the HF peak returned to a lower frequency, but power in the HF range remained reduced relative to baseline. In contrast, at rest, the power in the LF peak was greater than the HF power in the Wistar rat (Fig. 4-1C). Moreover, the power of both the LF and HF peaks at baseline were on average 10 fold higher in the Wistar compared to the SHR (note y-axis). At the onset of AJS, the power in the LF component decreased while the HF power increased in the Wistar relative to its baseline, but there was no general shift of the HF peak. By the end of the stress period, the LF component was reduced to approximately a fifth of the baseline, but the HF component returned to baseline levels and the HF peak shifted slightly to the right relative to baseline.

HRV Differences between Strains

Analysis of the resting or baseline HRV parameters identified significant differences between the SHR and Wistar. As shown in Table 1, data from all animals combined prior to manipulation of the CEA identified a significant elevation in HF power (P=0.003), LF power

(P<0.0001), the LF/HF ratio (P=0.0002), and total power (P=0.02) in the Wistar compared to the SHR, but no difference in the mean frequency of the HF Peak (P=0.121).

Figure 4-2 illustrates the average change in HRV parameters for the two strains before and during AJS following central microinjection of ACSF (Wistar, n=7; SHR, n=7). A two-way ANOVA of the HF power did not identify any significant strain effect (P=0.10), however, there was a significant effect of time (P<0.0001) and a significant interaction between time and strain (Fig 4-2A, P=0.02). Post hoc analysis of the time effect demonstrated that with both strains combined there was a significant decrease in HF power during both the initial and final periods of AJS relative to baseline (P<0.006). Post hoc analysis of the interaction between strain and time, however, only identified a trend for strain-related differences to be different at baseline (P=0.07). Analysis of the LF power demonstrated a significant effect of strain, time, and an interaction between both (P<0.04). Further analysis, demonstrated a significant difference between LF power at baseline between the SHR and Wistar (P=0.02). During the initial period of stress, both the Wistar and SHR demonstrated a significant decrease in LF power during the initial period of AJS compared to their own baselines (P<0.019). The LF/HF ratio was also greater in the Wistar compared to the SHR for all time periods and there was a strong trend for a strain effect for the LF/HF ratio (P=0.056), but no significant time effect (P=0.58) or interaction between time and treatment was identified (Fig. 4-2C, P=0.65). The total power demonstrated a significant interaction of strain (P=0.05) and time (P=0.0002) as well as an interaction between both factors (P=0.004). A strong trend towards an increased total baseline was evidident in the Wistar compared to the SHR (P=0.027). Nevertheless, the total power was significantly reduced during both the initial and final periods of AJS in both strains (P<0.020, initial; P<0.025, final). Finally, analysis of the HF peak location identified both a time effect (P=0.001) and a significant

interaction between time and treatment (P=0.003). Post hoc analysis identified that during the initial period of stress, the HF peak shifted to a higher frequency in the SHR relative to the preceding baseline (P=0.0001) and was significantly greater compared to the Wistar at the same time point (Fig. 4-2C, P=0.002). There was also a strong trend for the HF peak location to increase relative to baseline at the final time period in the Wistar rats (P=0.029).

Changes in HRV Parameters in Response to Y1 and GABA A Receptor Blockade in the CEA of the SHR.

The effects of microinjection of a Y1 receptor antagonist, GR231118 (n=6), compared to ACSF (n=7) into the CEA of the SHR on HRV parameters are displayed in Figure 4-3A. A two-way ANOVA identified a significant effect of time (P<0.0001), but did not identify a significant effect of treatment or interaction between time and treatment for the HF component (P>0.26). Post hoc analysis of the time effect demonstrated that the HF power decreased significantly from baseline during both the initial and the final time points irrespective of treatment (P<0.0002). The LF power demonstrated a significant time effect (P=0.021) and interaction between time and strain (P=0.019), however, no treatment effect was exhibited (P=0.88). Also, the LF frequency component was found to be significantly lower during the initial period of stress compared to baseline in the SHR-ACSF group (P=0.012). Analysis of the LF/HF ratio also identified a significant effect of time (P=0.0007), but there was no treatment effect or interaction between time and treatment (P>0.16), even though the ratio appeared to increase during stress with GR231118 compared to control. In contrast, a significant effect of time (P<0.0001) and an interaction between time and treatment (P=0.05) was identified in regards to total power. The total power was reduced in the SHR-ACSF group during the initial period of AJS (P=0.003) and in both treatment groups during the final periods of stress (P<0.02). Finally, GR231118 attenuated the HF peak shift during the initial period of AJS in the SHR, however, analysis only

identified a significant effect of time (P=0.03) and a trend for the interaction between time and treatment (P=0.09). Post hoc analysis of the time effect only demonstrated a trend for the HF peak location at the initial time period to be different from baseline with both groups combined (P=0.03).

In contrast to Y1 receptor blockade, administration of BIC (n=4) into the CEA of the SHR caused a slight rise in the power of the HF component, but a two-way ANOVA only identified a time effect (P=0.0002). There was no significant effect of treatment or an interaction between time and treatment (Fig. 4-3B, P>0.3). Post hoc analysis of the time effect demonstrated a significant decrease in HF power during both the initial and final period of AJS compared to baseline when both treatment groups were combined (P<0.006). Similarly, the LF component demonstrated only a significant effect of time (P<0.011) and no effect of treatment or an interaction between time and treatment (P>0.55). The time effect demonstrated a decrease in LF power during the initial period of AJS (P<0.024). Furthermore, the LF/HF ratio appeared to be slightly reduced following exposure to BIC. Yet, there was no treatment effect (P=0.68), only a trend for the effect of time to be significant (P=0.055), and no significant interaction between treatment and time was identified (P=0.97). Only a time effect was significant in regards to the total power (P<0.0001) while no strain effect or interaction between time and treatment was evident (P>0.2597). Total power was significantly reduced during the initial and final periods of AJS (P<0.003). Finally, the shift in the HF peak frequency peak location demonstrated both a significant effect of time (P=0.0008) and an interaction between time and treatment following BIC microinjection (P=0.05), however, there was only a trend for a treatment effect (P=0.08). Post-hoc analysis of the interaction between treatment and time demonstrated that following ACSF microinjection, the location of the HF peak shifted to a significantly higher frequency

during the initial period of AJS compared to baseline (P<0.0001), but following BIC microinjection into the CEA this upward shift relative to baseline was no longer significant (P=0.25). Accordingly, the HF peak frequency during the initial period of stress was significantly reduced in the BIC group compared to the ACSF group (P=0.008).

Changes in HRV Parameters in Response to Y1 and GABA A Receptor Blockade in the CEA of the Wistar

Figure 4-4A illustrates the effect of GR231118 microinjection into the CEA of Wistar rats (n=5) compared to the ACSF data (n=7). In the Wistar, Y1 receptor blockade reduced the power in the HF component at all time points, similar to the effect observed in the SHR. Yet, following a two-way ANOVA, there was no significant treatment effect (P=0.37) and no interaction between time and treatment was found (P=0.88). There was, however, a significant effect of time (P=0.0004) which identified that HF power decreased during both the initial and final period of AJS relative to baseline independent of treatment. The LF power also demonstrated a significant effect of time (P<0.0005), however, there was no effect of strain or an interaction between time and treatment (P>0.34). Post hoc analysis of the LF power exhibited a significant decrease during the initial and final period of AJS (P<0.004). The LF/HF ratio was only modestly reduced by GR231118 compared to ACSF therefore no significant effect of either time or treatment was identified (P>0.5) as well as no significant interaction between time and treatment (P=0.95). The total power demonstrated only a significant time effect (P<0.0003), but no effect of treatment or interaction of time and treatment (P>0.34). During both the initial and final periods of AJS, the total power was reduced (P<0.003). Similarly, GR231118 administered into the CEA did not change the HF peak location during stress and no significant effects of time, treatment, or interaction between time and treatment was identified (P>0.37).

Contrary to the effect observed in the SHR, BIC microinjection in the CEA slightly reduced the HF component in the Wistar (n=4) during AJS, compared to ACSF microinjection. Yet, a two-way ANOVA only identified a significant effect of time ($P<0.0001$). Additionally, there was no significant effect of treatment or interaction between time and treatment (Fig. 4-4B, $P>0.57$). Post hoc analysis of the effect of time demonstrated a significant reduction in HF power during both the initial and final periods of AJS relative to baseline ($P<0.001$). On the other hand, the LF power demonstrated a significant time effect ($P<0.0001$) and interaction between time and treatment ($P=0.003$). The LF power was lowered significantly in both treatment groups during the initial period of stress ($P<0.023$) and a strong trend towards a decrease during the final period of stress ($P<0.036$). BIC microinjection into the CEA appeared to have little effect on the LF/HF ratio during AJS and analysis only identified a small effect of time ($P=0.03$). Post hoc analysis of the effect of time, however, did not identify any significant differences during AJS relative to baseline ($P>0.05$). In contrast, a significant effect of time ($P<0.0001$) and interaction of time and treatment ($P=0.009$) was evident in the total power. Within both treatment groups, a significant decrease in total power was demonstrated during the initial period of stress compared to baseline ($P<0.024$) while during the final period of stress the ACSF group the total power was significantly reduced ($P<0.025$), yet there was only a strong trend in the BIC group ($P=0.0352$). Finally, BIC microinjection appeared to induce a downward shift of the HF peak location during AJS in the Wistar rats. Analysis of the effect of BIC identified a significant effect of treatment ($P=0.03$) and a trend for a time effect ($P=0.055$), but no interaction between time and treatment was identified ($P=0.88$). Post hoc analysis confirmed a significant treatment effect ($P=0.012$) following BIC microinjection in the Wistar rats.

Comparison between GABA A Receptor Blockade in CEA of the SHR with the Baseline AJS Responses in the Wistar and SHR

To evaluate whether disinhibition of the CEA in the SHR would modulate HRV to be more similar to the AJS in the Wistar administered ACSF, we compared the effects of BIC administration into the CEA on HRV parameters during the initial and final periods of AJS between 3 groups (SHR-ACSF, Wistar-ACSF and SHR-BIC). As shown in Figure 4-5A, a two-way ANOVA determined no significant effect of treatment or time or an interaction between time and treatment for either the HF component ($P>0.57$), the LF component ($P>0.16$), total power ($P>0.25$), or the LF/HF ratio ($P>0.26$; Fig. 4-5B). Even though there was a dramatic attenuation in the HF peak shift in the initial period of stress in SHR-BIC group compared to the SHR-ACSF group, analysis of the HF peak frequency also did not demonstrate an effect of time or treatment nor an interaction between time and treatment ($P<0.1$). It is apparent that the SHR-BIC group demonstrated a similar shift in the HF peak during the initial period of AJS compared to the W-ACSF group.

The Effects of ACSF, BIC, and GR231118 on the Correlation between HR and RMSSD

Figure 4-6 displays the relationship between HR and the variability within in R-R intervals or RMSSD for all treatment groups in the SHR (Figure 4-6A) and Wistar (Figure 4-6B). In the SHR-ACSF group, an inverse relationship between HR and RMSSD was identified, however, the r^2 value was low (Fig 4-6A, $r^2=0.29$) suggesting that even though the variability within R-R intervals decreases with increasing HR, the relationship between these two factors was not particularly strong in the SHR. GR231118 injected into the CEA of the SHR generated lower RMSSD values at higher HRs, but the fit for the data was better ($r^2=0.66$) compared to ACSF. BIC also increased the r^2 value or correlation between the HR and RMSSD compared to ACSF ($r^2=0.46$). Furthermore, the slope of the relationship was increased compared to both ACSF and

GR231118. The slope of the trend line proved to be significantly greater compared to zero for all treatment groups ($P<0.016$).

In contrast to the SHR, the Wistar rats exhibited a strong correlation between HR and RMSSD ($r^2=0.41$) following ASCF microinjection in to the CEA. Alternatively, GR231118 injected into CEA of the Wistars lowered the r^2 value compared to ACSF. Additionally, the slope was lower in GR231118 treated group and was no longer significant from zero ($P=0.0756$). Finally, BIC administered into the CEA demonstrated no relationship between HR and R-R variability in the Wistar; the r^2 value was zero and the slope of the relationship that was not significantly different from zero ($P=0.988$).

Discussion

Hypertensive individuals have increased cardiovascular reactivity when exposed to acute stress stimuli. In the previous chapter, we demonstrated an attenuation of stress-induced increases in MAP and HR by blocking Y1 and GABA A receptors in the CEA of the SHR. Associated with this attenuated response, we hypothesized that disinhibition of the CEA in the SHR must have induced an alteration in sympathovagal balance, specifically an increase in parasympathetic activity. Using HRV analysis, the current study identified six main findings. First, at rest there were noticable strain-related differences in HRV, including a significant reduction in both HF power, the LF/HF ratio, and total power in the SHR. Second, independent of strain, AJS induced a significant reduction in HF power and total power relative to baseline both at the initial and the final periods of AJS in both strains. Third, differences between strains in response to AJS were marked by a significant shift of the HF peak to the right in the SHR, but no change in the Wistar. Since the location of the HF peak is related to respiratory rate, this observation suggests that a unique component of the response of the SHR to AJS was a possible elevation in respiratory rate. Fourth, no significant treatment effects on HF power, LF power,

total power, or LF/HF ratio were identified in either strain following NPY 1 or GABA A receptor blockade. Nevertheless, in the SHR, disinhibition of the CEA with BIC eliminated the significant increase in the LF/HF ratio observed during AJS following GR231118 treatment. This observation suggests that one effect of GR231118 in the SHR may have been to modestly elevate sympathetic drive. Fifth, BIC microinjection in the SHR significantly reduced the rightward shift in the HF peak during the initial period of AJS. This observation suggests that disinhibition of the CEA in the SHR may have modulated the respiratory response to AJS. Finally, both GR231118 and BIC microinjected into the CEA strengthened the correlation between HR and R-R interval variability in the SHR, but decreased the correlation coefficient in the Wistar rats. This type of analysis suggested that disinhibition of the CEA appeared to improve cardiovascular variability in the SHR during AJS, but decreased variability in the Wistar rats. These observations suggest the exaggerated HR response to AJS in the SHR is mediated by a reduction in HRV and a possible increase in respiratory rate, and these changes in cardiorespiratory function are mediated in part by GABA release in the CEA. In contrast, disinhibition of the CEA in the Wistar rat induced a decrease in HR variability independent of any indication of a change in respiratory rate.

Baseline differences in HRV between strains. The results of the present study demonstrate that the sympathovagal balance of the autonomic nervous system at rest is strikingly different between the Wistar and the SHR. The HF power in the SHR at rest was significantly reduced compared to the Wistar rats. Since power in the HF range is reported to be indicative of vagal drive, this observation suggests that the SHR generally exhibits lower vagal tone at rest compared to the Wistar. This result is in agreement with an overall reduction in vagal tone that has been previously reported in the SHR (150, 172). Alternatively, the Wistar rat strain has

generally been reported to exhibit a large HF component at rest (22, 187). For comparison, in a previous study of Sprague Dawley rats, another normotensive strain, we reported a mean HF power of 0.08 msec² at rest, a value more in line with the SHR values in the present study (288). Interestingly the LF/HF ratio, which is typically considered an indicator of sympathetic drive, was also higher in the Wistar rats compared to the SHR. This observation fits the higher baseline HR reported in the previous two chapters for the Wistar strain and suggests that the balance of sympathetic drive to vagal drive in the Wistar, may actually be elevated, in general, compared to the SHR (see chapter 2 and 3). The total power was also higher in the Wistar compared to the SHR supporting the low HRV in the hypertensive model. Indeed, it should be noted that HRV components may be influenced by other inputs, including changes in respiration rate, tidal volume, anatomical orientation during testing, hormonal secretion, or a combination of all of these (1, 170, 236). Furthermore, the HF component is considered a pure indicator of parasympathetic activity, as identifed by acetylcholine-receptor blockade with atropine, parasympathetic blockade also elicits reductions of the LF component (1, 203). Additionally, systemic administration of the beta-adrenoceptor blocker propranolol has been shown to either reduce only the LF component or alter both LF and HF components (170, 298). Thus, it is apparent from the variation in blockade studies that neither HF nor LF is a pure representation of parasympathetic or sympathetic activity. Thus, although HRV can be an extremely useful tool in providing insight into the autonomic balance, caution should be taken when interpreting the results.

Differences in HRV during AJS between strain. In the normotensive animals, stress-induced tachycardia has been reported to be mediated via decreases in parasympathetic activity which is coupled with a parallel increase in sympathetic drive (22, 187, 263). Similarly, the

current study demonstrated strain-independent reductions in parasympathetic activity during AJS compared to baseline, with the Wistar exhibiting the greatest trend for a decrease in both the HF and LF components. This was coupled with a drop in the LF/HF ratio during the initial period of stress followed by a small increase in the LF/HF ratio back to baseline levels before the end of the stress. This suggests that the initial response to AJS in the Wistar rats involved a parallel decrease in activity in both the sympathetic and the parasympathetic limbs, possibly due to overcompensation of the sympathetic component followed by a rebalancing of autonomic inputs appropriate for the stress. In contrast, the SHR demonstrated a reduction in the HF and LF components during the initial period of stress relative to baseline and this was accompanied by an increase in the LF/HF component during the initial period of AJS. Additonally, both strains demonstrated reduced total power suggesting HRV is lowered during exposure to stress. Although not significant, by the end of the stress, power in the HF range remained reduced and LF/HF ratio was elevated relative to baseline. The change in HRV between the initial period of stress for the two strains is intriguing in that it may be indicative of differences in reflex control, possibly driven by baroreflex changes or hormone release. Stress has been demonstrated to lower baroreflex sensitivity (BRS). In fact, this suppression has been found to be enhanced in the SHR during AJS even though BRS is already low at baseline in this strain (217, 261). Differences in HR values reported in the previous chapter between strains and relative changes in HF power and the LF/HF ratio during the initial period of stress identified in this chapter suggests that adaptation of the autonomic system occurs rapidly during first few minutes of stress in the Wistar leading to the recruitment of compensatory mechanisms or a rapid restoration of BRS. The change in HRV between the two strains during AJS appears to hinge on the level of vagal tone and BRS.

The effects of Y1 receptor and GABA A antagonism in the CEA on HRV in the Wistar and SHR during AJS. Previously, we demonstrated that both Y1 and GABA A receptor antagonists microinjected into the CEA increased the HR response to AJS in the Wistar. In the present study, no significant changes in HRV were identified following antagonism of Y1 and GABA A receptors in the CEA of the Wistar, however, GR231118 administration into the CEA tended to reduce the HF component throughout the duration of stress. Similarly, BIC tended to decrease the HF component during the initial period of AJS, but by the end of stress, the HF component had increased to ACSF levels. The LF/HF ratio or total power did not change in either Wistar treatment group. The reduction of the HF component by GR231118 and BIC suggests that the CEA may play a more prominent role in regulating parasympathetic activity than modulating sympathetic drive in normotensive animals during stress. Indeed, the CEA has numerous connections to brain regions known to be involved in regulating vagal tone and baroreflex information (124, 128, 312, 314). Furthermore, lesions of the CEA have been shown to reduce both the gain and range of the baroreflex (210). CEA lesions have also been reported to attenuate the bradycardic response to fear conditioning, confirming the CEA is important in parasympathetic regulation in normotensive rats (307).

The CEA also appears to be important in regulating vagal tone in the SHR. For example, lesions of the CEA have been shown to attenuate the increase in HR during noise stress in the SHR (110). Based on the results of the previous chapter and the observation that microinjection of either GR231118 or BIC into the CEA during AJS attenuated the AJS-HR response in the SHR, we predicted the HF power would be increased during the AJS in the present study. Interestingly, although no significant changes in HRV parameters were identified in the SHR following manipulation of the CEA, the HF and LF component was only slightly reduced during

the final period of stress following Y1 antagonism and the LF/HF component increased throughout the duration of AJS. These findings were contrary to our original hypothesis. In contrast, when BIC was administered into the CEA there was a slight increase in both the HF and LF component during the initial period, but this increase was not maintained during the final period of stress. Also, the LF/HF ratio was lower in the SHR-BIC group compared to the SHR-ACSF group throughout stress, indicating a small reduction or no change in the sympathetic drive. Interestingly, the total power was increased in the SHR-BIC group suggesting an overall improvement in the regulation of HRV during AJS. As discussed in the previous chapter, both antagonists affected HR during AJS in the SHR in a similar direction, yet, the results of the present analysis suggest that change in HR was mediated by different changes in sympathovagal balance. One explanation for these differences autonomic control may be that by blocking Y1 receptors, this allowed released NPY to bind to other NPY receptors, which could lead to the variable responses displayed in both SHR and Wistar. For example, central activation of Y2 receptors has been shown to elicit anxiogenic behaviors and pressor responses versus the effects of Y1 receptor activation may be the exact opposite (317, 404, 422). Additionally, GABA A blockade maintained a reciprocal relationship between HF power and the LF component in both strains, suggesting GABA may have more specific effects in regulating vagal tone during stress.

Y1 and GABA A receptor antagonism effects on the location of the HF peak during AJS. In the current study, the only significant effect identified by HRV analysis during AJS was a shift in the HF peak frequency from approximately 1.5 Hz to 2.5 Hz in the SHR during the initial period of AJS. A similar shift was not observed in the Wistar rats. The location of the HF peak has been suggested to be an indicator of respiratory rate due to the shift that occurs with changes in breathing during either spontaneous breathing, paced breathing, or holding the breath

(96, 416). In general, stress causes a change in respiration which can influence vagal regulation of HR. In the SHR, both GR231118 and GABA A receptor antagonism in the CEA modulated the rightward shift of the HF peak during AJS. Furthermore, dishinibition of the CEA in the SHR normalized the location of the HF peak to that observed in the ACSF treated Wistar rats. The possible modulation of the HR response in the SHR may be related to a change in respiratory rate and may be related to the functional connection between the respiratory centers and the CEA (128, 377). In fact, it has been reported that lesions of the left amygdala decreased respiratory frequency during anticipation anxiety (227). Additionally, fMRI studies indicate that neuronal activity within the amygdala is in sync with every respiratory cycle (94). The results of the present study suggests that exaggerated inhibition of the CEA in the SHR may directly affect the respiratory cycle during stress and the resultant change in respiration may contribute to decrease in vagal drive and an elevation in HR during stress, as reported in the previous chapter for the SHR. The results of this chapter suggest that the potential effect of exaggerated respiratory responses to stress in hypertension may be particularly important during the initial response to stress.

Correlation of the variation in R-R interval with HR. In the current study, we also used the correlation between RMSSD and HR to further gauge changes in vagal tone. The RMSSD is one non-linear measure of HRV that has been found to be highly correlated with the HF peak, suggesting it is also an excellent measure of vagal tone (1). It should be noted, however, that even this HRV measure may be affected by respiration (25). Nevertheless, during AJS, in both the SHR and Wistar rats, there was a negative correlation between HR and RMSSD, suggesting within increasing HR, the variability within the HR was reduced. The correlation between R-R variation and RMSSD was inherently stronger in the Wistar compared to the SHR. Following

Y1 antagonism, the variation in HR was lowered even though the range in HR remained the same in both strains. Interestingly, the correlation between HR and RMSSD increased in the SHR and decreased in the Wistar, suggesting blockade of Y1 in the CEA may differentially affect variation of R-R intervals during AJS in both strains. On the other hand, BIC administered into the CEA increased the slope of the trend line in the SHR suggesting increased HRV at lower HRs. Moreover, in the SHR, BIC improved the correlation between HR and RMSSD to a value that was similar to the Wistar-ACSF group indicating that increased inhibition of the CEA strongly contributes to a reduction in HRV in the SHR. In contrast, BIC microinjected in the CEA of Wistar completely eliminated the relationship between HR and RMSSD.

The results of these two methods of analysis suggest that role of the CEA in regulating vagal tone appears to be reliant on modulation of the inherent inhibition that exists within the amygdala. Anatomically, the CEA is interconnected with many brain regions that modulate the autonomic responses to stress. Yet, the complexity of the amgydala may lie within the interconnections of neurons within the different subnuclei of the amygdala. Rich in GABA neurons, the amygdala is under constant inhibition which is generally thought to be suppressed during exposure to stressful stimuli. The normalization of the correlation between HR and RMSSD in the SHR by administration of BIC into the CEA provides compelling evidence for a dysfunction in the tonic inhibition of the CEA in this animal model of hypertension. Indeed, the SHR has been reported to have a reduced numbers of GABA A receptors in the CEA (200, 349). Furthermore, stimulation of the amygdala by glutamate in normotensive animals has been shown to elicit a depressor response which can be reversed by GABA (51). BIC-induced increases in HR and MAP are also reduced by glutamate receptor antagonist microinjection into the amygdala. These observations suggest that the excitation of cardiovascular system during the

defense response is modulated by a balance of GABA mediated inhibition and glutamate excitation (318, 359). The novel findings of the last two chapters suggest that an impaired relationship between GABA and possibly glutamate exists within the CEA of the SHR and may be one of the defects that can develop in hypertensive individuals thus contributing to an exaggerated autonomic response to stress and/or an alteration in regulation of parasympathetic activity.

Aside from glutamate and GABA, other neuromodulators may also contribute to the dysfunction within the amygdala in the SHR. For example, corticotropin releasing hormone (CRH) microinjected into the CEA increases HR, but does not affect peripheral NE secretion, suggesting the CRH-induced changes in HR are mediated by modulation of parasympathetic activity (410). During fear conditioning, central administration of CRH has been reported to increase release of GABA and glutamate, demonstrating its influence over excitatory and inhibitory balance within the amygdala (351). NPY neurons also innervate CEA projections to the dorsal vagal complex, indicating a possible mechanism by which Y1 antagonism could directly affect HRV (128). In addition, NPY in conjunction with glutamate has been reported to be anxiolytic (409). On the other hand, NPY acting through Y1 and Y2 can suppress GABA secretion and neurotransmission (369). Finally, NE, which is often co-released with NPY, reduces the glutamate-elicited depressor responses in the amygdala (305). A complex interplay between neurotransmitters and neuromodulators is evident in the CEA in relation to modulating autonomic activity and the results of our experiments suggest that changes in the normal balance of these neurochemicals may be the reason for the hypersensitivity of the cardiovascular system to stress in the SHR.

Methodological Considerations. Four methodological considerations need to be considered regarding the results of the present study. First, it was apparent administration of either drug in the SHR affected the initial or latter portion of the autonomic response to AJS. Yet, no significant changes in LF/HF ratio or HF power were identified in the present study. It is possible that with the low concentrations used here that either antagonist, but BIC in particular, may have worn off partially by the time the stress was actually applied. It is also possible that in the SHR the receptors may be more sensitive due to lower abundance, such as in the case of GABA A receptors, leading to a more robust response to the blockade. It is also possible that a similar inhibition of the stress response and changes in HRV may have been induced in the Wistar rats if higher doses of BIC were tested. Secondly, Wistar baseline HRV parameters were quite variable compared to the SHR, suggesting the parasympathetic activity in the Wistar may be more sensitive to fluctuations at rest. This would be in accordance with the reduced vagal drive evident in the SHR. Thirdly, measuring respiration would have further supported the possible increase in respiration frequency indicated by the significant attenuation of the HF peak shift in the SHR following BIC administration. Furthermore, the SHR has been reported to have an increased respiratory frequency and lower tidal volume compared to normotensive rats, implying respiratory drive is abnormal and may influence overall power in the baseline HRV profile (65, 106). Future studies evaluating the impact of tidal volume differences between strains and how they impact HRV are needed. Finally, giving atropine or propranolol prior to AJS and measuring their modulation of the stress response would have provided a more direct measurement of altered regulation of the autonomic nervous system in the SHR and is highly recommended for future studies

Summary. Combining the observations of the previous study and the current study, we have implicated the CEA as an important contributor to cardiovascular regulation during stress in the SHR. Specifically, heightened stress responses are often associated with hypertension may be related to a specific dysfunction within the CEA, possibly an impaired GABA system leading to dysregulation of the tonic inhibition over the output from the CEA. Additionally, the results of the present study demonstrated that BIC modified the location of HF peak, increased total power during stress, and improved the correlation between HR and R-R variation further implicating disinhibition of the amygdala as way to normalize the autonomic response to stress in the SHR. Future studies are need to evaluate changes in glutmatergic inputs to the CEA of the SHR, but there is now evidence to suggest the cardiovascular response to stress in the SHR may be related to an aberrant problem with excitatory and inhibitory input into the CEA.

Table 4-1. Baseline HRV Parameters

	SHR (n=17)	Wistar (n=16)
HF Power	0.108±0.015*	0.311±0.253
LF Power	0.055±0.012*	0.887±0.193
LF/HF Ratio	0.571±0.096*	3.122±0.612
Total Power	0.163±0.021*	1.198±0.241
HF Peak	1.641±0.06	1.422±0.127

*P<0.05 with respect to strain

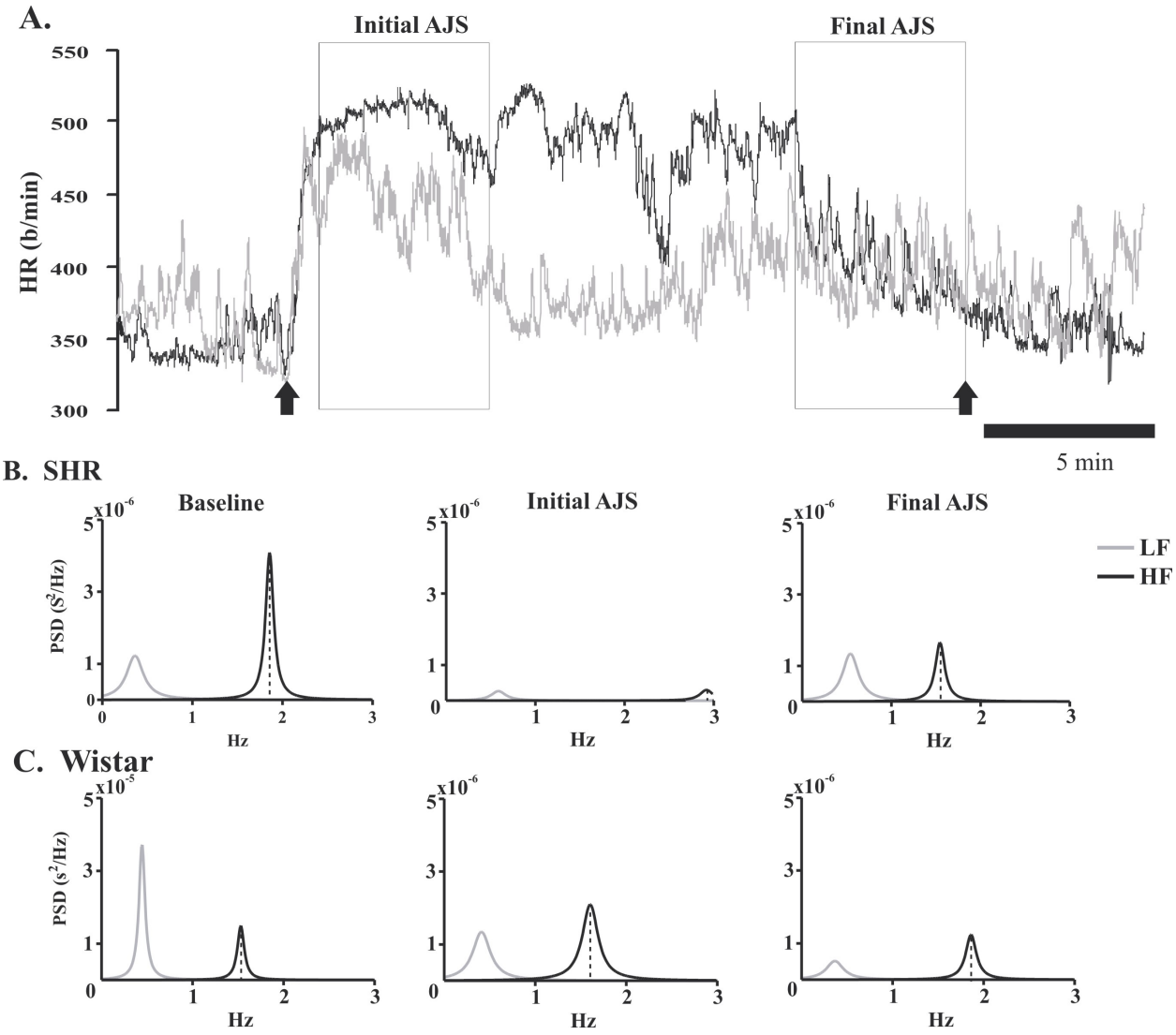

Figure 4-1. The effect of air jet stress on heart rate and heart rate variability analysis in a SHR and Wistar. A) Raw tracing of HR from an individual SHR, represented in black, and Wistar rat, represented in gray exposed to AJS following microinjection of ACSF into the CEA. Data sampled at 100 Hz. Arrows represent the beginning and end of AJS. Open boxes represent the time periods used for HRV analysis: the initial period is during min. 1-6 of AJS and the final period is min. 15-20 of AJS. Horizontal black bar reflects the time scale. Power spectral density (PSD) of the LF and HF components of HRV during baseline, initial AJS, and final AJS periods for either a (B) SHR or (C) Wistar exposed to AJS. Baseline period HRV analysis of data was taken approximately 30 min. prior to AJS. Note y-axis different for baseline curves. Vertical dotted lines represent the frequency at which the HRV components are at their peak.

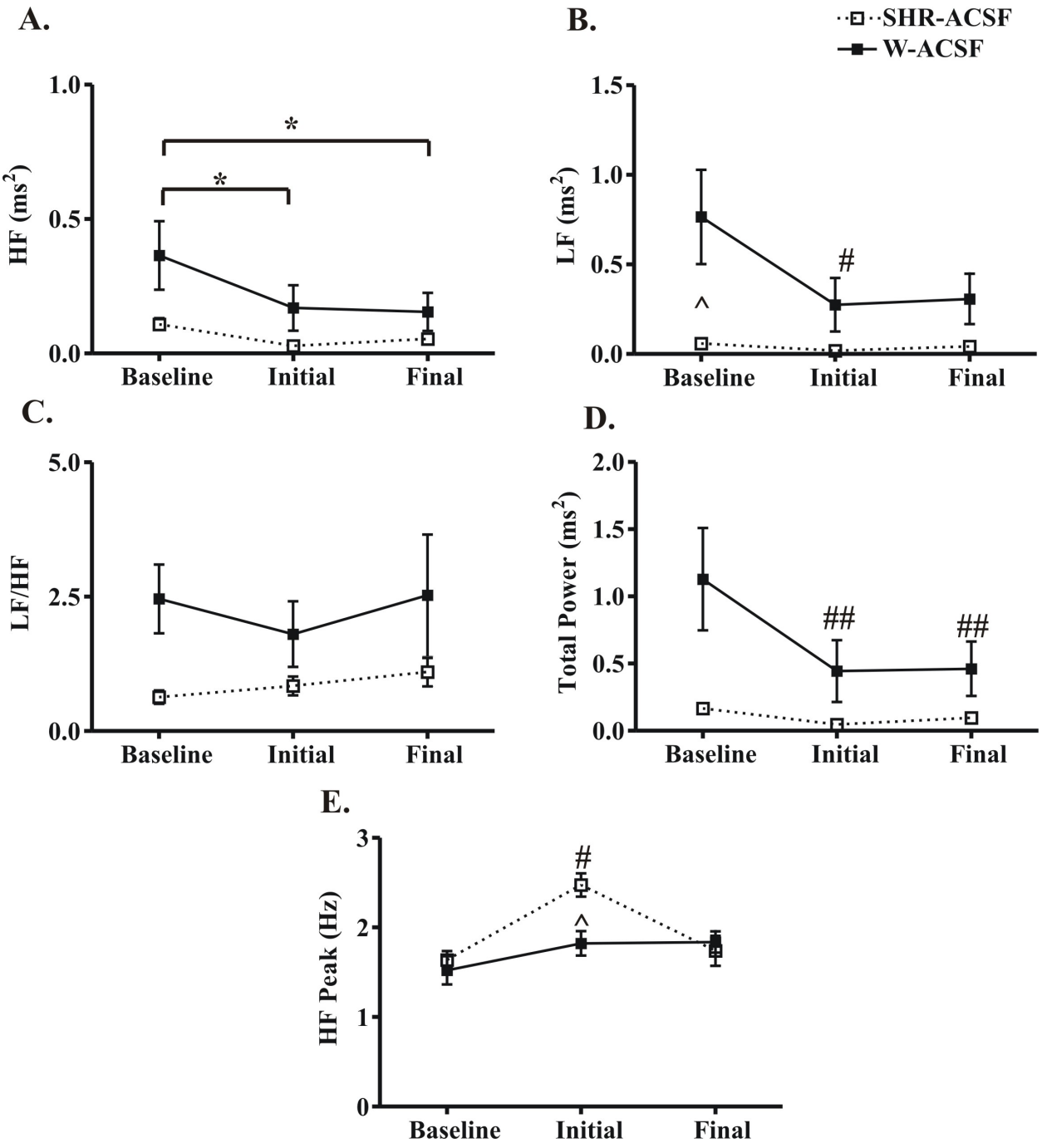

Figure 4-2. Comparison of HRV parameters before and during AJS between strain. A) HF power, B) LF Power, C) LF /HF ratio, D) Total Power, and E) location along x-axis of the HF peak in the SHR (n=7) and the Wistar (n=7) during AJS following microinjection of ACSF into the CEA. Data reflect mean±SEM. *P<0.025 with respect to baseline for strains combined. #P<0.025 with respect to baseline within SHR group. ##P<0.025 with respect to baseline within both strains. ^P<0.05 with respect to strain at time point.

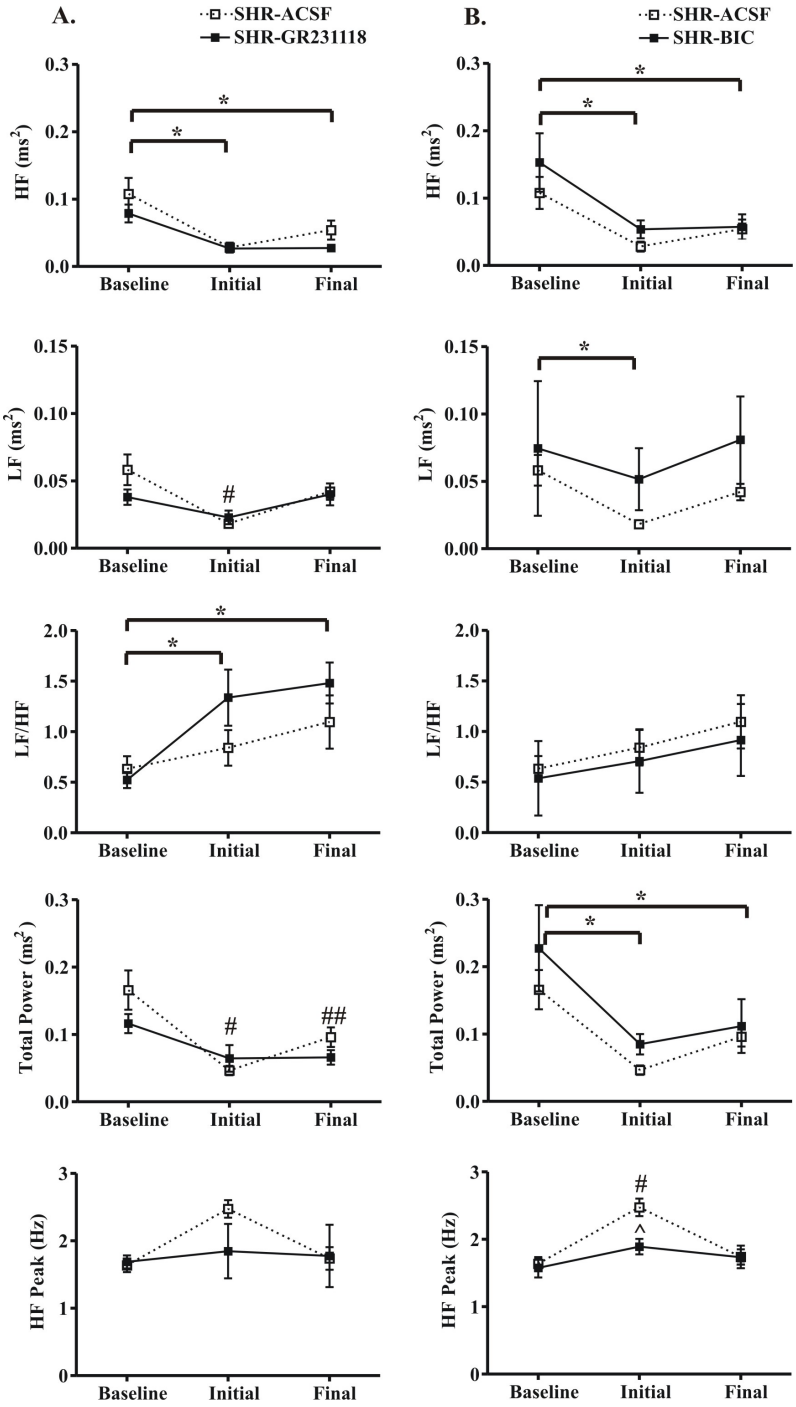

Figure 4-3. Changes in HRV parameters in the SHR following microinjection of the Y1 receptor antagonist, GR231118, or the GABA A receptor antagonist, bicucculline, before and during AJS. A) The HF power, LF power, LF/HF ratio, total power and HF peak frequency comparison between ACSF (n=7) and GR231118 (n=6) and (B) between ACSF (n=7) and BIC (n=4). *P<0.025 with respect to baseline for treatment groups combined. #P<0.025 with respect to baseline within SHR group. ##P<0.025 with respect to baseline withing both treatment groups. ^P<0.05 with respect to treatment at time point.

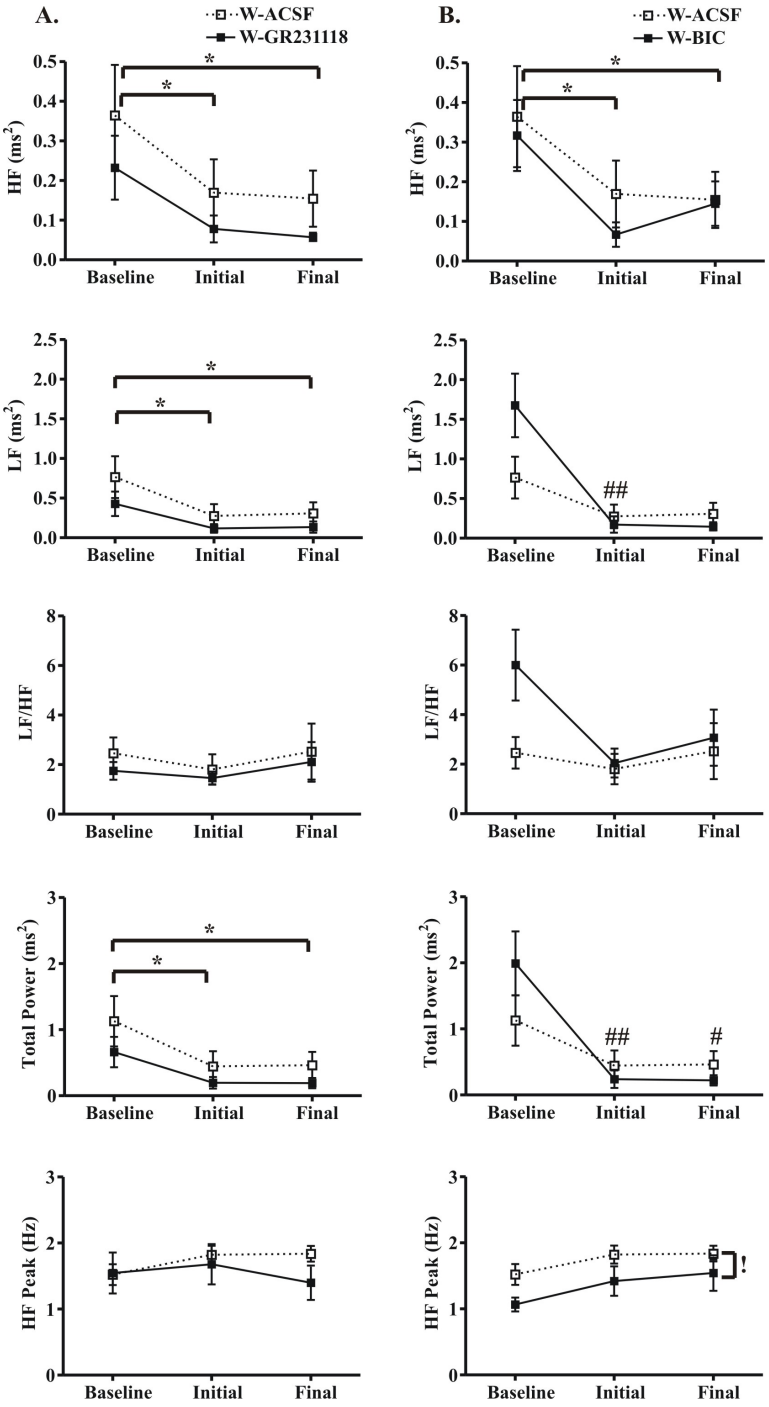

Figure 4-4. Changes in HRV parameters following microinjection of the Y1 receptor antagonist, GR231118, or the GABA A receptor antagonist, bicuculline, in the Wistar before and during AJS. A) The HF power, LF power, LF/HF ratio, total power, and HF peak frequency comparison between ACSF (n=7) and GR231118 (n=5) and (B) between ACSF (n=7) and BIC (n=4). *P<0.025 with respect to baseline for treatment groups combined. #P<0.025 with respect to baseline within SHR group. ##P<0.025 with respect to baseline within treatement groups. ^P<0.05 with respect to difference between treatment with no time interaction.

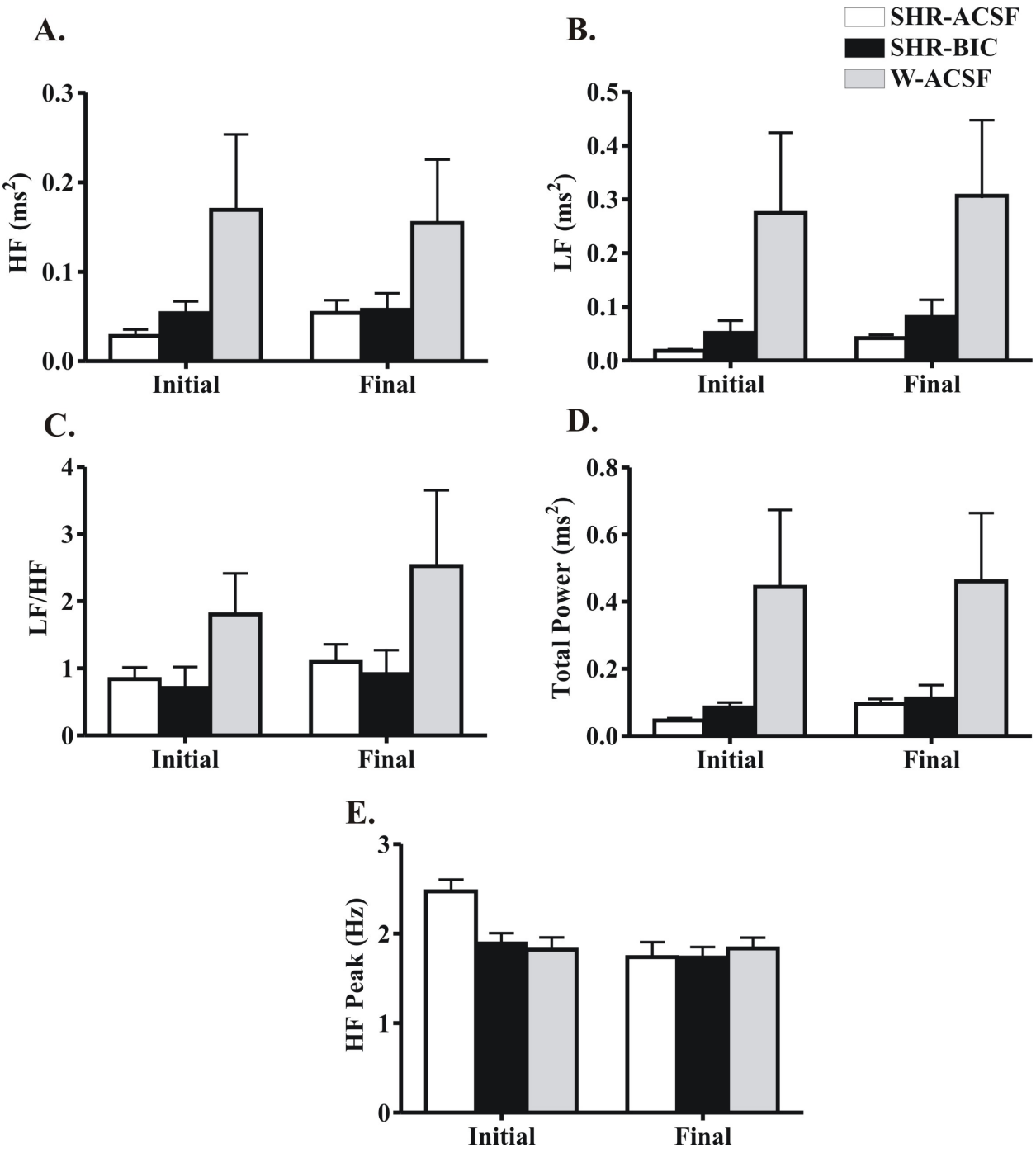

Figure 4-5. Comparison of the effects GABA A antagonism on HRV in the SHR versus control responses in the SHR and Wistar. A) The HF power, B) LF power, C) LF/HF ratio, D) total power, and E) HF peak frequency following microinjection of BIC into the CEA of the SHR (n=4), ACSF into the SHR (n=7) and ACSF into the Wistar (n=7).

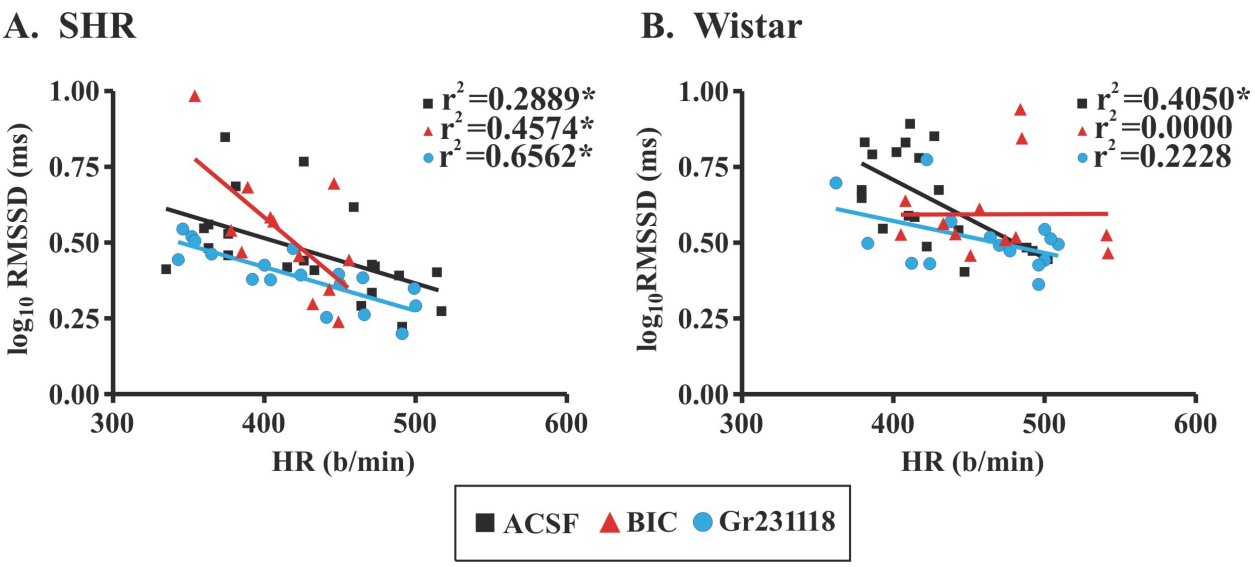

Figure 4-6. Correlations between 5 min. averages of HR and the root mean square difference among successive R-R intervals after CEA receptor blockade. A) HR versus $\log_{10}$ RMSSD in the SHR. B) HR versus $\log_{10}$ RMSSD in the Wistar. Black square represents ACSF (n=21/strain). Red triangle represents BIC (n=12/strain). Blue circle represents GR231118 (SHR, n=18; Wistar n=15). Points include initial AJS, final AJS, and recovery periods. r^2 represents the fit of the data to the trendline. *P<0.05 significance of slope from 0.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Summary

Specific Aim 1

Hyperreactivity of the cardiovascular system during stressful stimuli has been identified in both human and animals with hypertension. The amygdala has been characterized as the brain's epicenter for regulation of emotions. It has also been indicated in regulating the autonomic nervous system. Thus, the experimental focus of specific aim 1 was to identify how exposure to an acute psychological stress would alter the pattern of neuronal activation within the amygdala in an animal model of essential hypertension, the spontaneously hypertensive rat (SHR), compared to a normotensive control. Specifically, changes in corticotropin releasing hormone (CRH) neurons were monitored due to their importance in regulating neuroendocrine responses to stress, as well as producing anxiogenic behaviors. Furthermore, previous research has identified that the CRH system is altered in the hypertensive individual (141). It was hypothesized that an acute stressor, air jet stress (AJS), would cause activation of more CRH neurons in the amygdala of the SHR compared to the normotensive control. Additionally, more fos-like immunoreactive (FLI) cells, an early marker of neuronal activation, would be identified in select subnuclei of the amygdala and hypothalamus, as well as in the locus coeruleus (LC) of the SHR compared to control. Our AJS model generated a robust increase in both mean arterial pressure (MAP) and heart rate (HR) that was significantly greater in the SHR compared to the control. AJS also increased the level of FLI-positive neurons in the amygdala of the SHR during stress, however, the number of neurons activated by stress was not different compared to the normotensive control. The amygdala did, however, demonstrate a greater number of CRH neurons in the central nucleus of the amygdala (CEA) in the SHR compared to the Wistar and

more CRH-positive neurons were displayed following exposure to AJS compared to the noise control. Outside of the amygdala, exposure to AJS induced a significant increase in number of FLI-positive neurons the LC of the SHR compared the normotensive controls. Alternatively, no significant difference in FLI levels between strains was identified in the hypothalamus. Based on evidence of a strong interconnection between CRH neurons in the CEA and the rostral pole of the LC, the results of this study identified for the first time that the hyperreactivity to stress and increased arousal of the SHR may be related to dysfunction in CRH release in the amygdala contributing to the elevated activation of locus coeruleus (55, 174, 396, 397).

Specific Aim 2

Based on the results of Specific Aim 1, experiments in Aim 2 were undertaken to specifically evaluate the role different neurotransmitters in the CEA play in mediating an exaggerate response to stress in hypertension. Previous research has identified that under resting conditions, the amygdala is tightly regulated by inhibitory neurotransmitters. Yet, when exposed to stress the inhibitory tone appears to be reduced, possibly allowing for the amygdala to influence descending systems involved in neurohormone release, autonomic regulation, and behavioral action. In the SHR, extensive evidence has implicated both NPY and GABAergic systems within the brainstem and hypothalamus to contribute to autonomic dysregulation in this animal model of hypertension. Moreover, activation of either NPY 1 (Y1) or GABA A receptors in specific brain regions, such as the hypothalamus and brainstem, decreases both MAP and HR (5, 366). Yet, the role of these specific neurotransmitters in the CEA in modulating autonomic responses to stress or in mediating an exaggerated response to stress in hypertension had not been previously investigated. Specific Aim 2 concentrated on identifying whether dysregulation of NPY and/or GABA release in the CEA contributed in any way to the exaggerated cardiovascular response to stress of the SHR. The hypothesis to be tested was that Y1 and/or

GABA A receptor blockade in the CEA would modulate the cardiovascular response to acute stress in the SHR differently compared to a normotensive control, the Wistar rat. More specifically, it was hypothesized that blockade of GABA A and/or Y1 receptors in the CEA would be less effective in modulating MAP and HR responses to acute exposure to air jet stress in the SHR compared to the Wistar. In support of our original hypothesis, the results of Specific Aim 2 demonstrated that Y1 blockade within the CEA modestly attenuated of both MAP and HR during AJS in the SHR, but caused a slight increase in the Wistar, indicating that the NPY system was indeed altered in the CEA of the SHR. Similarly, antagonism of GABA A receptors in the CEA resulted in a decrease of both MAP and HR in the SHR compared to the augmentation of HR in the Wistar. The results provide new compelling evidence that a dysregulation of both NPY and GABA systems within the CEA may contribute to the increased number of CRH neurons in the SHR observed in specific aim 1 and subsequently leads to the exaggerated cardiovascular response to psychological stress, typical of the hypertensive state.

Specific Aim 3

Previous evidence has indicated that the SHR has increased sympathetic nerve activity and reduced vagal drive implying a dysregulation of the autonomic nervous system (172, 173). Additionally, the CEA sends several projections to the cardiovascular centers in the brainstem influencing the autonomic nervous system during various homeostatic challenges (125, 314). Thus, based on the results of Specific Aim 2, Specific Aim 3 was undertaken to evaluate the mechanism through which disinhibition of the CEA in the SHR attenuated the autonomic response to stress. We addressed this problem by using heart rate variability (HRV) analysis to determine changes in the autonomic system during stress following Y1 or GABA A blockade. Based on our previous results, it was hypothesized that blockade of Y1 and GABA A receptors in the SHR would induce an increase in parasympathetic drive or the HF component of HRV.

Additionally, it was hypothesized that receptor blockade would induce a reduction in the LF/HF ratio in the SHR during stress due to attenuation in the sympathetic response while the opposite response would be induced in the Wistar rat following manipulation of the CEA. The results of Aim 3 demonstrate that at rest, the SHR displayed a significant reduction in both the HF power and the LF/HF ratio compared to the Wistar. Furthermore, strain differences during AJS were indicated by opposite effects on the LF/HF ratio suggesting sympathetic activity may be regulated differently in the SHR. Y1 antagonism demonstrated variable effects on HRV parameters in both strains implying blockade of one receptor subtype may allow for activation of other NPY receptors in response to NPY release in the CEA during stess. Nevertheless, GABA A blockade in the CEA, appeared to increase vagal tone slightly in the SHR indicated by the increase in HF power and decrease sympathetic drive as indicated by a decrease in the LF/HF ratio. Additionally, BIC enhanced the correlation between HR and R-R interval variability, making the relationship appear more similar to the Wistar rat at rest. These observations provide intruiging evidence that disinhibition of the CEA may normalize the autonomic response to stress in the SHR to become more similar to a normotensive control. This observation provides the first evidence that disinhibition of the CEA improves HRV in the SHR during stress and further implicates dysfunction of GABAergic system in autonomic dysregulation in hypertension. Finally, HRV analysis identified for the first time that the response of the SHR to psychological stress also included a shift in the location of the HF peak to a higher frequency during the initial response to AJS. Since the location of this peak has been shown to be related to respiratory rate and a similar shift was not observed in the Wistars during stress, this suggested that the exaggerated cardiovascular response to stress in the SHR may be coupled with an exaggerated respiratory response. GABA A blockade in the CEA significantly attenuated the

shift in the HF peak during the initial period of stress indicating that a change in respiration rate may be contributing to the HRV parameters in the SHR.

Discussion

Role of CEA in the Response to Stress

Orchestration of the stress response requires cooperation between the peripheral tissues and the central nervous system to arrange synchronization of hormones, neurotransmitters, and inflammatory markers for an efficient, yet, appropriate way to return the body to homeostatic conditions. As discussed earlier, the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system, and the limbic system are the major regulators of the stress response. For the current investigation, the amygdala was chosen based on its unique position to integrate various sensory modalities into physiological responses. Furthermore, alterations within the amygdala have been implicated in various pathological states ranging from anorexia to depression to hypertension.

Even though the amygdala affects the autonomic responses to stressful stimuli, current literature has mainly focused on the amygdala's ability to modulate behavioral and cognitive reactions to stress. In fact, both the lateral and basolateral amygdala (BLA) have been designated the 'gate' within the amygdala, filtering information transmitted into the other subnuclei (207). Numerous studies have indicated the BLA as an important modifier of behavior and memory, yet, it is apparent the CEA also influences cognitive processes during stress (306, 316). In fact, lesions of the CEA have been shown to reduce immobility behavior during social defeat, decrease ethanol consumption following anxiety tests, and enhance hyperthermia during the cold swim test (243, 280, 307). Furthermore, microinjection of various neurotransmitters into the CEA has been shown to modulate behavioral responses to stress. For example, microinjection of a CRH antagonist into the CEA reduces anxiety during ethanol withdrawal and

emotionality during social defeat, indicating CRH as an anxiogenic neuropeptide (157, 300). Additionally, glutamate antagonists microinjected into the CEA have also been reported to reduce anxiety during the elevated plus maze test (EPM) (77). In contrast, microinjection of a GABA A receptor agonist in the CEA generated anxiolytic responses to social interaction and reduced anxiety during the elevated plus maze EPM (244, 326). NPY has also been shown to reduce anxiety in the EPM test and suppress alcohol self-administration when microinjected into the CEA (115, 152). In contrast, early markers of neuronal activation such as c-fos, have indicated that the CEA is activated during physical stressors such as hemorrhage, but not during processive stressors, like restraint, suggesting the CEA may have more influence during stimuli producing dynamic changes in the autonomic nervous system rather than cognitive stress (63, 73, 74). Although the above evidence demonstrates the CEA's ability to modulate behavioral responses during stress, it appears the amygdala may be very important in the translation of these behaviors to visceral responses.

Alternatively, the CEA is also known for it ability to modulate several autonomic nervous system functions during stress, such as cardiovascular reactivity, parasympathetic activity, and respiration. The vast influence of the CEA over the visceral function is marked by its connections to cardiovascular and respiratory centers within the pons and brainstem as well as within brain regions involved in the defense reaction such as the hypothalamus and the periaqueductal gray (PAG; (124, 125, 128, 313, 314, 401)). Modulation of cardiovascular function from the CEA appears to be dependent on the balance of various neurotransmitters released either locally or from afferent projection sites. Under anesthesia, electronic stimulation of the CEA has generally been reported to cause a depressor response (112). Glutamate microinjection also causes depressor and bradycardic response when injected into in the CEA

under anesthesia (51, 121). On the other hand, high doses of glutamate induced pressor responses when microinjected in the CEA of a conscious animal suggesting modulation of cardiovascular reactivity by the CEA is sensitive to ongoing levels inhibition (121). Aside from stimulation studies, changes in cardiovascular reactivity following manipulation of the CEA have been reported in response to other neuromodulators. For example, microinjection of carbachol, a cholinergic agonist, into the CEA elicits a pressor response (16). CRH microinjection into the CEA increases both MAP and HR during rest (133, 410). Similarly, a slight increase in MAP and HR was generated following microinjection of a GABA A receptor antagonist into the CEA (122). Glutamate also caused increases in MAP and HR in conscious rat following administration into the CEA (167). Even though plenty of evidence has implicated the CEA's involvement in regulating the cardiovascular tone, surprising few studies have investigated the role of the CEA in regulating the autonomic nervous system during stress.

The CEA's Role in the Stress Response of the SHR

In the early 1980s, the amygdala was first hypothesized to contribute to the development of hypertension in the SHR. Indeed, lesions of the CEA reduced the development of blood pressure in the SHR, although only modestly. This raised the possibility that the CEA may contribute somewhat to baseline autonomic tone, but may indeed be more important during stressful challenges (102). Indeed, the cardiovascular response to the open field test and noise stress was reduced in SHRs with a lesioned CEA (110, 324). Although, since that time is was apparent the CEA modifies cardiovascular reactivity in the SHR, most studies have mainly focused on the brain regions involved in regulating the HPA axis or the autonomic nervous system, and have ignored the role of the amygdala. As discussed previously, the HPA axis and the sympathetic nervous system have been indicated as being altered in the SHR and may contribute the cardiovascular reactivity during stress. Yet, some evidence also suggests that changes in the

anatomical structure within the amygdala of hypertensive individuals exists, however, no correlation between the amygdala and cardiovascular hyperreactivity during stress has been investigated (114). Furthermore, potential involvement of several neurotransmitters including NPY, GABA, and glutamate in dysregulation of the CEA had not been previously evaluated, despite evidence that these neurotransmitter systems are significantly altered in other brain regions in the SHR. The above studies addressed the involvement of the CEA in regulating the cardiovascular system during stress in the SHR. The results indicate for the first time a possible disruption the inhibitory tone within the CEA which may alter CRH secretion and subsequently cause the exaggerated cardiovascular response elicited by stress in the SHR. Since GABA A blockade of the CEA reduced both MAP and HR in the SHR and appeared to normalize HRV parameters during stress, we concluded other regulatory systems may be suspect in altering inhibitory tone and CRH secretion within the amygdala. Two potential neuromodulators are discussed below.

Role of Angiotensin II in GABA A Mediated Imbalance within the CEA

Noted as one of the main contributors to essential hypertension, the renin-angiotensin system extends its influence into the regulation of various brain regions (41). Following conversion from angiotensin 1 via angiotensin converting enzyme (ACE), circulating angiotensin II (AngII) has the ability to modulate brain circuits involved in autonomic regulation indirectly via actions on circumventricular organs located with the brainstem and forebrain (344). Additionally, ACE and Ang II have been found within many brain regions, including the amygdala, indicating endogenous formation and secretion (46, 402). In fact, local microinjection of Ang II antagonists into the CEA has been shown to reverse increases in MAP and HR induced stimulation of the CEA by glutamate. This indicates that Ang II modulates excitatory neurotransmission in the CEA (198). Similarly, ICV pretreatment with Ang II antagonists has

been shown to reverse GABA-mediated decreases in MAP and HR in three different rat strains suggesting, Ang II also stimulates inhibitory action (303). The potential impact of local AngII on GABA and glutamatergic neuronal function in the CEA is illustrated in Figure 5-1.

AngII also has the ability to modulate the release of stress peptides within the brain. Following stimulation of the PVN, AngII antagonism reduced CRH-elicited secretion of cortisol and ACTH (296). Additionally, angiotensin receptor 2 (AT2) knock-out mice have increased anxiety which has been associated with modulation of norepinephrine secretion within the amygdala (258). AngII's versatility as a neuromodulator suggests that it performs an important role in maintaining homeostasis and an alteration in the regulation of Ang II could disrupt many central mediated functions.

Hypertensive individuals typically have high levels of circulating AngII which appears to alter central mechanisms within the brain, possibly leading to a hypersensitivity to stressful stimuli. In fact, chronic administration of an ACE inhibitor via ICV reduced increases in corticosterone and ACTH levels after cold stress and ether stress in the SHR (23). Furthermore, a transgenic mouse model of hypertension demonstrated increased amygdalar activity in response to ANG II and GABA A receptor antagonists which reversed the inhibitory transmission caused by Ang II (8). The current study demonstrated GABA A blockade caused attenuation in the exaggerated cardiovascular response to stress. As mentioned above, Ang II mediates GABA-mediated depressor responses. In the same study, it was noted that the SHR had increased sensitivity to GABA (303). Additionally, chronic Ang II administration into the NTS leads to increased expression in GABA B receptors (423). The SHR also demonstrated increased expression in GABA B receptor activation in the NTS suggesting that Ang II's influence could contribute to a dysregulation of the GABAergic systems in a hypertensive

individual (361). Furthermore, GABA B receptor activation in the hypothalamus has been shown to enhance glutamate and inhibit GABA transmission in the SHR (212). Couple the studies describing glutamate receptor stimulation in the CEA inducing a pressor response and evidence that Ang II also aids glutamate transmission, it may be insinuated that the attenuation of the cardiovascular response in the SHR by GABA A receptor antagonism may be due to enhanced activation of both GABA B and glutamate receptors mediated by AngII. Thus, an imbalance of inhibitory and excitatory transmission in the amygdala may be influenced by the high levels of Ang II in the SHR which in turn may contribute to the cardiovascular hyperreactivity to stress seen in these animals. Additional studies are needed to evaluate how Ang II blockade within the amygdala specifically modulates GABA or glutamate release in the CEA.

Role of Corticosterone in the CRH Mediated Imbalance within the CEA

The HPA axis modulates the neuroendocrine response to stressful stimuli resulting in secretion of glucocorticoids, in particular corticosterone in rats or cortisol in humans (CORT). Although, CORT acts through a negative feedback pathway to prevent further secretion of CRH and ACTH; it can also act through a positive feedback mechanism to increase extrahypothalamic CRH levels (221, 373). In fact, adrenalectomy in rats has been shown to reduce CRH immunoreactivity in both the bed nucleus of the stria terminalis (BNST) and CEA indicating that both CORT and catecholamines are important in regulating CRH activity outside the PVN (327). Furthermore, subsequent microinjection of CORT into the CEA generated increases in CRH mRNA expression and induced anxiogenic responses to the EPM (341). Likewise, acute CORT administration reduced freezing during fear conditioning and subsequently lead to increased c-fos generation in both the hypothalamus and the MEA (352). Aside from stress, adjustments in blood pressure also affected secretion of CORT into the CEA. For example, in response to

peripherally induced increases in blood pressure, CORT levels have been reported to rise in the CEA. In contrast, during hypotension decreases in CORT release in the CEA was observed (80). Thus, the increased number of CRH neurons in the SHR during AJS in the current study may be due to either the high levels of circulating CORT exhibited in the SHR or the elevated blood pressure response observed in the SHR. Due to the influences of CORT on the output of the amygdala, it may be suggested that glucorticoids indirectly affect the CEA's regulation of the autonomic response to stress through modulation of CRH release. Figure 5-1 describes the consequences of CORT activity on CRH neuronal function in the CEA.

In addition to direct modulation of CRH in the CEA, CORT's interaction with GABA may also provide another mechanism for mediating an imbalance between excitation and inhibition in the CEA of the SHR. CORT has been shown to increase the excitability of neurons within the BLA by reducing GABA A receptor activity (86). Furthermore, chronic CORT administration into the BLA increases freezing behavior during contextual fear conditioning; this response was reduced by pretreatment with a GABA agonist indicating an interaction (54). Lastly, subcutaneous injections of GABA B receptor agonists have been reported to stimulate increases in HPA axis activity (145). In the SHR, the increased levels of CORT may be a reason for the low GABA A expression in the brain (83, 142, 200). Accordingly, high GABA B expression in the SHR may facilitate the high levels of CORT that are evident in the SHR, leading to the low CRH and ACTH levels in the PVN while causing a parallel elevation in CRH in the CEA as demonstrated in this study (141, 142). Thus, similar to Ang II, CORT has the ability to adjust the intrinsic inhibition within the amygdala which could lead to the exaggerated cardiovascular response to stress in the SHR.

The Role of the Parabrachial Nucleus in Mediating Abnormal Stress Responses in the SHR

The parabrachial nucleus (PBN) is a major center within the pons transmitting cardiovascular, respiratory, and nociception sensory information between the lower brainstem (NTS) and spinal cord to the forebrain. In fact, the PBN has dense ascending projections to the CEA demonstrating its capacity to intercommunicate visceral information with the emotional centers of the brain (195, 328). Alternatively, the CEA sends projections back to the PBN exhibiting yet another region the amygdala can modulate (242, 292). Interestingly, a portion of the CRH and GABA neurons in CEA send projections to the PBN (169, 270). Moreover, CRH infusion into the CEA increases neuronal activity in the PBN (411). Additionally, CRH administration into the PBN has been reported to increase arginine vasopressin release, suggesting the PBN also has the ability to modulate hormone release (43). The existence of an efferent projection from the PBN to the CEA suggests that the PBN may provide a feedback over CRH neurons in the CEA, which would support the current study's increased neuronal activation of CRH neurons following acute stress in the SHR (Fig. 5-1). The notion of a feedback system can be supported by the dense population GABA interneurons within the subnuclei of the amygdala that can serve as an interface between the major input and output neurons within the CEA (368). Additional studies regarding the relationship between the PBN and the CEA are warranted in that they could uncover a direct link between behavioral and cardiovascular regulation during stress.

Aside from its connections with the amygdala, the PBN is better known for modulating both baroreflex function and respiration. In fact, lesions of the PBN produced plasma increases in NE and renin as well as increased baroreflex sensitivity (164, 322). Furthermore, chemical and electrical stimulation of the PBN has been shown to markedly attenuate the baroreflex mediate depression of MAP and renal sympathetic nerve activity suggesting the PBN provides

an inhibitory influence over baroreflex activity (146). Stimulation of the PBN can also generate a pressor response indicating the PBN connects directly with the cardiovascular centers in the brain (199, 247). Due to the reduced baroreflex sensitivity in the SHR, it is possible the PBN may be exerting too much inhibitory tone on baroreflex circuits, leading to activation of sympathoexcitatory regions in the brainstem, including the RVLM. Nevertheless, the contribution of the PBN to baroreflex dysfunction in the SHR remains to be evaluated. The PBN also adjusts ventilatory function during various respiratory challenges (241). In the current study, GABA A receptor antagonism reduced the respiratory rate during the initial portion of AJS. It is possible the disinhibition of CEA modulated descending excitation of respiratory signals integrated by the PBN (139). Overall, the PBN maintains a considerable influence over the cardiovascular and respiratory systems and could possibly influence the amygdala's regulation over the autonomic nervous system during stress.

Correlation between Anxiety and the Cardiovascular System

The connection between the mind and body has become increasingly important within the clinical setting as more studies provide evidence pertaining to psychosocial states leading to progression of cardiovascular diseases. Diagnosis of cardiovascular pathologies can also trigger negative affective behaviors that can lead to further progression of the disease (117, 129). The psychosocial demands that affect the cardiovascular system include level of anxiety, social isolation, personality traits, and work stress (310). For example, individuals with high trait anxiety demonstrate a disruption in the activation of the amygdala to recognize emotional differences within faces and this is coupled with low HRV (248). Similarly, pre-atherosclerosis patients demonstrate individual differences in the reactivity of their amygdala which may correlate with level of risk for development of artherosclerosis (113). Psychological disorders and high stress levels can also lead to increased development of inflammation and infection

which can exacerbate or generate issues within the vasculature (185, 342). Both depression and anxiety increase proinflammatory cytokines including IL-6 (79, 220). Additionally, IL-6 and C-reactive protein levels are both used as risk factors for myocardial infarction (302). In fact, the level of circulating cytokines in patients with cardiovascular disease positively correlates with an increase risk of death (140). Finally, both psychological and cardiovascular pathologies demonstrate reduced HRV and withdrawal of vagal drive (1, 135, 231, 345). Due to the impact of psychological state on the cardiovascular system, therapies are now including behavioral and life style changes. Exercise, a strong support system, breathing exercises, and a positive attitude have all been shown to increase HRV, reduce morbidity, lower blood pressure, and improve immune function in patients with poor cardiovascular health (10, 185, 282, 417). Essentially, negative psychological states in combination with stress can lead to the development of various cardiac pathologies which can be adjusted by behavioral modification.

Conclusion

Established literature and the current study have demonstrated the exaggerated cardiovascular reactivity to stressors in the SHR may be due to dysfunction within the amygdala. Our results suggest an alteration in GABA receptor sensitivity or GABA release within the amygdala contributes to the exaggerated stress response. This was indicated by the attenuation of the cardiovascular response to an acute stress by GABA A blockade. Furthermore, we now propose that an upregulation of CRH projections from the CEA to the PBN may contribute to the increased cardiovascular and respiratory responses observed during stress in the SHR (Fig 5-1). Particularly, the high levels of Ang II in the SHR can affect the balance of GABA and glutamate in the amygdala while CORT excites CRH neurons leading to the exaggerated response to stress. Future studies regarding the role of the amygdala in the regulation of the cardiovascular response to stress in the SHR should address the role of Ang II and CORT as well as glutamate.

Furthermore, stress experiments should include both behavioral and cardiovascular measures since they are both highly interconnected. In summary, the amygdala has demonstrated the capability to alter the integrity of the autonomic response to stress in the SHR indicating negative emotions and behaviors can either lead to the development or maintenance of cardiovascular pathologies.

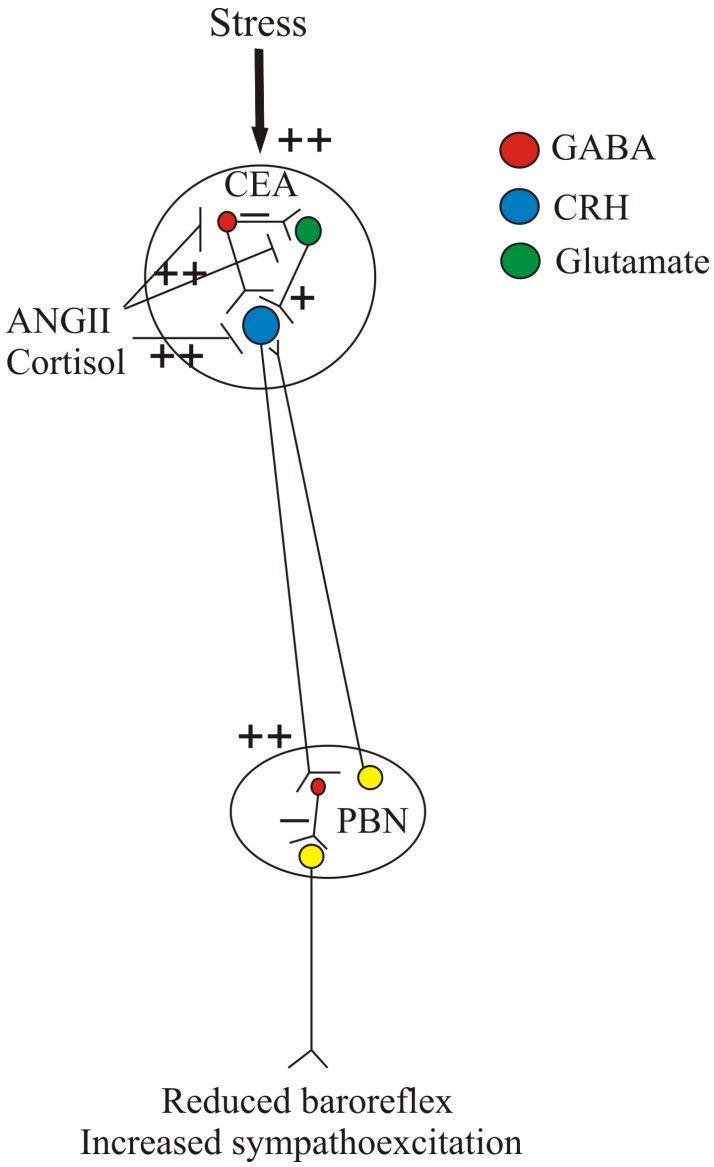

Figure 5-1. Hypothesized pathway involved in the cardiovascular response to stress in the SHR. Stress causes excitation of the CEA leading to activation of glutamate and CRH neurons. At the same time, GABA interneurons within the amygdala are inhibited. CRH efferents project to the PBN activating GABA interneurons reducing descending excitation of baroreflex circuits, leading to sympathoexcitation. In the SHR, high levels of both Ang II and CORT increase excitation of CRH driving the exaggerated cardiovascular response. Ang II can also activate GABA activity. Additionally, the dysfunction of GABA neurons allows for increased glutamate activity which can activate CRH neurons. Finally, a feedback pathway may exist from the PBN regulating CRH activation within the CEA. CEA, central nucleus of amygdala; GABA, γ-aminobutyric acid; CRH, corticotropin releasing hormone; PBN, parabrachial nucleus; AngII, angiotensin II; CORT, cortisone.

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BIOGRAPHICAL SKETCH

Karen Porter was born and raised in Cortez, CO. After graduating with a Bachelor of Arts degree in Environmental, Population, and Organism Biology from University of Colorado, Boulder in May 2003, she worked at Array Biopharma, Inc. and Gene Check, Inc. to prepare for graduate school. Karen joined the laboratory of Dr. Linda Hayward in May of 2005 and studied stress-induced cardiovascular hyperreactivity in a hypertensive model. She was awarded a Pre-doctoral Fellowship from the Florida/Puerto Rico affiliate of the American Heart Association during her graduate career. Karen received her Ph.D. in Biomedical Sciences-Physiology and Pharmocology from the Department of Physiology and Functional Genomics in the College of Medicine in December 2009 while concurrently starting a post doctorate position in the Department of Nutrition and Exercise Physiology in College of Pharmacy at Washington State University, Spokane.