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CENTRAL NUCLEUS OF THE AMYGDALA AND THE DEVELOPMENT OF HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS

by

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ABSTRACT

Electrolytic lesions of the central nucleus of the amygdala (ACe) have been shown to attenuate the development of hypertension in spontaneously hypertensive rats (SHR). Whether this was due to destruction of local neurons and/or fibres of passage is unknown. In the present study, neuronal perikarya in the ACe of 4 week-old SHR were selectively destroyed with ibotenic acid. Three separate experiments were conducted, in which mean arterial pressure (MAP), heart rate and blood pressure responses to acute mental stress were measured in groups of lesioned and sham-lesioned SHR. In Experiment 1, in which rats were fed *ad lib.*, lesioned SHR had a significantly lower average MAP (173 mmHg ± 7 S.E.) vs. sham-lesioned SHR (201 ± 4), 15 weeks post-operation ($p<0.05$, t-test). These results show that the attenuation of the development of hypertension in young SHR is due to the selective destruction of neurons in the ACe. The lesioned animals in Experiment 1 also had significantly lower body weights (BW) from 5 weeks post-operation onwards ($p<0.05$, two-way repeated-measures ANOVA). Therefore, in Experiment 2, food intake (and hence BW) among the lesioned and sham-lesioned rats was equalized. Average MAP in the lesioned SHR at 7 and 15 weeks post-operation was not different vs. sham-lesioned SHR, but was significantly higher (190 ± 9) vs. sham-lesioned SHR (164 ± 5) 22 weeks post-operation ($p<0.05$, t-test). These results indicate that destruction of neuronal perikarya in the ACe in young SHR merely delays the development of hypertension, due to a reduced BW gain. In Experiment 3, the effect of a high salt diet in ACe-lesioned SHR was examined. No significant differences in MAP were measured between lesioned and sham-lesioned rats 4 or 11 weeks post-operation.

TABLE OF CONTENTS

LIST OF FIGURES

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LIST OF ABBREVIATIONS

ACe	central nucleus of the amygdala
ADH	antidiuretic hormone
ADN	aortic depressor nerve
Ang II	angiotensin II
AV3V	anteroventral third ventricle
BNST	bed nucleus of the stria terminalis
BP	blood pressure
BW	body weight
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CSN	carotid sinus nerve
CVLM	caudal ventrolateral medulla
DLH	D,L-homocysteate
DMH	dorsomedial nucleus of the hypothalamus
DMV	dorsal motor nucleus of the vagus nerve
ECF	extracellular fluid
GABA	γ-aminobutyric acid
HR	heart rate
HRP	horseradish peroxidase
IML	intermediolateral cell column
LHA	lateral hypothalamic area
LV	left ventricle
MAP	mean arterial blood pressure
NA	nucleus ambiguus
NTS	nucleus tractus solitarius
PBN	parabrachial nucleus
PBS	phosphate buffer solution
PVN	paraventricular nucleus of the hypothalamus
RV	right ventricle
RVLM	rostral ventrolateral medulla
SHR	spontaneously hypertensive rat
SPN	sympathetic preganglionic neuron
WKY	Wistar-Kyoto rat

1 INTRODUCTION

Hypertension is a state of chronically elevated mean blood pressure (BP). The following sections outline the possible role of the central nervous system (CNS) and salt intake in its pathogenesis. An overview of the development of a rat model of genetic hypertension, the spontaneously hypertensive rat (SHR), will be presented first. This section also discusses some of the structural changes that may be the cause, or consequence, of hypertension, and the role of the kidney in long term BP control.

1.1.1 Spontaneously hypertensive rats

The SHR was bred from Wistar-Kyoto stock in the 1960s by Okamoto and Aoki [1963]. As the name implies, these rats develop hypertension spontaneously, i.e., without the need for external stimuli. The mean arterial blood pressure (MAP) of SHR starts to rise (relative to normotensive Wistar-Kyoto control rats) at four weeks of age. BP continues to increase until it levels off at 190 - 200 mmHg, when the rats are 16 to 20 weeks of age [Oxamide and Aoki 1963; Okamoto 1969].

A major role for a genetic defect in the CNS of SHR causing the development of hypertension was convincingly shown in grafting studies from two labs [Eilam *et al.* 1991; Deschepper *et al.* 1994]. In both studies, hypothalamic tissue was taken from 16 day-old SHR embryos and implanted into the 3rd ventricle of 9-10 week old normotensive Wistar-Kyoto rats. Four to six weeks after placement of the graft, BP in the hosts was 30-50 mmHg higher than in sham-operated rats. In one of the studies the host rats were followed for four months in which the BP elevation was shown to be maintained [Eilam *et al.* 1991]. These studies suggest that a genetically-linked dysfunction in the CNS of SHR results in the

development of hypertension in these animals.

As an example of CNS dysfunction, the activity of the sympathetic nervous system is increased in weanling SHR [Yamori 1976]. This general increase in sympathetic activity has also been demonstrated in 12 week-old SHR [Engelmann *et al.* 1987]. Many studies support the hypothesis this increase in sympathetic activity in young SHR contributes to the development of hypertension. Neonatal sympathectomy in SHR can prevent hypertension [Provoost and de Jong 1978; Oparil and Cutilletta 1979]. Similarly, sympathectomy in one week-old SHR, using a combined treatment of nerve growth factor antiserum and guanethidine, resulted in significantly lower pressures compared to intact control rats at 28 weeks of age [Lee *et al.* 1986]. Collis *et al.* [1980] also reported an increased quantal release of norepinephrine terminals in the kidneys in young SHR vs. age-matched Wistar-Kyoto rats (WKY) during electrical stimulation of the renal nerve. These results support previous findings that suggested that young SHR may develop hypertension in response to increased sympathetic activity during early growth [Johnson and Macia 1979]. In another study, the induction of ornithine decarboxylase was used as an enzymatic marker in the heart to study the development of functional sympathetic neurotransmission [McCarty 1986]. It was found that sympathetic control of the heart appeared earlier in SHR (at 2 days of age) than in WKY pups (8 days), and that heart rate (HR) is more responsive to sympathetic stimulation from two to eight days of age in SHR vs. WKY. These findings suggest that the SHR have a transient period in their development where they exhibit both an increased sympathetic outflow to the heart as well as an increased sensitivity to sympathetic activity and increased quantal release [McCarty 1986].

SHR hypertension may also be a result of mechanisms beyond the CNS. Renal nerve dysfunction has been suggested to be involved in the development of hypertension in young SHR [Liard 1977; Winternitz *et al.* 1980]. In the study by Winternitz *et al.* [1980], the renal sympathetic nerves of young (7 week-old) SHR were shown to be involved in the development of hypertension. Bilateral renal denervation delayed the onset of hypertension as shown by measurement of systolic BP two weeks after operation, and kidney sodium excretion was lower in the denervated SHR after 7 weeks. Thus, it was concluded that the renal sympathetic nerves contribute to the development of hypertension in young SHR by causing increased sodium retention [Winternitz *et al.* 1980].

The kidney receives extensive and exclusive adrenergic innervation of the afferent and efferent arterioles, the proximal and distal tubules, the ascending loop of Henle and the juxtaglomerular apparatus [Barajas 1978]. The increased sodium retention by the SHR kidney suggested by Winternitz *et al.* [1980] may be due to the abnormal distribution of adrenoreceptors in the kidneys of these animals. Pharmacological stimulation of renal α_2-adrenoreceptors in adult Sprague-Dawley rats increased sodium reabsorption in the kidney [Smyth *et al.* 1984]. These α_2-adrenoreceptors were found to be increased in number in young (10 to 13 week old) SHR [Sanchez and Pettinger 1981]. These findings were supported by Sripanidkulchai and Wyss [1987], who found that the number of binding sites of two types of α_2-adrenoreceptors appeared to be increased in neonatal SHR. An up-regulation of the number or affinity of α_2-adrenoreceptors in the SHR kidney may contribute to the pathogenesis of hypertension through increased sodium retention.

1.1.2 Central nervous system and hypertension

The CNS plays a role in the regulation of every aspect of the cardiovascular system, and abnormalities in its function have been implicated in the pathogenesis of hypertension. Outlined below are some aspects of CNS regulation of the cardiovascular system, and how irregularities in its function may be involved in the pathogenesis of chronic high BP.

Some studies suggest that a resetting of the baroreceptor reflex to a higher threshold causes hypertension to develop. Sinoaortic denervation in dogs has been shown to cause hypertension [Hering 1924], suggesting that a malfunction in some property of baroreceptors may induce or aggravate the development of the disease. In another study, baroreceptor resetting was shown to occur over several days in dogs [Sleight *et al.* 1977], which suggests that the baroreflex works to keep BP down in opposition to experimentally induced hypertension. This slow resetting was thought to indicate that the baroreflex is powerful enough to control BP over a prolonged period, and that its dysfunction would be a strong enough mechanism to cause hypertension.

Whether baroreceptor dysfunction is a cause or a consequence of hypertension is as yet unresolved [Sleight 1991]. Some studies suggest that the baroreceptor reflex is <u>not</u> involved in long term BP control, and that hypertension causes the characteristic "resetting" of the level about which the baroreflex functions. For example, McCubbin *et al.* [1956] experimentally elevated BP in dogs by inducing renal hypertension through cellophane perinephritis. They removed one kidney, and wrapped the other in cellophane, which leads to the formation of a fibrous capsule around the kidney that acts to compress the organ and cause intrarenal ischemia [Page 1939]. The dysfunctional kidney then activates mechanisms

that raise systemic BP to increase renal perfusion. McCubbin *et al.* [1956] showed that, instead of continuing to discharge at an elevated frequency which would trigger various mechanisms to reduce BP (see section 1.2.1), baroreceptors resumed a discharge rate more appropriate to a normal BP. This is most likely caused by a rapid resetting of baroreceptor sensitivity as demonstrated by Krieger [1970], who suggested that the baroreflex acts instead to maintain hypertension. In a study by Cowley *et al.* [1973], sinoaortic denervation was used to eliminate any control function of the baroreflex, and was reported to increase only the lability of pressure in dogs, without resulting in hypertension. In another study in rabbits, chronic renal hypertension caused a reduction in baroreceptor sensitivity associated with lesions and thickening of arterial walls after 7 to 20 weeks [Angell-James 1973]. This suggested that a high BP may lead to changes in arterial vessel wall structure which in turn reduces the sensitivity of baroreceptors. It should be noted that this presents a possible secondary mechanism for baroreceptor desensitization, as its time course differs from the one presented above. All of these studies suggest that baroreflex dysfunction is not the cause, but an effect of hypertension. It is generally thought that the short-term control of the cardiovascular system mediated by the baroreflex is not able to cause hypertension through long-term malfunction; however its dysfunction may be involved in the pathogenesis of the disease.

Nucleus tractus solitarius (NTS) (see section 1.3.2) malfunction has also been implicated in the pathogenesis of hypertension. Bilateral electrolytic lesions of the NTS in the rat have been reported to abolish baroreceptor reflexes and elicit a sharp (though transient) rise in BP [Doba and Reis 1973; Zandberg *et al.* 1978]. These studies suggested

that abnormal NTS function may serve to initiate a rise in BP during the development of hypertension. However, it must be noted that the NTS was ablated electrolytically in these studies, which may have affected cardiovascular areas beyond the NTS by destroying fibres of passage.

Abnormal levels of central neurotransmitters have been found to exist in the NTS of SHR, suggesting that altered neurochemical processes may be involved in its dysfunction. Studies have measured higher norepinephrine, dopamine and epinephrine levels of the A2 region of the NTS in 16 week-old SHR vs. WKY [Versteeg *et al.* 1976]. However, as the hypertension in these SHR was clearly established (systolic BP 196 ± 2 mmHg vs. 125 ± 1 in WKY), it is unclear whether these altered catecholamine contents were cause or consequence of the hypertension. The glutamate [Okamoto 1969] content in the NTS of young SHR is also higher vs. young WKY. This evidence suggests that abnormal neurotransmitter levels in young SHR may contribute to NTS dysfunction and eventually lead to the development of hypertension. These lesion and neurotransmitter studies require refinement before it can be determined whether the NTS is involved in the development of hypertension in SHR.

Another central area that may be involved in the development of hypertension is the paraventricular nucleus of the hypothalamus (PVN). The CNS controls kidney function through a feedback loop between the PVN and renal sympathetic neurons [Wyss *et al.* 1990]. In a study by Krukoff and Calaresu [1982], metabolic activity in the parvocellular PVN (which projects to autonomic sites in the brainstem and spinal cord [Swanson and Kuypers 1980; Kuypers and Maisky 1975]) and magnocellular PVN (involved in the synthesis,

storage and release of antidiuretic hormone (ADH)) was found to be significantly higher in adult (300 g) SHR vs. normotensive rats. Increased activity in the PVN could lead to increased sympathetic outflow to the kidney, and thus lead to inappropriately high levels of renin release and sodium retention [Sripanidkulchai and Wyss 1987; Slotkin *et al.* 1987] - finally causing the development of hypertension.

The rostral ventrolateral medulla (RVLM) (see section 1.3.3) has also been implicated in the pathogenesis of hypertension in SHR. γ-aminobutyric acid (GABA) injection into the RVLM in young anaesthetized, paralyzed SHR caused smaller depressor responses vs. those in age-matched normotensive control rats [Kubo *et al.* 1986]. This presents the possibility that a similarly decreased responsiveness to endogenous GABA in the sympathoexcitatory RVLM, which receives GABAergic input from the caudal ventrolateral medulla (CVLM) [Willette *et al.* 1984a; Agarwal *et al.* 1989; Agarwal *et al.* 1990], may be involved in SHR hypertension.

Other CNS regions that have been implicated in the development of hypertension are circumventricular organs lacking a blood-brain barrier: the anteroventral third ventricle (AV3V) region and the area postrema. The AV3V area in the rostral hypothalamus is involved in fluid homeostasis [Johnson 1985]. This function has been established mostly through ablation studies. Electrolytic destruction of the AV3V typically causes acute adipsia associated with a deficit in ADH release, with no direct effect on other processes such as eating, grooming, and locomotor activity [Buggy and Fisher 1977]. In other studies, ablation of the AV3V caused a decrease in BP due to systemic dehydration and prevented the development of hypertension in some models of hypertension [Panneton and Loewy 1980;

Brody *et al.* 1978]. In contrast to these studies in normotensive rats, AV3V destruction in SHR did not attenuate the development of high BP [Wyss *et al.* 1990]. This would suggest that SHR are unresponsive to AV3V function, since the destruction of this circumventricular area did not reproduce the effects on BP reported in other animal models. It was suggested by Wyss *et al.* [1990] that some disturbance in AV3V osmoregulatory function may be involved in the pathogenesis of hypertension.

The area postrema is located in the floor of the 4th ventricle in the dorsal brainstem, and is closely associated with the NTS . It receives afferent input from the buffer nerves [Ciriello *et al.* 1981; Panneton and Loewy 1980]. Low-intensity (< 100 μA) stimulation of the area postrema in the anaesthetized rat results in a short-latency decrease in both BP and HR [Ferguson and Smith 1991]; these effects are not due to current-spread to the juxtaposed NTS. Vagotomy abolished the bradycardic response, but not the drop in BP; in addition, combined α- and β-blockade had no effect on either HR or BP. When atropine was given, both the depressor and the tachycardic response to electrical stimulation of the area postrema were abolished. This evidence strongly suggests mediation of the depressor response by muscarinic ACh receptors. Electrolytic lesions of the area postrema in normotensive rats cause long-lasting (>one week) lowering of resting BP (by about 15 mmHg) and HR (by about 90 bpm), and a significantly more sensitive baroreflex control of HR [Skoog and Mangiapane 1988]. It is rather difficult to reconcile these findings with the effects of electrical stimulation of the area postrema as discussed above. Lesions in the area postrema of 5 week-old SHR attenuate the development of hypertension and cause significant bradycardia when measured up to 16 weeks of age [Mangiapane *et al.* 1989]. It was

concluded that the area postrema may be involved in maintaining an increased neurogenic drive that increases BP in SHR [Mangiapane *et al.* 1989].

1.1.3 Kidney

The autonomic nervous system is involved in acute BP control, reacting within seconds and minutes through the baro- and chemoreflex to quickly rectify potentially harmful changes in the level of BP. It has been argued that the baroreflex has no function in long term pressure control (see section 1.3.2). The kidney, on the other hand, functions to maintain BP over long periods of time, and renal malfunctions do result in chronic hypertension. This section briefly outlines mechanisms through which the kidney controls systemic BP, and how hypertension can result from renal dysfunction.

Blood pressure and volume are controlled by the kidney through the mechanisms of pressure natriuresis (sodium excretion) and diuresis (water excretion) [Selkurt 1949]. Both salt and water excretion by the kidney are increased during increases in BP, and pressure natriuresis and diuresis activity declines as BP returns to normal. These two processes are important to long term BP control because they both function until the set-point of BP (as dictated by the CNS) is reached, providing an "infinite gain" control. It is believed that pressure natriuresis and diuresis act through direct increases in glomerular filtration rate and filtered sodium load [Hall *et al.* 1984]. Various experiments have demonstrated the importance of the pressure diuresis and natriuresis mechanisms in maintaining BP during experimental hypertension induced by infusion of aldosterone, angiotensin II (Ang II), vasopressin, or norephinephrine and adrenocorticotropic hormone [as reviewed by Hall *et al.* 1986].

A decrease in kidney perfusion pressure (due to, for example, systemic blood volume reduction, a change to upright posture or renal artery constriction) or increased sympathetic activity stimulates renin release from the granular cells of the afferent arterioles of the juxtaglomerular apparatus [Taugner *et al.* 1979]. In a well known sequence, renin then cleaves angiotensinogen to angiotensin I, which in turn is cleaved by angiotensin converting enzyme to Ang II. Ang II acts to increase systemic BP (and therefore kidney perfusion) in various ways. Ang II has long been know as a potent vasoconstrictor, increasing peripheral resistance, which along with an increase in cardiac output, increases BP [Page and Helmer 1940]. Ang II also increases BP by directly stimulating renal sodium and water retention through the cells of the proximal convoluted tubule [Liu and Cogan 1988].

Ang II also increases sodium and water retention by triggering the release of aldosterone from the adrenal cortex. Aldosterone has long been know to cause an increase in sodium retention by the kidney [Simpson and Tait 1952]. An autoradiographic labelling study in the rat nephron has shown that aldosterone promotes sodium reabsorption (and potassium excretion) at the cells of the late distal tubule and the cortical collecting duct [Farman and Bonvallet 1983]. Sodium and water retention increases systemic fluid (and eventually) blood volume. The vasoconstrictive action of Ang II works to increase BP within minutes of a drop in pressure, whereas aldosterone levels will increase to promote sodium retention 1-2 hours after a drop in BP is detected.

Ang II and increased stimulation of baroreceptors and osmoreceptors are some of the activators of the release of ADH (vasopressin) from the magnocellular PVN [as reviewed by Schrier *et al.* 1979]. ADH then not only acts to increase BP by increasing water

permeability in the cortical and medullary collecting duct [Abramow and Dratwa 1974], but it has long been known to be a potent peripheral vasoconstrictor [Oliver and Schafer 1895].

A negative feedback system limits the action of all these hormones: as BP increases, renal perfusion increases, and circulating renin (and therefore Ang II, aldosterone and ADH) decreases.

A series of experiments by Bianchi *et al.* [1974a] were designed to determine if the kidney could cause an hereditary type of hypertension. Three to four month-old Milan SHR, a breed of rat that spontaneously develops high BP starting at one month of age [Bianchi *et al.* 1974b], and normotensive Wistar rats of the same age were used in kidney transplantation studies. Three months after surgery, Wistar rats that were recipients of SHR kidneys had developed significantly higher tailcuff systolic BP vs. Wistars that received transplanted Wistar kidneys. Also, SHR recipients of Wistar kidneys were found to have significantly lower BP than SHRs that received SHR kidneys. Serum urea, used as an index of renal function because its concentration increases as glomerular filtration rate decreases, was higher in SHR that received SHR kidneys than in SHR receiving Wistar kidneys. This indicated that glomerular filtration rate was reduced in the hypertensive rats when compared to the normotensive SHR with Wistar kidneys. Thus, it was concluded that higher BP of rats given SHR kidneys was due to a functional abnormality of SHR kidneys present before transplantation [Bianchi *et al.* 1974a]. To determine whether this renal abnormality was the cause or the result of hypertension, Bianchi *et al.* [1974a] also performed transplantation of kidneys from young (100 g) pre-hypertensive SHR into age-matched normotensive Wistars. These experiments showed that Wistar recipients of SHR kidneys had higher BP than

Wistars receiving Wistar kidneys, three months after surgery. It was suggested by Bianchi *et al.* [1974a] that adult SHR do not possess an external factor that re-establishes hypertension if they have kidneys from normotensive rats. The pathogenesis of hypertension is probably not due to a single dysfunction (for example kidney malfunction), but is most likely a result of multiple abnormalities in SHR physiology. Bianchi's conclusion is likely overstated conclusion, as many researchers support the theory that other factors (for example CNS dysfunction) lead to SHR hypertension (see sections 1.1.1, 1.1.2).

1.1.4 Cardiovascular remodeling in hypertension

One of the first signs of structural adaptation of the vascular wall to increased BP occurs within hours, with increased cellular uptake and protein incorporation of amino acids [Folkow 1986]. More often, the adaptation to high BP occurs over days or weeks, such as those in the resistance vessels, heart, kidney, baroreceptors and veins [Folkow 1993]. A major structural change occurs in the proximal resistance vessels in hypertension [Folkow 1990]. As the small arteries compensate for higher internal pressure, they undergo "remodeling" (a rearrangement of a normal amount of vessel media around a smaller lumen) [Mulvany 1993]. The smaller lumen of a vessel necessitates an increase in pressure for the maintenance of "normal" blood flow. The adaptation of the resistance vessels is pronounced enough that this structural change alone may account for almost all of the rise is resting BP in SHR [Folkow *et al.* 1973]. The heart is also affected in hypertension, it undergoes growth that results in left ventricular concentric hypertrophy [Friberg 1985]. This response to increased pressure in the aorta is complicated by increased cardiac output demands which by itself results in left ventricular eccentric hypertrophy [Folkow 1993].

1.1.5 Salt

An inherited defect in sodium handling, such that the kidney requires a higher than normal perfusion pressure to maintain extracellular fluid (ECF) volume, has also been suggested as a cause of hypertension [Guyton *et al.* 1974]. The importance of ECF volume and its relationship to BP is exemplified by the fact that there is a significant increase in ECF volume prior to the development of renal hypertension [Lendingham 1953]. This increase in ECF volume is accompanied by a rise in cardiac output and BP [Lendingham 1953]. To reduce cardiac output to normal, total peripheral resistance increases to maintain a state of elevated BP (this response is typical of the chronic stage of hypertension [Guyton *et al.* 1974]). An antihypertensive effect is seen through the reduction of sodium intake, or the use of diuretics, to decrease ECF [Murphy 1950].

Sodium is involved in the pathogenesis of the SHR model of genetic hypertension. A study by Winternitz and Oparil [1982] showed that three weeks of an increased sodium intake in young SHR (starting at 7 weeks of age) increased BP by 25%, increased peripheral resistance due to higher sympathetic activity (as demonstrated by increased plasma norepinephrine levels and an exaggerated depressor response to ganglionic blockade) and decreased the time course of the development of high BP vs. young SHR on a normal sodium diet [Winternitz and Oparil 1982]. The increase in sympathetic vasomotor activity clearly points to the role that neurogenic factors may play in the development of hypertension in this model. In another study, a low sodium diet (starting at 4 weeks of age) was shown to reduce BP by 15 % in 15 week-old SHR [Berk and Finkelstein 1982], suggesting that a hypersensitivity to salt may exacerbate the development of hypertension

in genetically predisposed subjects. SHR also show increased preference for higher salt in their diet compared to normotensive rats [Sripanidkulchai and Wyss 1987], which suggests that SHR may be genetically "programmed" to increase their uptake of salt, thereby aggravating the development of the disease.

1.2 Central nucleus of the amygdala

A complex neuronal network in the CNS is involved in the control of the cardiovascular system (see Fig. 1). The CNS receives and processes visceral sensory information and sends out signals to effector organs to regulate BP, HR, cardiac output, blood volume and arterial blood gas tensions. Imbalances in the regulatory function of the CNS have been implicated in the pathogenesis of hypertension. The current study focuses on one of these CNS centres, the central nucleus of the amygdala (ACe).

1.2.1 Amygdala

The amygdala, a part of the limbic system, is a subcortical region in the anterior part of the temporal lobe [Rolls 1992]. It has long been implicated in the control of affective behaviour and associated cardiovascular effects. The function of the amygdala was first appreciated when Kluver and Bucy [1939] reported on the effects of temporal lobectomy in the monkey. Removal of the temporal lobe, including the amygdala, resulted in the development of a global hypoemotionality, an overall lack of fear and a general inability to recognize familiar stimuli [Kluver and Bucy 1939]. Another study reported a marked attenuation of defensive behaviour in cats following bilateral amydalectomy [Schreiner and Kling 1953].

More sophisticated techniques in more recent studies have implicated the amygdala

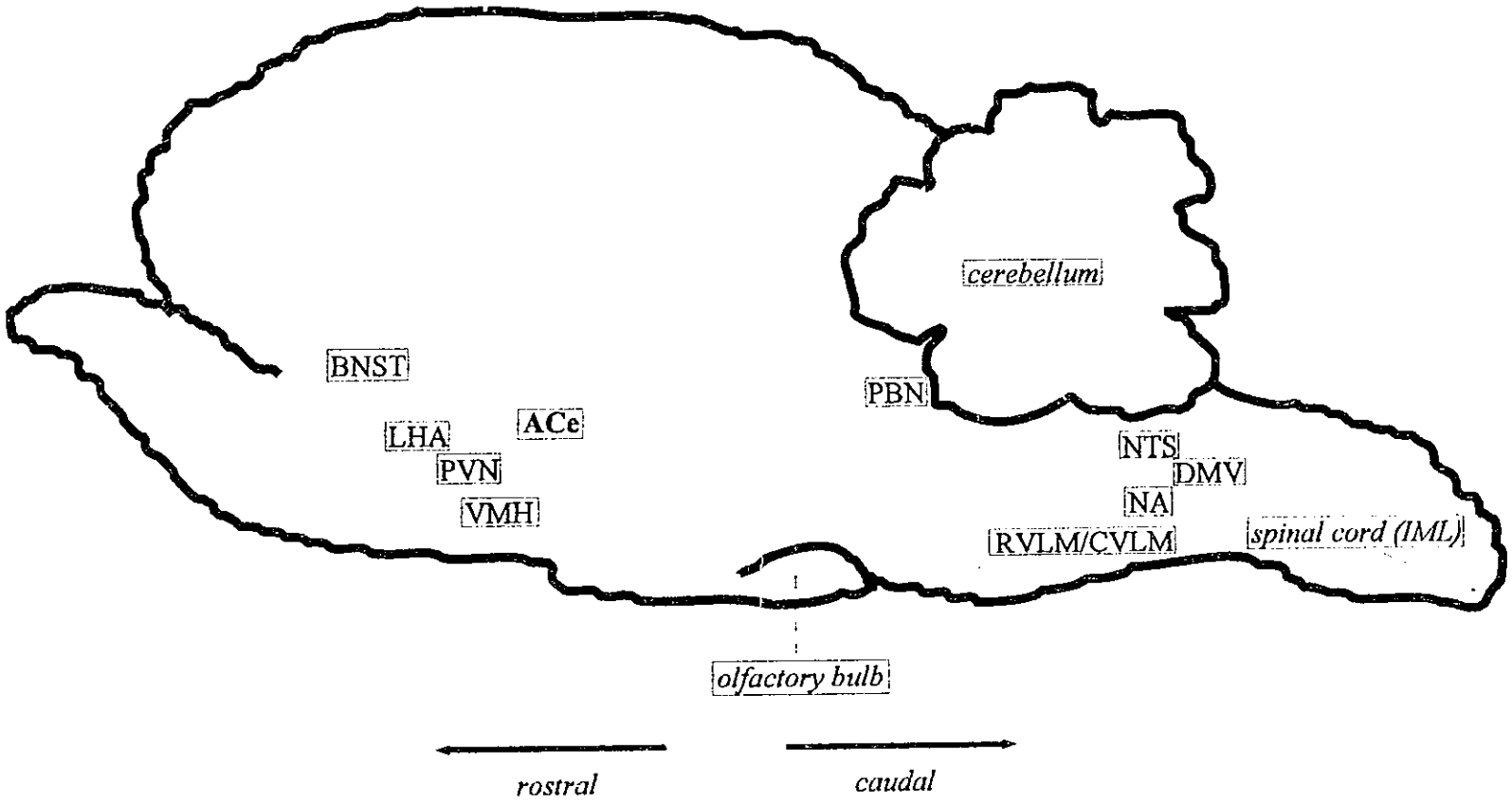


Fig. 1: Diagrammatic representation of parasagittal view of rat brain showing central areas involved in cardiovascular regulation. BNST = bed nucleus of the stria terminalis; LHA = lateral hypothalamic area; PVN = paraventricular nucleus of the hypothalamus; VMH = ventromedial hypothalamus; ACe = central nucleus of the amygdala; PBN = parabrachial nucleus; RVLM/CVLM = rostral/caudal ventrolateral medulla; NTS = nucleus of the solitary tract; NA = nucleus ambiguus; DMV = dorsal motor nucleus of the vagus; IML = intermediolateral cell column

in learning and memory functions. For example, destruction of the amygdala in monkeys by coagulation [Aggleton and Passingham 1981] resulted in an inability of the animals to display appropriate emotional behaviour in response to environmental stimuli. The amygdala has therefore been described as that part of the brain that extracts the emotional content from environmental stimuli and orchestrates a suitable behavioral and associated autonomic response [Kesner and Di Mattia 1987]. Such a view fits with the implication of amygdaloid function in the expression of fear and startle behaviour. Electrical stimulation of the amygdala in conscious humans elicits general responses of fear and anxiety [Chapman *et al.* 1954]. In rats, electrical ablation of the ACe attenuates fear-like behaviour [Roozendaal *et al.* 1991].

The amygdala may also affect intestinal function. Electrical stimulation of the centromedial area of the amygdala in the cat increases gastric motility and acid secretions [Zawoisky 1967]. Another study reported that stomach erosions are produced upon electrical stimulation of the ACe, suggesting a link between the ACe, stress, and the development of stomach ulcers [Henke 1988].

Investigations of the cardiovascular concomitant of emotional behaviour have shown that electrical stimulation of the ACe in conscious animals elicits an arterial pressor response, an increase in HR and muscle blood flow, and a decrease in mesenteric, renal and cutaneous flows [Hilton and Zbrożyna 1963; Galeno and Brody 1983; Gelsema *et al.* 1987; Timms 1981]. These effects, similar to those elicited naturally by psychological stressors, accompany behavioral changes that resemble arousal or fear [Hilton and Zbrożyna 1963; Galeno and Brody 1983; Gelsema *et al.* 1987].

The behavioral and autonomic responses to stimulation of the ACe in experimental animals resemble the defence reaction (see section 1.1.3). This response was later studied in more detail and associated with the "defence area" of the brain by Hilton and Zbrożyna [1963].

1.2.2 Cardiovascular control by ACe

Anatomically, the ACe has been implicated in cardiovascular regulation because it is connected to other CNS areas thought to be involved in cardiovascular control and because it receives baro- and chemoreceptor information from the periphery via the buffer nerves (see ACe connections below).

Physiologically, stimulation of the ACe elicits changes in BP and HR that vary with the method of stimulation and the subject's state of consciousness. Electrical stimulation of the ACe in the conscious cat causes pressor responses resulting from tachycardia and an increase in total peripheral resistance [Morin *et al.* 1952]. Similar increases in BP and HR were recorded in conscious rats after electrical stimulation of the ACe [Gelsema *et al.* 1987; Galeno and Brody 1983]. Electrical stimulation of the ACe in anaesthetized cats and rats has been shown to cause bradycardia - this was thought to be due to a stimulation-induced decreased sympathetic and increased parasympathetic tone [Faiers *et al.* 1975; Bonvallet and Gary Bobo 1972]. Galeno and Brody [1983] showed that electrical stimulation of the ACe in anesthetized SHR caused different hemodynamic responses in different vascular beds. In anesthetized rats, ACe stimulation caused a depressor response accompanied by a decrease in hindquarter vascular resistance, and little or no change in renal or mesenteric resistance. The discrepancies between results of studies in conscious vs. anaesthetized

animals are likely to be due to anaesthesia, which selectively affects changes in regional blood flow that bring about increases in BP [Gelsema *et al.* 1987; Timms 1981].

Another possible explanation for the inconsistent results of the above studies is that electrical stimulation of CNS targets cannot be isolated to activation of target cell bodies - fibres passing through the area are also affected. For example, bed nucleus of the stria terminalis (BNST) projections to hypothalamic and brainstem areas involved in cardiovascular control run through the ACe [Holstege *et al.* 1985], and the stria terminalis runs from the BNST through the caudal pole of the ACe [De Olmos *et al.* 1985; Paxinos and Watson 1986]. Thus, the cardiovascular effects of electrical stimulation of the ACe may, to an unknown extent, be due to activation of fibre bundles passing through the ACe.

To clarify the role of ACe neurons in cardiovascular control, Gelsema *et al.* [1987] stimulated the area in conscious and anaesthetized rats using both electrical stimulation and an excitatory amino acid, D,L-homocysteate (DLH). Neuroactive chemicals like DLH selectively activate local neurons, but avoid stimulating fibres of passage [Goodchild *et al.* 1982]. Electrical stimulation of the ACe in conscious rats resulted in behaviour that resembled fear, which was accompanied by pressor responses and tachycardia, similar to those seen by previous investigators. However, chemical stimulation with DLH in conscious rats elicited no BP, HR or behavioral changes. Similarly, the often reported depressor responses to electrical stimulation in anaesthetized rats were seldom seen upon chemical stimulation of the ACe . One conclusion of this study was the ACe may not be involved in the control of BP and HR since chemical activation of its neurons (with DLH) did not elicit cardiovascular effects [Gelsema *et al.* 1987].

1.2.3 Defence reaction and defence area

The defence reaction is an innate response elicited by environmental stimuli that are perceived as a threat to the survival of the individual. The response allows for a heightened level of activity to respond to the threat. The cardiovascular component of the defence reaction includes sympathetically mediated tachycardia, vasodilation in cerebral, coronary and skeletal muscle beds, and vasoconstriction in cutaneous and splanchnic beds. Increased cardiac contractility [Rosen 1961], cardiac output [Kylstra and Lisander 1970] and renal vasoconstriction [Abrahams *et al.* 1960] are also part of the defence reaction. These various cardiovascular effects usually lead to a rise in BP as a result of the increases in both cardiac output and total peripheral resistance, the latter being the net effect of vasodilation and constriction in the various beds. Other autonomic effects are an increased rate of respiration, pupillary dilation, and piloerection [Abrahams *et al.* 1960].

Hilton and Zbrożyna [1963] showed that the immediate cardiovascular effects accompanying the defence reaction could be mimicked by electrical stimulation of sites distributed over a large area of the brain of anaesthetized cats, stretching rostrally from the hypothalamus through the central mesencephalic grey to sites in the brainstem [Abrahams *et al.* 1960]. These sites were collectively named "the defence area" of the brain. The most rostral of these sites was later found in the amygdala: here the weakest stimulation current resulted in the largest cardiovascular responses [Hilton and Zbrożyna 1963].

1.2.3 Defence reaction and hypertension

In man, even mild mentally arousing stimuli, such as forced mathematical calculations [Brod *et al.* 1959] and computer games [Trap-Jensen *et al.* 1980] seem to elicit

the defence reaction. Such stimuli have been used in clinical studies to evaluate the status of central circulatory control in individuals, as several aspects of the defence reaction have been found altered in the prehypertensive and hypertensive state. In some studies, forced mental arithmetic causes larger and more sustained increases in HR and BP, not only in borderline hypertensive [Falkner *et al.* 1981], but also in normotensive [Widgren *et al.* 1992] individuals with a positive family history of hypertension vs. age-matched normotensives with a negative family history. An exaggerated reactivity to acute mental stressors has long been hypothesized to precede, and thus possibly contribute, to the development of hypertension, coronary heart disease, or both [Rostrup *et al.* 1993; Hines and Brown 1936]

It has been hypothesized that frequent activation of the defence reaction may contribute to the development of hypertension in SHR. Young SHR show intensified defence reactions (higher BP and HR responses) to threatening stimuli compared to control rats [Hällback and Folkow 1974; Lundin and Hällback-Nordlander 1980]. SHR also show exaggerated cardiovascular responses to environmental stressors, such as vibration or loud noise [Yamori *et al.* 1969]. It has been hypothesized that frequent elicitation of these defence responses, including increased sympathetic activity and elevated systemic BP, may be sufficient to cause structural vascular changes in SHR that maintain hypertension once developed [Folkow 1982].

Galeno and Brody [1993] showed that the stimulation of the ACe elicits physiological responses characteristic of the defence reaction. In the study, electrical stimulation of the ACe elicited a pressor response, tachycardia and renal and mesenteric vasoconstriction in conscious 12 to 16 week-old WKY and SHR. There were no significant

differences found between the hemodynamic responses evoked in WKY vs. SHR [Galeno and Brody 1983], suggesting that ACe dysfunction was not involved in the development of hypertension in SHR. These results were in direct contrast to the conclusions of Galeno *et al.* [1982], who suggested that ACe dysfunction was involved in the development of high BP in young SHR.

The ACe also appears to be involved in the endocrine response to stressful stimuli. Increases in corticosterone and renin during a stress response were suggested to be at least partly mediated by the ACe when ibotenic acid ablation of local neurons inhibited these increases after immobilization stress (produced by placing rats in small Plexiglas restrainers, inverting the restrainers for 20 minutes prior to decapitation) [Van de Kar *et al.* 1991]. In a study by Beaulieu *et al.* [1987], electrolytic lesions in the ACe attenuated noradrenergic activity in the hypothalamus and BNST during immobilization stress. It was concluded that the ACe may stimulate the noradrenergic system prior to secretion of adrenocorticotropic hormone.

The ACe may also partially control kidney function during stress responses. Air-jets directed at the head of SHR (used in the laboratory as a "mental stressor") invoked a stress response that increased MAP and renal sympathetic nerve activity, and decreased urinary sodium output [Koepke *et al.* 1987]. These effects could be blocked by prior injection of an α_2-adrenoreceptor antagonist (rauwolscin) into the ACe - demonstrating that pharmacological blockade of adrenergic receptors within the ACe could blunt renal nerve activity. These results present the possibility that the ACe is not only involved in acute cardiovascular responses to stressful stimuli, but also in long-term cardiovascular control,

through control of kidney function mediated by renal sympathetic nerve activity.

1.2.4 Connections and neurotransmitters

ACe connections

The ACe receives input from a number of areas throughout the brain, including those thought to be involved in cardiovascular control. The identification of NTS and buffer nerve projection to the ACe suggests that the ACe receives ongoing information about systemic BP. The ACe is reciprocally connected to the NTS: anterograde tracers injected into the NTS and retrogradely transported horseradish peroxidase (HRP) injected into the ACe have demonstrated efferent pathways from the NTS that presumably synapse with ACe neurons [Ricardo and Koh 1978]. HRP, or True Blue (a retrograde fluorescent tracer) has shown projections from the ACe back to the NTS [van der Kooy *et al.* 1984]. Activity from both the aortic depressor nerve (ADN) and carotid sinus nerve (CSN) reaches the ACe; recording of single unit activity in the ACe has shown that electrical stimulation of the ADN or CSN can alter the firing frequency of neurons in the ACe [Cechetto and Calaresu 1983]. It has also been shown that chemoreceptor activation in anaesthetized cats both inhibited and excited ACe neurons, while baroreceptor activation excited those neurons found to alter their firing frequency during chemical stimulation of the buffer nerves (see section 1.2.1) [Cechetto and Calaresu 1984]. Input to the ACe also comes from other cardiovascular areas such as the BNST [Russchen 1982; Krettek and Price 1978], paraventricular nucleus of the hypothalamus (PVN) [Hopkins and Holstege 1978], lateral hypothalamic area (LHA) [Krettek and Price 1978], parabrachial nucleus (PBN) [Swanson and McKellar 1979], nucleus ambiguus (NA) [Volz *et al.* 1990], the ventral tegmental area, locus coeruleus, the

raphe nuclei, periaqueductal grey [Otterson 1981] and the substantia nigra [Bunney and Aghajanian 1976].

Besides projecting to the NTS, the ACe also sends efferents to other cardiovascular areas in the brainstem. H^3-leucine injected into the ACe of cats autoradiographically labeled terminals in the PBN and locus coeruleus of the pons [Hopkins and Holstege 1978]. Parasympathetic output may be affected by the ACe through its efferent projection to the dorsal motor nucleus of the vagus nerve (DMV) [Hopkins and Holstege 1978]. The ACe also sends efferents to the LHA and BNST. Both of these areas are thought to be involved in cardiovascular regulation, since neuronal activation of these areas with glutamate has been shown to produce depressor responses and bradycardia in anaesthetized [Gelsema and Calaresu 1987; Hopkins and Armour 1982] and conscious rats [Gelsema *et al.* 1993]. These ACe connections present anatomical support for a functional role of the nucleus in cardiovascular regulation [Roder and Ciriello 1993].

ACe neurotransmitters

Many neurotransmitters have been identified within ACe neurons. Glutamate and aspartate are found in high levels throughout the amygdala, including the ACe [De Olmos *et al.* 1985]. The ACe is also rich in GABA - this may be due to a high degree of GABAergic afferent input to the area [Otterson 1982]. Somatostatin, neurotensin, corticotropin releasing factor, enkephalin, substance P, vasoactive intestinal peptide and galanin have all been identified within the neurons of the ACe [Cassell and Gray 1989a]. They have also been shown to be present in its projections to other areas [Cassell and Gray 1989a; Cassell and Gray 1989b].

1.3 Central nervous control of the cardiovascular system

This section (1.2) provides a basic overview of the areas in the CNS that form the central controlling network of the cardiovascular system. Not all areas of the brain suggested to be involved in cardiovascular regulation are discussed since emphasis has been placed on the functional role and anatomical interconnections of those areas implicated in the development of hypertension. Identification of the neurotransmitters involved in the function of these areas, and a detailed description of the effector mechanisms through which they bring about changes to the cardiovascular system, were considered beyond the scope of this thesis.

The following section describes the anatomy and physiology of one of the most-studied and best understood mechanisms by which the CNS maintains BP: the baroreceptor reflex. The ACe, the area of the brain that is the focus of the current study, is associated with the baroreceptor reflex - it projects to and influences the activity of many nuclei involved in the baroreflex (see section 1.1.4).

1.3.1 Baroreceptor reflex

There are five components to any physiological reflex: 1. receptors, which monitor the ongoing status of the controlled variable, 2. afferents, that convey receptor signals centrally, 3. a central processing area, that receives input from the receptors and determines what action is to be taken to maintain the controlled variable within preset limits, 4. efferents, which conduct controlling signals peripherally, and 5. effectors, which mediate changes to the controlled system.

The baroreceptor reflex exists to prevent excessive changes in BP in response to

changes in bodily postures and states. Maintaining an optimum BP provides the necessary constancy of tissue perfusion to maintain an appropriate environment for the survival and function of cells.

The function of the baroreceptor reflex can be described as follows. An increase in BP activates the baroreceptors. This increases baroreceptor afferent impulse traffic. Reflexively, vagal discharge to the heart increases, thereby slowing the heart. At the same time, sympathetic output to the heart and arterial resistance vessels (arterioles) decreases. The decreased cardiac drive leads to a cardiac slowing and a decrease in ventricular contractility, while the decreased vasoconstrictor drive widens the vessels, reducing total peripheral resistance and thereby reducing BP.

The baroreceptor reflex function of the carotid sinus was first described by Hering [1924], who reported that electrical stimulation of the carotid artery at the point of its bifurcation resulted in bradycardia and systemic hypotension in the dog. Decreased stimulation of receptors as BP falls in the upper body and head results in increased sympathetic and decreased parasympathetic outflow [Izzo *et al.* 1990]. This leads to peripheral vasoconstriction and increased cardiac output that increases and maintains adequate blood flow to the CNS [Izzo *et al.* 1990]. The receptors of the baroreflex are mechanoreceptors that respond to deformation of the wall of the blood vessels they innervate, the most prominent of which are the carotid sinus and aortic arch [Koch 1992]. A change in BP causing vessel wall deformation triggers a change in baroreceptor cell membrane polarization and a subsequent change in the firing frequency of the sensory neuron [Angell-James 1971; Bronk and Stella 1932]. This response does not occur if BP

changes without a change in vessel wall calibre [Angell-James 1971]. Baroreceptors are rate sensitive, i.e. the change in their activity (firing frequency) for a change in steady pressure is less than that obtained with equivalent sinusoidal pressure changes [Bronk and Stella 1935; Harada *et al.* 1992].

Afferent signals from the baroreceptors in the carotid sinus are conveyed to the brain stem via the CSN, a branch of the glossopharyngeal nerve [De Castro 1928], and signals from baroreceptors in the aortic arch travel via the ADN, a branch of the vagus [Cyon and Ludwig 1992]. These "buffer" nerves, so named because they function to resist changes in BP, terminate primarily in the NTS [Bronk and Stella 1932; Ciriello *et al.* 1981]. Histological evidence suggested that the projection of first-order afferents of the glossopharyngeal and vagus cranial nerves was to the NTS in guinea pigs and cats [Cottle 1964; Allen 1923]. Using the Nauta technique of silver impregnation of degenerating axons [Nauta 1957], Cottle [1964] observed axonal and terminal degeneration in the NTS after transection of the baroreceptor nerves in the cat. Autoradiography has shown that injection of H^3-proline into the ganglia of the glossopharyngeal and vagus nerves labels terminals throughout the NTS of the monkey [Beckstead and Norgren 1979]. This projection has been confirmed using retrograde tracing with HRP injected into the NTS in cats [Ciriello *et al.* 1981]. In addition to these anatomical studies, NTS neurons have been shown to increase their uptake of H^3 2-deoxyglucose upon electrical stimulation of the aortic nerve, or by stimulating aortic baroreceptors with pulsatile increases in BP in the rat [Ciriello *et al.* 1983]. This indicates that NTS neurons respond to baroreceptor discharge by increasing their metabolic activity. Based on this and other information (see section 1.2.2), the NTS is

considered the primary termination point of peripheral baroreceptor afferents, and is thought to be a key area in the processing of baro- and chemoreflex information.

Chemoreceptors monitor the tensions of blood gases (oxygen, carbon dioxide, and hydrogen ions) flowing through the chemosensitive regions of the carotid sinus and aortic arch. Low blood tension of oxygen, or high tensions of carbon dioxide and/or hydrogen cause chemoreceptors to fire at high frequencies. In the dog, stimulation of carotid body chemoreceptors with hypoxic blood [Bernthal *et al.* 1951], with nicotine, cyanide or other drugs [Heymans *et al.* 1931] caused bradycardia. Decreased contractility of the dog heart was observed by Downing *et al.* [1962] upon infusion of hypoxic blood. Stimulation of chemoreceptors with drugs (sodium cyanide, lobeline, sodium citrate) in the cat has also been shown to cause hyperventilation [Landgren and Neil 1952]. Chemoreflex-elicited decreases in cardiac contractility, HR and BP reduce the rate of systemic blood flow, allowing for a greater amount of time for gas exchange in the lungs and tissues. Compared to stimulation of those in the carotid body, activation of aortic chemoreceptors has been shown to be less effective: injection of hypoxic and hypercapnic blood to stimulate the aortic bodies in dogs triggered only 15% of the increase in ventilation seen during similar activation of the carotid bodies [Landgren and Neil 1952].

Regarding the central connections of chemoreceptor afferents, early studies did not differentiate between the central projection pathways of chemoreceptors and baroreceptors [Ciriello *et al.* 1981; Panneton and Loewy 1980]. A study of the NTS in the cat was the first to show that chemoreceptor afferents terminate in different subnuclei of the NTS from baroafferents [Donoghue *et al.* 1984]. Chemoreceptive afferents were isolated by identifying

those afferents that increased their rate of firing in response to increased arterial carbon dioxide tension [Donoghue *et al.* 1984]. An anatomical study by Claps and Torrealba [1988] in cats using retrograde transport of HRP found a similar pattern of projection of chemoreceptor fibres to the NTS. They injected HRP into the carotid body, which contains chemosensory dendrites, and avoided injection of the tracer into the carotid sinus, which contains barosensory nerve endings. These studies suggested that there are subnuclei in the NTS that integrate information from chemoreceptors separately from those receiving baroafferent signals.

1.3.2 Nucleus of the solitary tract

The first histological studies used degeneration techniques on intracranial rootlets to determine the termination sites of the glossopharyngeal and vagus nerves. Intracranial sectioning of the rootlets in the cat showed that the majority of afferents from these cranial nerves terminate in the NTS [Cottle 1964]. The results of these early transection studies have been confirmed by more recent neuroanatomical techniques (see 1.1.1) in the cat [Ciriello *et al.* 1981] and rat [Koch 1992]. To date, the NTS is regarded as the major visceral sensory relay cell group in the brain, receiving input from virtually all the major organs of the body [Loewy 1990]. The NTS has many subnuclei that receive organ- or modality-specific projections from visceral fibres, such as the dorsolateral/medial nucleus (cardiovascular receptors), the interstitial nucleus (respiratory tract receptors), the ventral/ventrolateral nuclei (pulmonary receptors) and the parvicellular nucleus (gastrointestinal receptors) [Loewy 1990].

NTS connections

The NTS is connected to many areas in the CNS suggested to be involved in cardiovascular regulation. In the brainstem, NTS neurons project to the RVLM and CVLM [Ross *et al.* 1985] and to both vagal nuclei: the NA and the DMV, the nuclei that mediate parasympathetic activity to the heart (and other viscera) via the vagus [Ross *et al.* 1985; Ricardo and Koh 1978; Nosaka *et al.* 1979]. Recently, an anatomical study showed that neurons of the rat-NTS project to the upper (mainly cervical) spinal cord, where descending solitariospinal fibers converge upon phrenic motor nuclei in the ventral horn [Mtui *et al.* 1993]. Some descending fibers end in/around the intermediolateral cell column (IML) of the upper thoracic cord, but were found only sparsely distributed in the lower thoracic segments. This specific distribution of terminals, combined with the unique origin of these fibers in the NTS (mainly from subnuclei receiving pulmonary mechanoreceptor and carotid chemoreceptor afferents) suggest a role for these fibers in respiratory control rather than cardiovascular control, although the latter cannot be fully excluded [Mtui *et al.* 1993]. In earlier studies concerning NTS connections with the forebrain, anterograde radiographic tracers injected into the NTS labelled the ACe, the dorsomedial and arcuate nuclei of the hypothalamus, the BNST and the median preoptic area [Ricardo and Koh 1978]. Retrograde tracing using HRP confirmed the presence of these NTS projections [Ricardo and Koh 1978]. Electrophysiological techniques have also demonstrated a reciprocal connection between the NTS and PBN [Jhamandas and Harris 1992] and the PVN [Coffey *et al.* 1988]. The high degree of connectivity demonstrated between the NTS and other cardiovascular areas gives anatomical support to its suggested role as an integrative centre for autonomic

control of the cardiovascular system.

1.3.3 Ventrolateral medulla

The ventrolateral medulla is the most critical area in the brain for the maintenance of resting systemic BP. Early studies showed that serial transverse sections of the medulla, starting rostrally and progressing caudally, had no effect on BP until a superficially located area in the RVLM was severed from the spinal cord, which caused BP to fall to spinal levels [Dittmar 1870; Owsjannikow 1871]. Chemical manipulation and electrolytic ablation of the ventral surface of the medulla was used for the first time almost a century later to more clearly define the cardiovascular role of the area. Topical application of the inhibitory neurotransmitter glycine within Perspex rings placed onto the ventral surface of the medulla in anaesthetized cats caused depressor responses that led to the definition of the "glycine-sensitive area" [Guertzenstein and Silver 1974]. Electrical stimulation of the glycine-sensitive area produced sharp, acute rises in BP in the cat, whereas bilateral electrolytic lesions in the area resulted in BP falling to severely hypotensive levels [Guertzenstein and Silver 1974]. It was concluded that the glycine-sensitive area played a key role in the maintenance of BP. Ten years later, the RVLM was described as the most sensitive area in the ventrolateral medulla from which increases in BP could be elicited by both chemical and electrical stimulation [Ross *et al.* 1984]. This finding implied that local cell bodies, rather than fibers of passage, were responsible for the hypertensive effects of local electrical stimulation.

Other evidence also suggested a role for the RVLM in the baroreceptor reflex. Activity of sympathoexcitatory neurons in the RVLM was shown to be synchronous with

the cardiac cycle and to be inhibited by electrical stimulation of arterial baroreceptors, exhibiting their barosensitive nature [Sun *et al.* 1988]. Granata *et al.* [1985] showed that the reflex depressor effect and bradycardia elicited in the rat by electrical stimulation of the left vagus (containing the bulk of the aortic baroreceptor afferents), or by mechanical stretch of the carotid sinus (activating baroreceptors) was abolished after placing bilateral electrolytic lesions in the RVLM containing the C1 cell group, synthesizing epinephrine (see RVLM neurotransmitters below). Such lesions also caused a precipitous drop in resting BP and HR, similar in size to the effect of spinalization. Their landmark study showed that the RVLM (and possibly the C1 group of neurons) mediate(s) the fall in BP and HR to baroreceptor activation, and that this area is responsible for maintaining resting levels of BP.

RVLM connections

The functional evidence pointing to an integrative role of the RVLM in cardiovascular control is supported by the identification of heavy projections from the NTS [Ross *et al.* 1985], PBN, spinal cord, central gray area, area postrema and hypothalamic defence areas [Ross *et al.* 1985; Loewy and Burton 1978] ending in the RVLM [Andrezik *et al.* 1981]. In addition, the RVLM was shown in the cat [Amendt *et al.* 1978] and rat [Zagon and Smith 1993] to project directly to the IML, the site of sympathetic preganglionic neurons (SPNs) in the lower cervical, thoracic and upper lumbar spinal cord that project to the heart and blood vessels.

Caudal ventrolateral medulla

Various studies have identified a group of neurons in the caudal part of the "medullary cardiovascular centre" that contain noradrenaline [Swanson and Hartman 1975;

Dahlstrom and Fuxe 1964]; this group of neurons came to be known as the "A1" cell group [Dahlstrom and Fuxe 1964]. The area of the A1 cells was first defined as a vasodepressor ("nicotine sensitive") area after topical application of nicotine in the cat resulted in a drop in systemic BP [Feldberg and Guertzenstein 1976]. Electrical stimulation of the CVLM in cats inhibited the discharge of sympathetic renal and splanchnic nerves, suggesting a sympathoinhibitory function for this area [Coote and MacLeod 1974]. Subsequent work (in the rabbit) showed that electrolytic or excitotoxic destruction of the area, or injection of inhibitory neurotransmitters (GABA, glycine) into the CVLM caused large elevations in BP [Blessing and Reis 1982; Willette *et al.* 1984a; Blessing and Reis 1983]. These experiments suggested the presence of tonically active sympathoinhibitory neurons in the CVLM.

CVLM connections

While its functional role was indirectly defined with pharmacological techniques, neuronal connections through which the CVLM mediated its effects remained undetermined. Most projections to the spinal cord from the ventrolateral medulla, as discussed earlier, were shown to be from the RVLM [Ross *et al.* 1984]. When a heavy projection of neurons was shown to exist from the CVLM to the RVLM [Ross *et al.* 1984; Willette *et al.* 1984b], it was suggested that CVLM function was mediated through the RVLM. This suggestion was supported by the finding that injection of tetrodotoxin, to inhibit RVLM function, abolished the decrease in BP, HR and sympathetic activity induced by chemical activation of the CVLM [Granata *et al.* 1985]. Subsequent experiments in which the GABA antagonist bicuculline was injected into the RVLM of the rabbit showed a dose-dependent decrease in the magnitude of the vasodepressor response to glutamate injection into the CVLM [Blessing

1988]. This suggested that the vasodepressor effects of CVLM activation were mediated by inhibition of RVLM function through a GABAergic projection [Blessing 1988]. Using extracellular single unit recording techniques, Agarwal and Calaresu [1992] showed that injections of kynurenic acid into the CVLM of anaesthetized rats, which block the glutamate-mediated excitatory effect of NTS afferents on CVLM neurons [Agarwal *et al.* 1990], also block the inhibitory response of cardiovascular neurons in the RVLM to experimentally induced BP elevations (systemic injections of phenylephrine) [Agarwal *et al.* 1989; Agarwal *et al.* 1990]. This finding added the first electrophysiological support to the notion of a multisynaptic central pathway through the NTS, CVLM, RVLM and IML, in that order, subserving the sympathetically mediated effects of the baroreceptor reflex.

The central components of the vagally mediated cardiac baroreflex follow in the next section.

1.3.4 Nucleus ambiguus and dorsal motor nucleus of the vagus

The majority of parasympathetic (vagal) neurons to the heart arise from the ventrolateral NA, although some originate in the DMV. This has been concluded from experiments in which the cardiac branches of the vagus nerves were saturated with retrogradely transported HRP [Nosaka *et al.* 1979; Hopkins and Armour 1982]. Confirming these anatomical findings, electrical stimulation of the cardiovagal nerves has been shown to activate neurons in both the NA and DMV [McAllen and Spyer 1976; Nosaka *et al.* 1982]. Further electrophysiological studies showed that neurons in these nuclei that were orthodromically activated by stimulation of the baroreceptor nerves could be antidromically excited by stimulation of the cardiac branches of the vagus nerve [McAllen and Spyer 1978;

Ciriello and Calaresu 1980]. This combination of responses suggests that these neurons are involved in the cardiac baroreceptor reflex.

A cardioinhibitory function of neurons in the NA and DMV could be concluded from the bradycardic effect of electrical [Nosaka *et al.* 1979; Calaresu and Pearce 1965] and chemical [McAllen and Spyer 1978; Machado and Brody 1988] stimulation of its neurons. Neurons in the NA and DMV were shown to be tonically active, being excited by ongoing baroreceptor activity and therefore showing a pulse-synchronous rhythm in their activity [McAllen and Spyer 1978; Jordan and Spyer 1986].

Although the DMV may play some role in cardiac control [Nosaka *et al.* 1979; Sporton *et al.* 1991], Kerr [1969] showed that parasympathetically mediated visceral (including cardiac) effects could still be elicited from the vagus nerve after electrolytic ablation of the DMV and subsequent degeneration of its efferents. This suggests a less important role for DMV vs. NA vagal neurons in cardiac control. However, the somata of the cardiac vagal neurons in the NA are larger than those in the DMV, which may correlate with different functions of the two nuclei in cardiac control [Nosaka *et al.* 1979].

NA and DMV connections

The NA is reciprocally connected to other neuronal cell groups involved in cardiovascular control, including the NTS [Kalia *et al.* 1979] and PBN [King 1980]. It receives projections from the BNST, SI, ACe, zona incerta, dorsomedial nucleus of the hypothalamus (DMH), and PVN, and from the lateral and posterior hypothalamus [Luiten *et al.* 1985; Luiten *et al.* 1987]. The DMV receives descending input from the insular cortex [Shipley 1982], the ACe [Hopkins and Holstege 1978; Schwaber *et al.* 1982], PVN [Luiten

et al. 1985], LHA [Berk and Finkelstein 1982], DMH, posterior hypothalamus, mesencephalic gray, PBN, NTS, medullary reticular formation, nucleus raphe obscurus [Luiten *et al.* 1987] and from the A5 cell group [Loewy *et al.* 1979]. This connectivity provides the anatomical substrate through which these cardiovascular control nuclei may alter parasympathetic output via NA and DMV neurons.

1.3.5 Intermediolateral cell column

Sympathetic nervous control of the cardiovascular system is mediated via pre- and postganglionic neurons. The somata of SPNs are located in the thoracolumbar spinal cord. The IML is one of four regions in the thoracolumbar spinal cord where SPNs are clustered, along with the lateral funicular nucleus, the intercalated cell group and the central autonomic nucleus [Cabot 1990]. It has been shown that SPNs are likely to be organized in function-specific clusters, i.e. that SPNs with a vasomotor function are located separately from those with a non vasomotor function [Jänig 1988]. Furthermore, function-specific SPNs may possibly be further subdivided into target-organ specific clusters [Jänig 1988].

Sympathetic nerve activity to blood vessels (vasomotor activity) depends greatly upon supraspinal control; this can be concluded from the BP-lowering effect of cervical bulbo-spinal transection. However, MAP in such experiments is not reduced to the much lower vascular filling pressure, but remains around 60 mmHg [Dittmar 1870; Owsjannikow 1871]. Systemic administration of a sympathetic blocking agent will lower BP further [Taylor and Weaver 1993]. This implies that SPNs are capable of generating spontaneous sympathetic outflow as a result of endogenous pacemaker activity and/or spinal reflexes.

IML connections

In a study designed to demonstrate central nuclei controlling sympathetic output, Strack *et al.* [1989] injected pseudorabies virus as a retrograde tracer into various sympathetic ganglia of rats to identify the spinal and supraspinal areas that innervate all major sympathetic ganglia. This study showed labeling in the SPNs in the spinal cord, and in projection neurons of the parvocellular PVN, the A5 cell group, the caudal raphe region, the RVLM and the rostral ventromedial medulla. Tracer injections into the superior cervical ganglion (mediating sympathetic innervation of the head) and into the stellate ganglion (mediating sympathetic innervation of the heart) identified projections from the central gray, LHA and zona incerta. Apart from these central nuclei projecting onto IML neurons, the NTS has also been shown to project directly to the IML [Loewy and Burton 1978], a finding recently confirmed by Mtui *et al.* [1993] using retrogradely transported HRP and anterograde (*Phaseolus vulgaris*) tracing. However, as discussed earlier (see section 1.1.2) this solitariospinal projection is unlikely to contribute greatly to cardiovascular control.

1.4 Rationale

The attenuated development of hypertension following ACe lesions in SHR has been attributed in the past to their diminished cardiovascular reactivity to acute stressors, and to the subsequent decreased incidence and duration of periods of elevated BP, HR and plasma catecholamines [Folkow 1982; Folkow *et al.* 1982; Galeno *et al.* 1982]. The argument was based on the following experimental evidence. First, cardiovascular responses to acute stressors are known to be exaggerated in SHR vs. normotensive rats [Hällback and Folkow 1974; Yamamoto *et al.* 1987]. Hällback and Folkow [1974] demonstrated that SHR

exhibited exaggerated cardiovascular responses to alerting stimuli such as loud noise and vibration. Second, Galeno *et al.* [1982] demonstrated that the increase in HR and BP, and the decrease in renal and mesenteric blood flow following exposure to acute stressors were all significantly smaller in ACe-lesioned vs. sham-lesioned SHR. Finally, Folkow *et al.* [1982] showed that the increase in MAP in amygdala-lesioned SHR exposed to a strong stressor was smaller to that in intact SHR.

Although two independent studies have suggested a role for the ACe in the development of hypertension in young SHR (Folkow *et al.* [1982]; Galeno *et al.* [1982]). Electrolytic ablation of brain tissue destroys cell bodies as well as fibres of passage [Goodchild *et al.* 1982]. There are fibres of passage within the ACe that connect areas involved in cardiovascular control [Holstege *et al.* 1985; De Olmos *et al.* 1985]. The interruption of these connections could affect cardiovascular variables (HR, BP). Also, Gelsema *et al.* [1987] showed that chemical stimulation (which only affects cell bodies) of the ACe in conscious rats does not elicit the depressor responses observed during electrical stimulation (which affects cell bodies and fibres of passage) of the same area. Therefore the results from these previous electrolytic studies are difficult to interpret and require a re-investigation of the role of the ACe in the development of hypertension in SHR by using an excitatory amino acid to selectively destroy its neurons. Excitotoxins provide more selective lesions compared to mechanical, electrolytic or radio-frequency techniques because they do not affect fibres of passage, and are specific to receptors on neuronal soma [Goodchild *et al.* 1982].

The purpose of this research is to test the hypothesis that the ACe is <u>not</u> involved in

the development of hypertension when its afferent and efferent connections are left intact following lesion placement.

BP measurements, obtained through carotid artery cannulas implanted up to 22 weeks after initial operation or sham-operation, will be compared between lesioned and sham-lesioned SHR. BP recordings will allow for comparison of MAP and HR between lesioned and sham-lesioned rats while the animals are at rest, and also during air-stress stimulation of the SHR. Because increases in BP can lead to the development of cardiac hypertrophy [Friberg 1985; Folkow 1993], measurements of cardiac anatomy will be obtained to determine if differences in BP between lesioned and sham-lesioned rats correspond to differences in the degree of cardiac hypertrophy between treatment groups. Also, because the ACe has been linked to feeding and body weight (BW) gain [Miñano *et al.* 1982; Hajnal *et al.* 1992], a group of SHR will be pair-fed to eliminate any possible BW differences between lesioned and sham-lesioned animals that may affect BP measurements [Wright *et al.* 1981]. Finally, another group of lesioned and sham-lesioned rats will be pair-fed a high salt diet to determine whether ACe ablation affects BP in SHR on high salt. This experiment was based on the following: 1. the development of hypertension is aggravated in SHR on a high sodium diet [Winternitz and Oparil 1982]; 2. neurogenic factors have been suggested to be involved in sodium-aggravated hypertension in SHR [Winternitz and Oparil 1982; Pawloski-Dahm and Gordon 1993]; 3. the ACe may be involved in the regulation of dietary sodium intake in rats [Zardetto-Smith *et al.* 1994]; 4. increased sodium intake may be positively correlated to increases in aggression. Increases in aggression may be mediated by the ACe through the defence reaction [Hilton and Zbrożyna 1963].

2 METHODS

2.1 General procedures

Experiments were done in male SHR (Taconic IBU3 colony, Germantown, N.Y.). All rats were housed individually in a climate controlled room under a 12 hour light/dark schedule. Tap water was made available *ad libitum*. BW was measured weekly to measure each animals' rate of growth.

2.2 Optimum lesion production and visualization

Five rats were used in preliminary studies to determine: 1. the optimum volume of ibotenic acid to be injected into the ACe for adequate ablation of the ACe, 2. the optimum thickness of cryostat-cut brain slices for lesion verification, 3. the quality of cresyl violet staining of sections for visualization of landmarks within the brain tissue, 4. the effect a 10% sucrose solution after perfusion of the brain with fixative to improve tissue sectioning technique.

Initial injectate volumes of 100 and 150 nl ibotenic acid (10 μg/μl) were selected to destroy the cells of the ACe. These volumes and their concentration were based on a survey of studies where the ACe was discretely and successfully ablated with ibotenic acid [Van de Kar *et al.* 1991; Lorenzini *et al.* 1991; Jellestad and Grahnstedt 1985; Jellestad *et al.* 1986; Riolobos and Garcia 1987].

Four sets of brain slices were collected from each brain with 4 different section thicknesses (15, 20, 30, 40 μm). The optimum thickness was determined by noting the ease of cutting, collecting, staining and utilizing the sections for identification of landmarks within the brain tissue.

Following dehydration and delipidization in a series of ethanol baths, cresyl violet was used to stain the brain tissue sections. This was done in order to compare results with previous studies [Van de Kar *et al.* 1991; Jellestad and Grahnstedt 1985; Jellestad *et al.* 1986; Riolobos and Garcia 1987; Hitchcock *et al.* 1989] which successfully destroyed the ACe with ibotenic acid and used cresyl violet to visualize the lesions. Cresyl violet was also compared to thionin to determine which stain produced better visualization of the lesioned brain tissue.

Following perfusion and removal of the rat brain from the skull, the brains were stored in a 10 % sucrose in 100 mM phosphate buffer solution (PBS). This procedure was performed in order to remove water from the brain tissue to limit damage due to ice crystal formation during freezing (cryoprotection).

After Experiment 1, it was noted that the clear demarcation of lesions in brains from animals with relatively short survival times (up to 7 weeks) was not as clear in animals with longer survival times (15 weeks); it was decided that an additional method for histological verification of ibotenic acid lesions could facilitate lesion verification for rats in the 22 week survival group in Experiment 2. An immunohistochemical technique (see below) was adopted for the visualization of a peptide neurotransmitter. It has been reported that several peptides are abundant in the cell bodies and/or afferent terminals of the ACe, and are sparse in surrounding areas [Cassell and Gray 1989a; Sar *et al.* 1978; Lind *et al.* 1985; Haring *et al.* 1991; Skofitsch and Jacobwitz 1985; Nguyen *et al.* 1986; Yasui *et al.* 1991]. It was hypothesized that these peptides may serve as good markers for the ACe, and that immunohistochemistry may allow differentiation of lesioned vs. sham-lesioned tissue based

on the distribution of these peptides following operation. Brain slices from 3 rats were therefore incubated with antibodies to either calcitonin-gene related peptide (CGRP), substance P, met-enkephalin or preprotachykinin to determine the best marker for the ACe in the current experiments.

Injection site determination

To determine the proper site of injection of ibotenic acid in the ACe of 4 week-old rats, 6 SHR were used in a preliminary study. The study was needed because the commonly used, detailed stereotaxic atlas of rat brain is based on brains of adult (250-300 g BW) rats [Paxinos and Watson 1986]. For these preliminary experiments, each rat was anaesthetized using urethane (Sigma, St. Louis, MO), and placed in a Kopf stereotaxic frame. The incisor bar was set such that the top of the skull was level between bregma and lambda . An incision was made to expose the top of the skull, after which holes were drilled to expose the area of the brain overlying the estimated location of the ACe. A glass micropipette (tip size 50 μm O.D.) filled with a 20 % solution of India ink in distilled water was then lowered into the area of the ACe. The site was marked with 5 nl of India ink (for methodology, see Injections below). After completion of a series of injections on either side of the brain, each rat was perfused through an intracardial needle with 150 ml of 10 mM PBS followed by 100 ml of fixative containing 4 % paraformaldehyde in 100 mM PBS. The brain was excised and post-fixed overnight in the paraformaldehyde fixative, frozen and then sectioned on a cryostat (AO Reichert Model 855, AO Reichert Scientific Instruments, Buffalo, NY). Alternating 30 μm thick slices were collected to make two complete sets of brain sections. One set of sections was melted directly onto coated microscopy slides (Fisher Scientific) for

visualization of India ink marks; the second set was stained with cresyl violet for visualization of landmarks within the brain tissue.

2.3 Surgery

Ablation of the ACe or sham-operation was done at 4 weeks of age (average BW approximately 93 g). Each rat was anaesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, 65 mg/kg BW, i.p.) and placed in a Kopf stereotaxic frame. The incisor bar was set such that the top of the skull was level between bregma and lambda, as in the preliminary experiments. Body temperature of the rats was maintained by a heating pad during surgery. The shaved scalp was cleaned with a 1% iodine solution prior to making an incision to expose the skull. The appropriate coordinates for destruction of the ACe in 4 week-old SHR were derived from the preliminary experiments. Small holes were drilled bilaterally, 2.1 mm caudal to bregma and 3.7 mm lateral to the midline to expose the brain overlying the ACe. A glass micropipette (tip size 50 μm O.D.) filled with a solution of ibotenic acid (10 μg/μL, Regis Chem. Co., Morton Grove, IL) in artificial cerebrospinal fluid (composition in mM: NaCl 133.3, KCl 3.4, $CaCl_2$ 1.3, $MgCl_2$ 1.2, NaH_2PO_4 0.6, $NaHCO_3$ 32.0, glucose 3.4; pH 7.4), or filled with artificial cerebrospinal fluid only, was lowered 7.5 mm below the surface of the brain into the ACe. Animals of the lesioned group received 100 nl of ibotenic acid solution bilaterally. The micropipette was left *in situ* for 10 min. to prevent backflow [Schwarcz *et al.* 1979a]. Control (sham-lesioned) animals received identical treatment, but only vehicle was injected. After completion of the bilateral injections the skull was cleaned and the scalp was sutured using 3-0 silk (Ethicon, Peterborough, Ontario). Penicillin G (30 000 I.U., i.m. Derapen, Ayerst Labs, Montreal,

Quebec) was injected after surgery to prevent infection.

Injections

Solutions were ejected from the micropipette over a period of 5 min. by applying pulses of pressurized nitrogen using a pressure ejection system (Picospritzer General Valve Corp., Fairfield, N.J.). Volumes were measured by viewing the movement of the fluid meniscus in the micropipette barrel (456 µm I.D.) using an operation microscope equipped with a previously calibrated eyepiece micrometer (Olympus, Tokyo, Japan).

2.4 Experiments

2.4.1 Experiment 1 - The effect of ACe ablation on the development of hypertension in SHR fed *ad libitum*

The pilot Experiment (Experiment 1) contained 15 lesioned and 15 control animals. Six additional 4-5 week-old SHR were used to determine pre-experimental MAP. Historical controls were used to record BP in 4-5 week-old SHR because chronic intra-arterial cannulas do not remain patent for prolonged periods of time. Cannulas eventually become blocked, rendering BP measurement inaccurate or impossible, and pressure measurement would require additional surgery to insert a second cannula. The rats were divided into a 7 week (N=6 lesioned, 6 control SHR) and a 15 week (N=9 lesioned, 9 control SHR) survival group. Normal rat chow was made available to Experiment 1 animals *ad libitum*.

2.4.2 Experiment 2 - The effect of ACe ablation on the development of hypertension in pair-fed SHR

Experiment 2 contained 24 lesioned and 24 control animals. Each group of 24 was divided into three survival periods of 7, 15 and 22 weeks, with 8 SHR in each sub-group. Six additional rats were used to determine pre-experimental MAP in 4-5 week-old SHR.

Since sham-lesioned SHR in Experiment 1 were found to be consistently heavier than the lesioned rats, it was assumed that they ate more, and each sham-lesioned rat was therefore given a limited amount of food - determined by the amount consumed by its lesioned partner the previous day. The food intake of each pair was matched to minimize weight differences between the lesioned and control rat groups.

2.4.3 Experiment 3 - The effect of ACe ablation on the accelerated development of hypertension in SHR induced by a high sodium diet

Experiment 3 contained 21 lesioned and 21 control SHR. A 4 week survival group consisted of 8 lesioned and 8 control SHR, and an 11 week survival group had 13 lesioned and 13 control animals. Animals in Experiment 3 were given an 8% NaCl diet (Harlan Teklad, Madison, WI), and were pair-fed to minimize weight differences between groups.

2.5 Measurements

Pulsatile pressure and MAP were measured via a carotid artery cannula made from PE-50 tubing. The cannula, filled with heparinized saline (100 I.U./ml) was inserted under halothane anaesthesia (Ayerst Labs, Montreal, Quebec, 2.5% in O_2). Cannulas were tunneled under the skin and exteriorized at the back of the neck. Rats were allowed to recover for a minimum of 24 hours before measurements were made.

For BP measurements, the carotid cannula was connected to a pressure transducer. The bridge output signal of the transducer was amplified (Transbridge TBM4, World Precision Instruments, Sarasota, FL) and fed to an IBM-AT compatible computer equipped with an A/D card (DT2821-F-16SE, Data Translation, Marlboro, MA) and programmed by a data acquisition program (DataSponge, Bioscience, Calgary, Alberta). BP and HR (obtained off-line by counting BP waves during recording period) were measured under

resting conditions, with the rats left undisturbed in individual cages. BP was monitored after an accommodation period of at least one half-hour. Once all rats were sitting or lying motionless, two minutes of pulsatile BP was recorded (250 Hz sampling rate). This sample was analyzed to determine resting BP and HR.

For measurement under stressed conditions, rats were placed in individual restraining cages (Centrap, Fisher Scientific) and two air nozzles were directed at their nose and hindquarters, respectively (see Figure 2). Timing and duration of the jets of air were controlled by remotely opening and closing electromagnetic valves placed in the nozzle input lines. Three air-jets lasting 5 s each were applied, separated by a minimum 5 minute recovery period. For off-line analysis, BP was recorded and stored for 60 s each before, during and after the air-jet. The cardiovascular effects of this mental stress [Ely *et al.* 1985] were calculated as the difference between the average level of MAP and HR during the 5 s immediately preceding the air-jet (baseline levels) and the peak levels of these variables reached during or immediately following the air-jet. In addition, the response integral was calculated for Experiment 2, being the product of time and the positive difference between instantaneous MAP levels during air-stress application and the base-line level (i.e. the area under the curve).

2.6 Cardiac anatomy

After final MAP determination, the animals in Experiments 2 and 3 were deeply anaesthetized with urethane (≥ 1.4 g/kg BW, i.p., Sigma, St. Louis, MO) and saturated KCl was injected into the carotid artery cannula to arrest the heart in diastole. The heart was then rapidly excised and placed into ice-cold saline to remain in diastole and to remove the blood

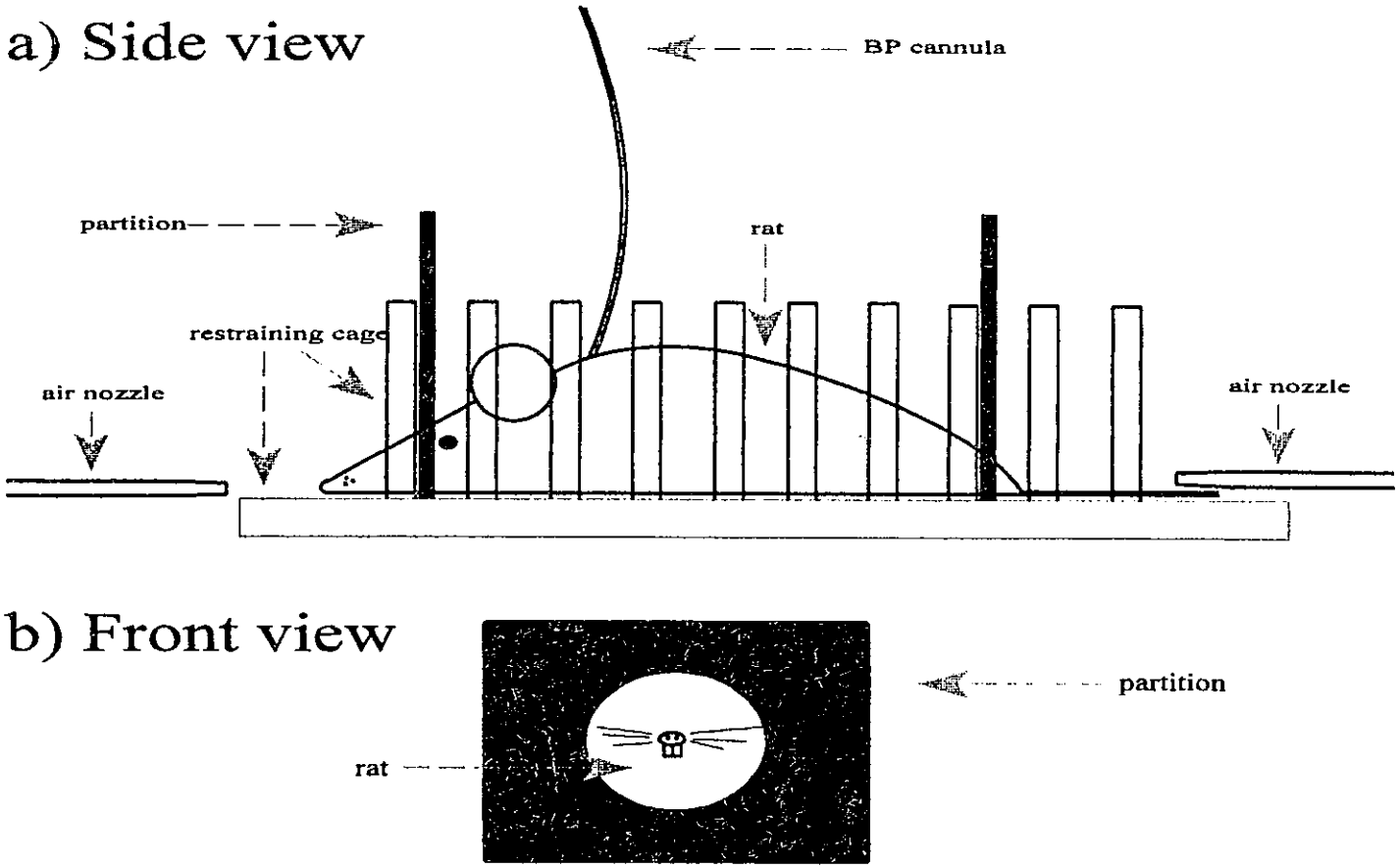


Fig. 2: Air-stress set-up.

from the cardiac cavities and from the surface of the tissue. After removal of the atria and great vessels, the ventricles were blotted dry and the right ventricle (RV) was dissected along its septal insertion from the left ventricle (LV). LV and RV mass were determined, and LV long (top of the ventricle to ventricle apex) and short (top of the ventricle to bottom of the shortest ventricle wall) axes were measured . A transverse midlevel slice of cardiac tissue was then obtained by two transverse cuts at 1/3 and 2/3 of the length of the LV. The slice was viewed under a light microscope using a calibrated ocular. LV wall thickness was measured at eight points around the circular section and the average was calculated. The internal diameter of the LV was measured from the farthest points of the major (anterior-posterior) and minor (septal-lateral) internal diameters. Dry weight of total ventricular mass was measured after drying the tissue at 50 °C for a minimum of 48 hours.

2.7 Histology

After final MAP determination, the rats were perfused through the heart (Experiment 1) or aorta (Experiments 2 and 3) with 150 ml of 10 mM PBS followed by 100 ml of fixative containing 4% paraformaldehyde and 0.4% picric acid in 100 mM PBS. The brain was post-fixed overnight at 4 °C and then stored in 10% sucrose in 100 mM PBS (containing 0.02% sodium azide) for minimum one week for cryoprotection. To minimize damage as a result of ice-crystal formation, the brain was frozen by slowly lowering it (over 1 min.) into a beaker of 2-methylbutane (BDH) which had been previously cooled to approximately -70 °C on dry ice. Fifteen to 20 µm thick sections were cut on a cryostat.

Sections were stained with cresyl violet to determine the extent of damage due to ablation. The 2 % cresyl violet stain was prepared in an acetate buffer (2 g sodium acetate,

3 ml acetic acid in 1 l water). Dehydration and delipidization of the tissue sections was accomplished by dipping the slides for 2 min. each in 70, 80, 90 and 100 % ethanol prior to and after cresyl violet staining. The slides were then dipped in xylene, and cover-slipped with Permount (Fisher Scientific).

Additional series of brain sections were obtained from rats in Experiments 2 and 3 for immunohistochemical verification of the lesion. For this, glass-mounted sections were kept frozen at -80 °C until use, at which point they were left at room temperature to air dry. The slides were then washed in 10 mM PBS. Subsequently, individual sections (6/slide) were covered with 30-35 µl of a 1:400 solution of primary antibody (Rabbit anti-CGRP, RPN.1842, Amersham Canada Ltd, Oakville, Ontario; in 0.3% Triton X-100 in PBS). The sections were left to incubate overnight at 4 °C in a humidifying box. Following three 5-min. washes in PBS, each section was covered with 30-35 µl of a solution of fluorescent secondary antibody (indocarbocyanine (Cy3)- conjugated donkey anti-rabbit IgG, Jackson ImmunoResearch, West Grove, PA; 1:100 in 0.3% Triton X-100 in PBS) for a 40 min. incubation in darkness at 37 °C in a humidifying box. After three 5-min. washes in PBS the slides were coverslipped in 0.1 % p-phenylenediamine (0.1 M $NaCO_3$, phenylenediamine, glycerol) mountant. The slides were stored at -80 °C until viewing under a fluorescent light microscope (Zeiss Axioskop, Germany). Histological analysis of the ablations were verified by individuals blind to the experiments (Drs. Staines and Gelsema).

2.8 Statistics

Group comparisons for BW gain were done using a two-way repeated measures ANOVA. All other group comparisons were done using Student's t-test. In all cases the

level of significance was set at $p<0.05$. Values are presented as means ± S.E.

3 RESULTS

3.1 Optimum lesion production and visualization

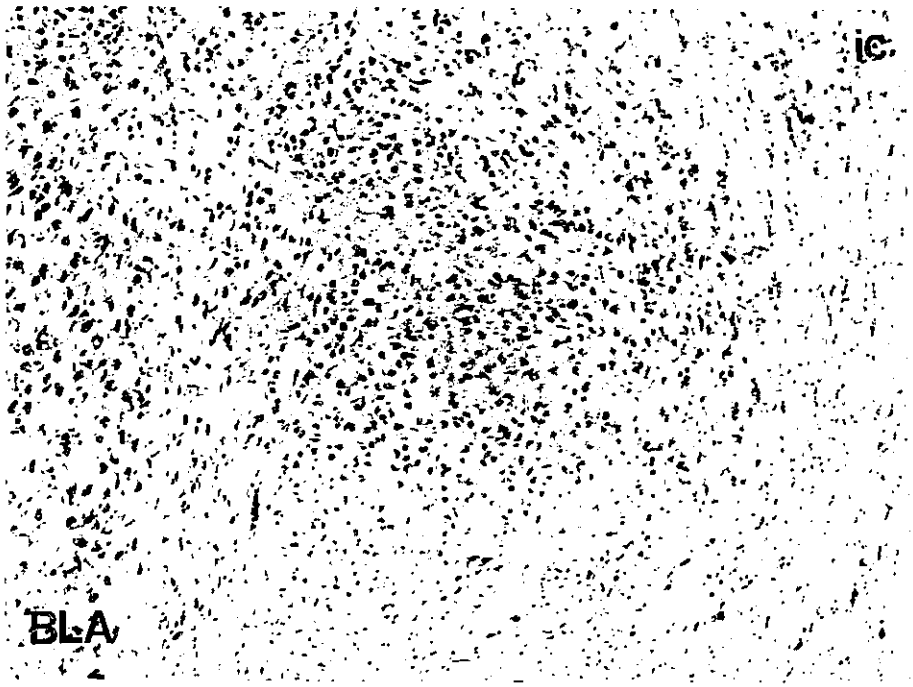
It was determined that placing the tip of the micropipette 2.1 mm caudal, 3.7 mm lateral and 7.5 mm ventral to bregma coincided with the centre of the ACe in 4 week-old SHR.

A volume of 100 nl ibotenic acid (10 μg/μl) provided adequate destruction of the neurons of the ACe without damaging areas beyond the target area. Injection volumes of 150 nl destroyed the ACe, but too often resulted in damage to areas beyond the ACe, such as the basolateral amygdala and the cortex ventral to the ACe.

It was found that 20 μm thick sections of brain tissue were relatively easy to cut, to mount upon slides, to stain and to use for identification of landmarks within the brain tissue. Sections 15 μm thick provided detailed visualization of lesions and landmarks within the brain, but sections were fragile and difficult to manipulate for mounting on slides without damaging the tissue. Thicker 30 and 40 μm sections had good integrity, but provided too few sections and lacked the detail for lesion and landmark verification. Thicker sections were also more difficult to uniformly stain, and required longer incubation times during the immunohistochemical protocol.

Cresyl violet was found to provide good staining for the identification of landmarks and lesions within the brain tissue (see Figure 3 a,b). Cresyl violet provided better contrast between different structures in the brain than thionin, and also allowed direct comparison of tissue appearance with results from other studies that used cresyl violet to stain areas ablated with excitotoxins (e.g. [Jellestad and Grahnstedt 1985; Riolobos and Garcia 1987]).

a)



b)

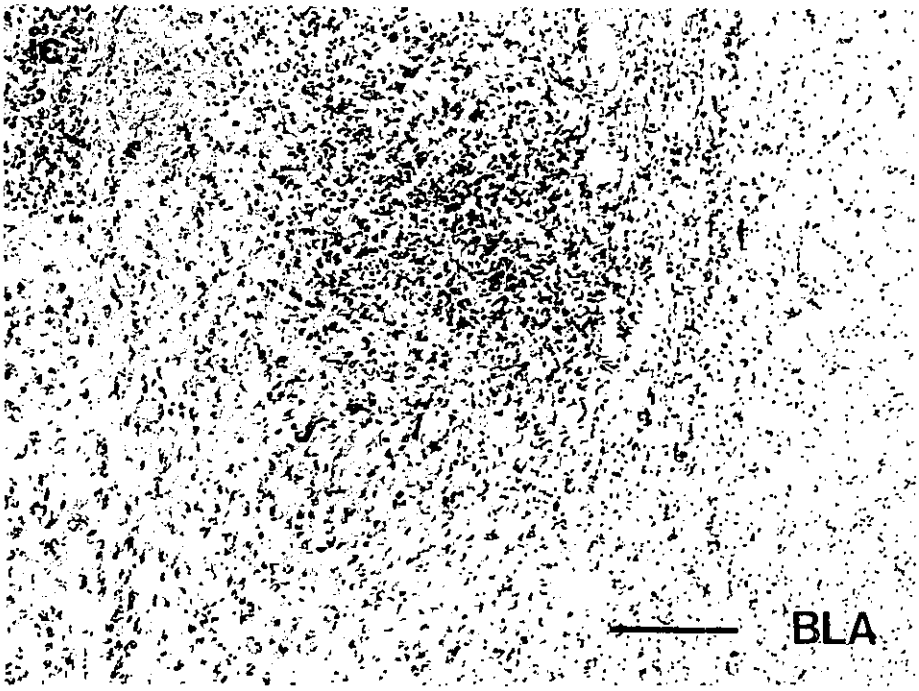


Fig. 3: a.b - Photomicrographs of cresyl violet stained transverse sections through ACe in sham-lesioned (a) and lesioned (b) rat. Note increased gliosis in lesioned ACe. ic - internal capsule; BLA - basolateral nucleus of the amygdala. Bar - 100 μm.

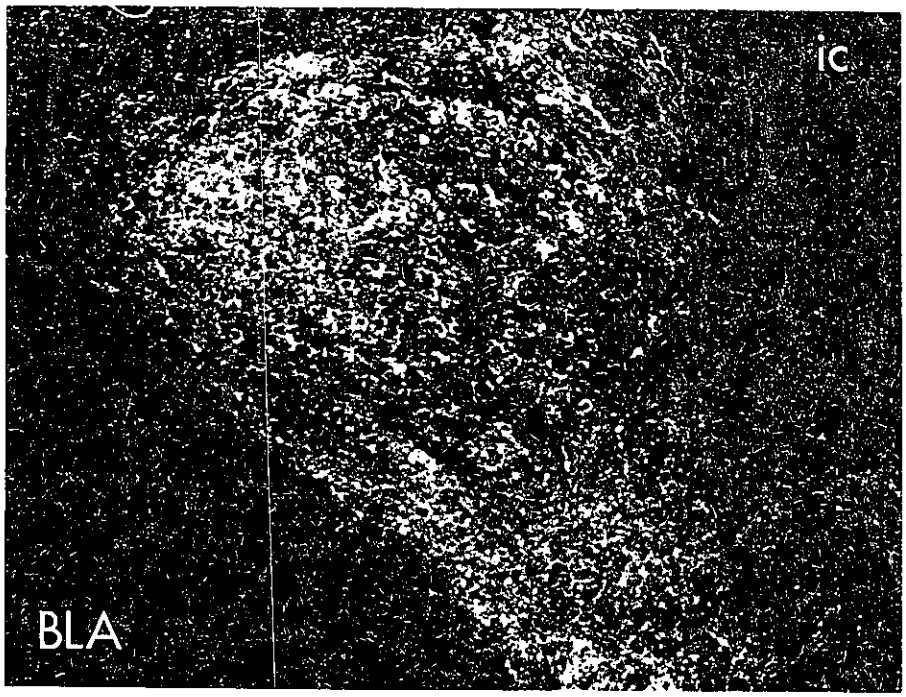
The post-fixing procedure involving the storage of perfused brains in a 10 % sucrose solution overnight prior to cutting resulted in sections with greater structural integrity and limited damage from ice crystal formation during freezing, compared to brains post-fixed in paraformaldehyde solution.

The immunohistochemical technique using antibodies to CGRP proved to be an adequate marker for the ACe when sections were viewed under a fluorescent microscope. Higher concentrations of CGRP were noted in ACe vs. surrounding tissue than when the brain slices were incubated with substance P, met-enkephalin or preprotachykinin. Nerve terminals in the ACe, especially in its lateral subdivision, were easily recognizable from any adjacent areas of the rat brain, which were almost or completely devoid of CGRP-immunoreactive fibres and terminals. The distribution of the nerve terminals clearly delineated the extent and characteristic ovoid shape of the ACe in sham-lesioned SHR. CGRP immunohistochemistry in lesioned animals showed a decreased density of CGRP in nerve terminals in lesioned vs. sham-lesioned rats, and clearly outlined the collapsed, misshapen form of the lesioned ACe under fluorescent light (see Fig. 3 c,d,e,f).

3.2 Pre-experimental BP

BP records from three 4-week old SHR were removed from Experiment 1 and 2 due to poor pressure signals. Because there was no statistically significant difference in MAP values between the remaining 5 rats from Experiment 1 and the 4 from Experiment 2, these 9 rats were combined into one group yielding a pre-experimental resting MAP of 113 ± 3 mmHg.

c)



d)

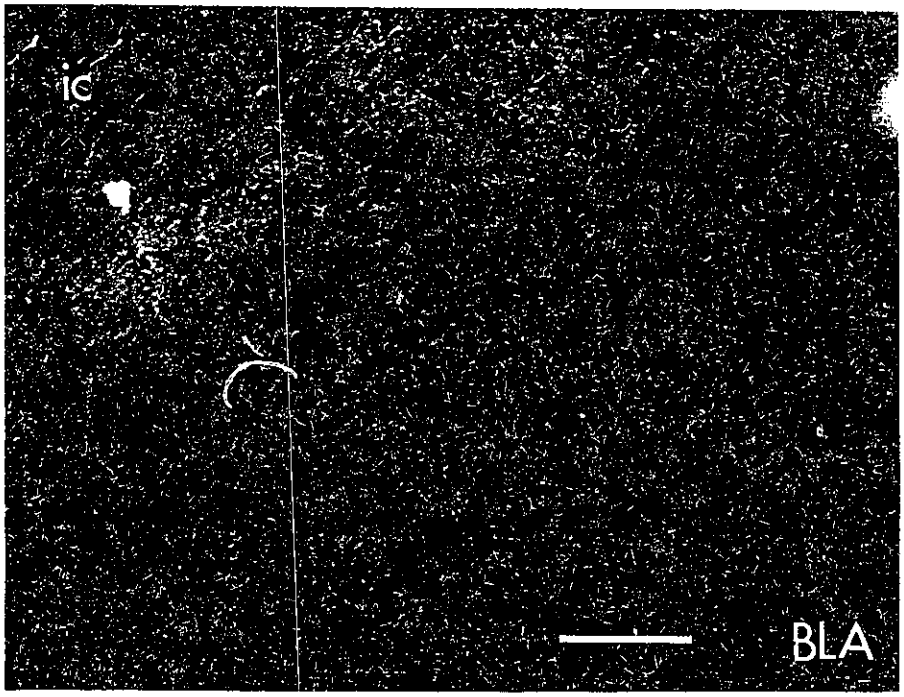
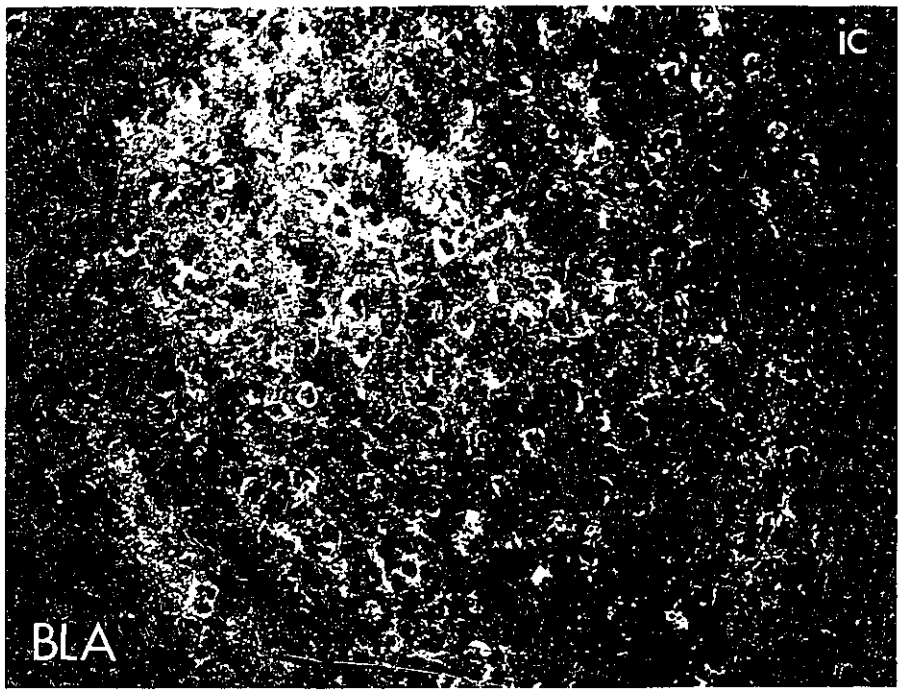


Fig. 3: c,d - Immunofluorescence photomicrographs of transverse sections through ACe of sham-lesioned (c) and lesioned (d) rat after incubation with CGRP antiserum. Note reduced CGRP concentration in lesioned ACe. ic - internal capsule; BLA - basolateral nucleus of the amygdala. Bar - 200 µm.

e)



f)

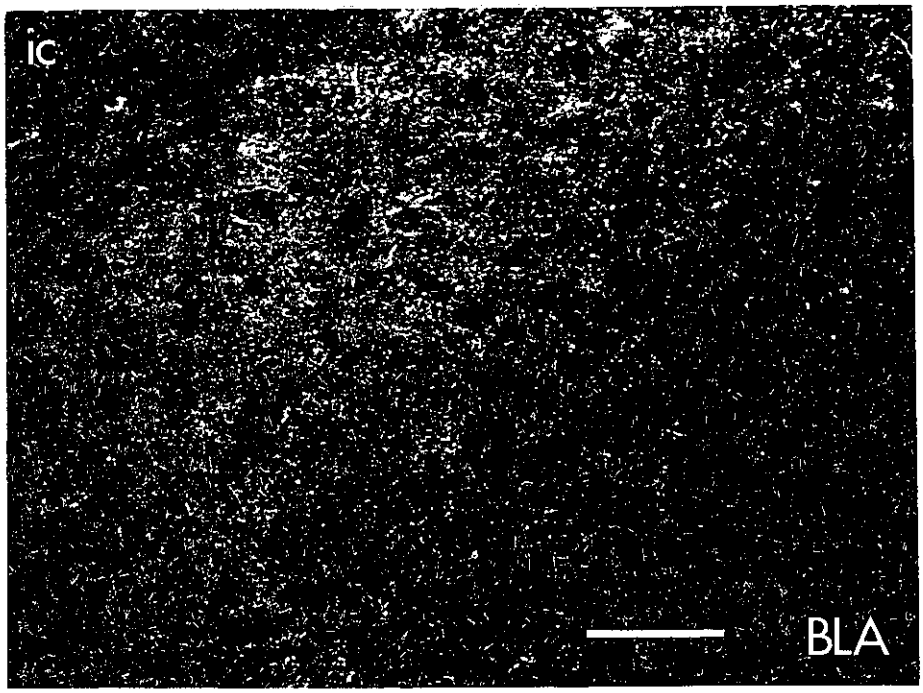


Fig. 3: e,f - Immunofluorescence photomicrographs of transverse sections through ACe of sham-lesioned (e) and lesioned (f) rat after incubation with CGRP antiserum. Note reduced CGRP concentration in nerve terminals in lesioned ACe. ic - internal capsule; BLA - basolateral nucleus of the amygdala. Bar - 100 µm.

3.3 Experiment 1

Clear BP signals could not be obtained from 2 sham-lesioned rats following cannulation. These SHR were removed from analysis.

Seven weeks after operation, resting MAP in the lesioned animals (178 ± 5 mmHg, n=6) was less than that in the control rats (195 ± 11 mmHg, n=5), but the difference was not statistically significant. Fifteen weeks after operation, however, resting MAP in the lesioned animals (173 ± 7 mmHg, n=9) was significantly lower than in the sham-lesioned SHR (201 ± 4 mmHg, n=8) (see Fig. 4). Resting HRs in the lesioned (342 ± 8 bpm) and sham-lesioned (351 ± 12) rats were not significantly different after 7 weeks, nor was there any difference after 15 weeks between the lesioned (365 ± 17) and sham-lesioned (363 ± 15) animals.

BW gain in the lesioned rats was less than in the sham-lesioned rats. A two-way repeated-measures ANOVA of the weekly BWs in the lesioned vs. sham-lesioned rats showed a significant difference between the mean BWs from 5 weeks post-operation onwards (see Fig. 5); during this period lesioned rats weighed 3.6 - 7.1% less than sham-lesioned rats.

There were no significant differences in the amplitude of the BP response to air stress between lesioned and sham-lesioned rats at 7 or 15 weeks (see Fig. 6). Technical and equipment limitations precluded analysis of the BP data to determine resting HRs and the stress-response integral in Experiment 1.

3.4 Experiment 2

Five animals died prematurely following operation. Due to the paired feeding schedule, BWs in the lesioned and sham-lesioned rats were not significantly different

EXPERIMENT 1

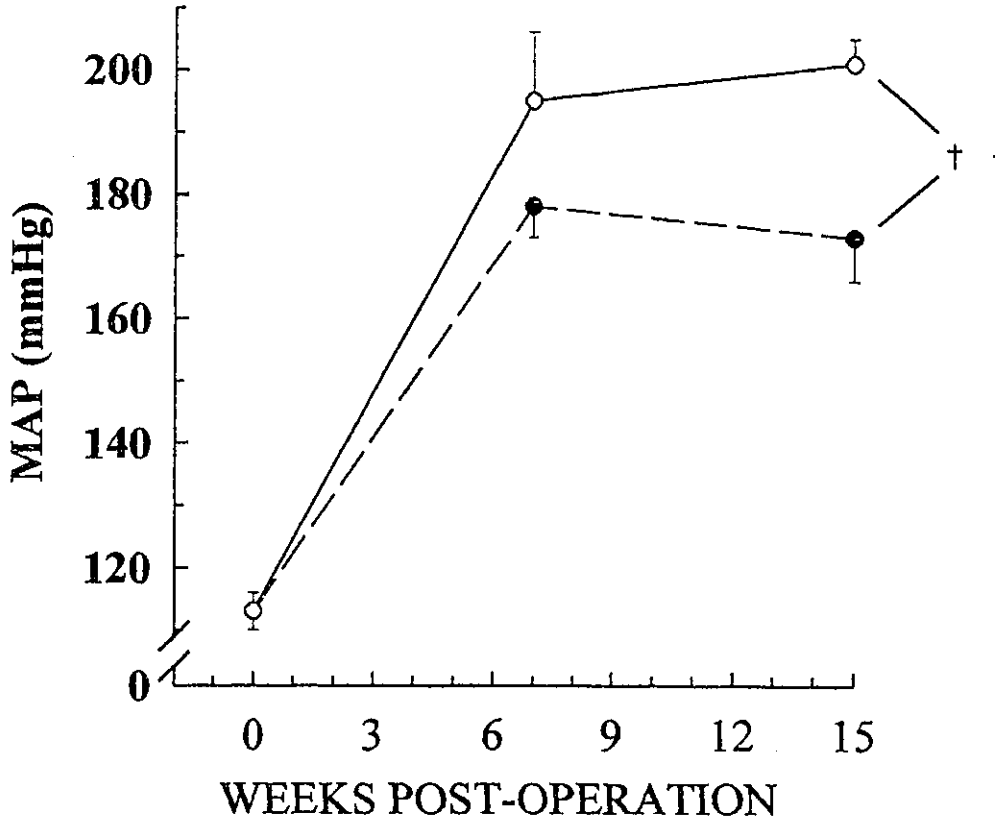


Fig. 4: Average mean arterial pressure (MAP) in Experiment 1 pre-experimental, lesioned (black circle) and sham-lesioned (white circle) rats. † = $p<0.05$ vs. sham-lesioned rats, Student's t-test. N = 6 lesioned, 5 sham-lesioned at 7 weeks; 9 lesioned, 8 sham-lesioned at 15 weeks. Error bars are S.E.

EXPERIMENT 1

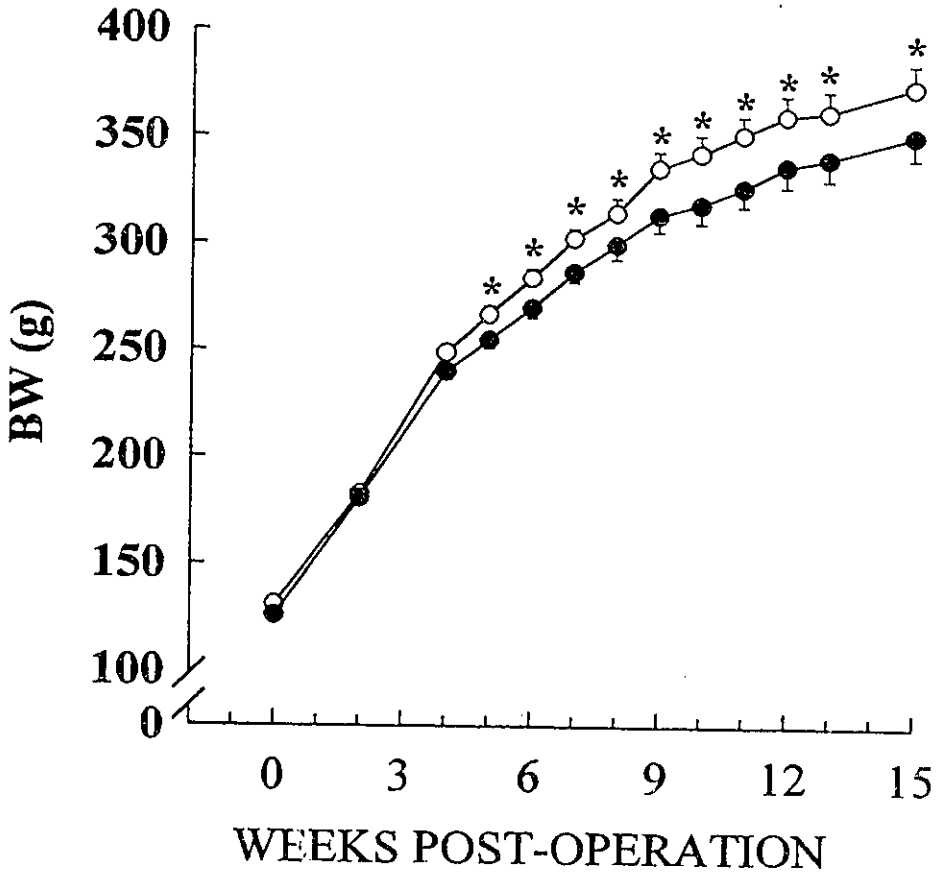


Fig. 5: Average body weight (BW) in Experiment 1 lesioned (black circle) and sham-lesioned (white circle) rats through 15 week experimental period. * = $p<0.05$ vs. Sham-lesioned rats, two-way repeated measures ANOVA. N = 15 lesioned, 13 sham-lesioned weeks 0 to 7; 9 lesioned, 8 sham-lesioned weeks 8 to 15. Error bars are S.E.

EXPERIMENT 1

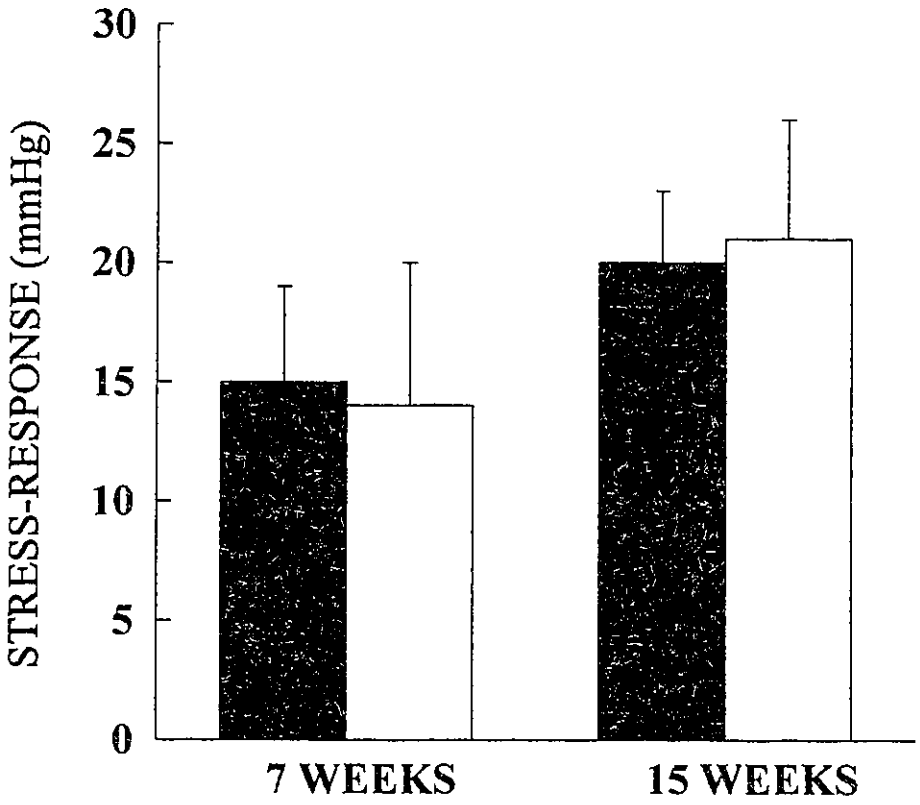


Fig. 6: Average peak MAP response to air-stress in Experiment 1 lesioned (black bar) and sham-lesioned (white bar) rats. N = 6 lesioned, 5 sham-lesioned at 7 weeks; 9 lesioned and 8 sham-lesioned at 15 weeks. Error bars are S.E.

throughout the 22 post-operative weeks (see Fig. 7).

Seven weeks after operation there was no significant difference in resting MAP levels between lesioned (147 ± 15 mmHg, n=5) and sham-lesioned rats (161 ± 8 mmHg, n=6). Similarly, 15 weeks after operation resting MAP in the lesioned rats (167 ± 11 mmHg, n=6) was not significantly different from that in the control rats (167 ± 6, n=7). Finally, 22 weeks after operation, resting MAP in the lesioned rats (190 ± 9 mmHg, n=5) was significantly higher than that in the sham-lesioned rats (164 ± 5 mmHg, n=7). Fig. 8 summarizes these findings.

There were no significant differences in resting HRs at 7 (lesioned 336 ± 7; sham-lesioned 351 ± 13) 15 (lesioned 367 ± 11; sham-lesioned 361 ± 15) or 22 weeks after operation (lesioned 358 ± 21; sham-lesioned 347 ± 35).

The average peak amplitude of the BP responses to acute air-jet stress in the lesioned rats was not significantly different vs. the sham-lesioned rats 7 and 22 weeks post-operation (see Fig. 9). At 15 weeks post-operation, the lesioned rats responded with significantly smaller peak BP responses to air-jet stress, while the average response durations were not different among the lesioned and sham-lesioned rats, nor was the response amplitude-time integral (see Fig. 10). The pressor responses to air-jet stress were usually accompanied by a transient bradycardia. In none of the three survival groups were there significant differences in the average (absolute or percentile) magnitude of the bradycardic responses between lesioned and sham-lesioned rats.

EXPERIMENT 2

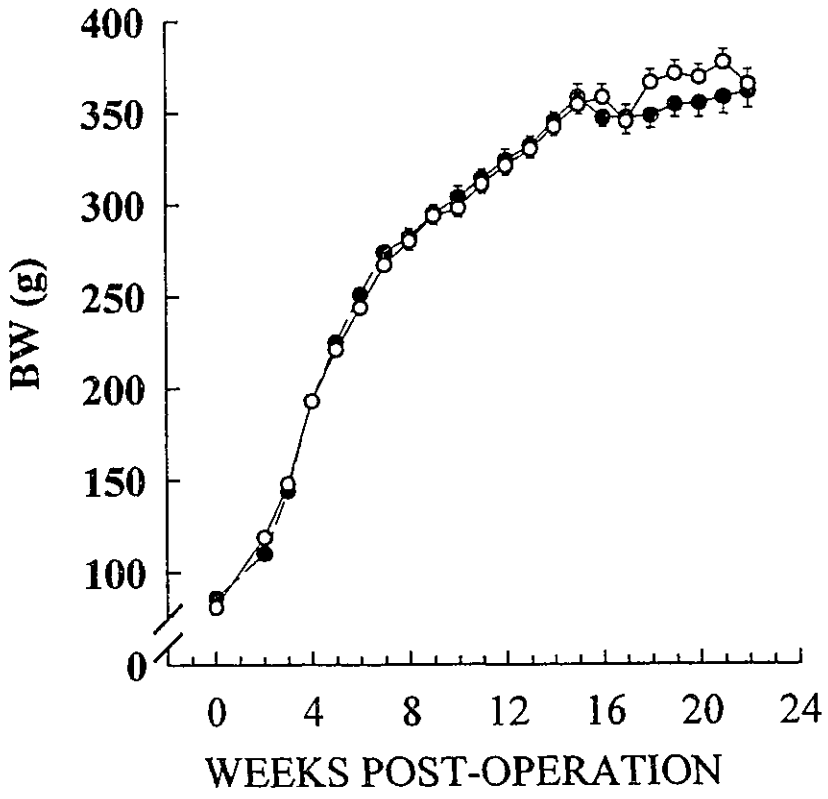


Fig. 7: Average body weight (BW) in Experiment 2 (pair-fed) lesioned (black circle) and sham-lesioned (white circle) rats through 22 week experimental period. N = 16 lesioned, 20 sham-lesioned weeks 0 to 7; 11 lesioned, 14 sham-lesioned weeks 8 to 15; 5 lesioned, 7 sham-lesioned weeks to 16 to 22. Error bars are S.E.

EXPERIMENT 2

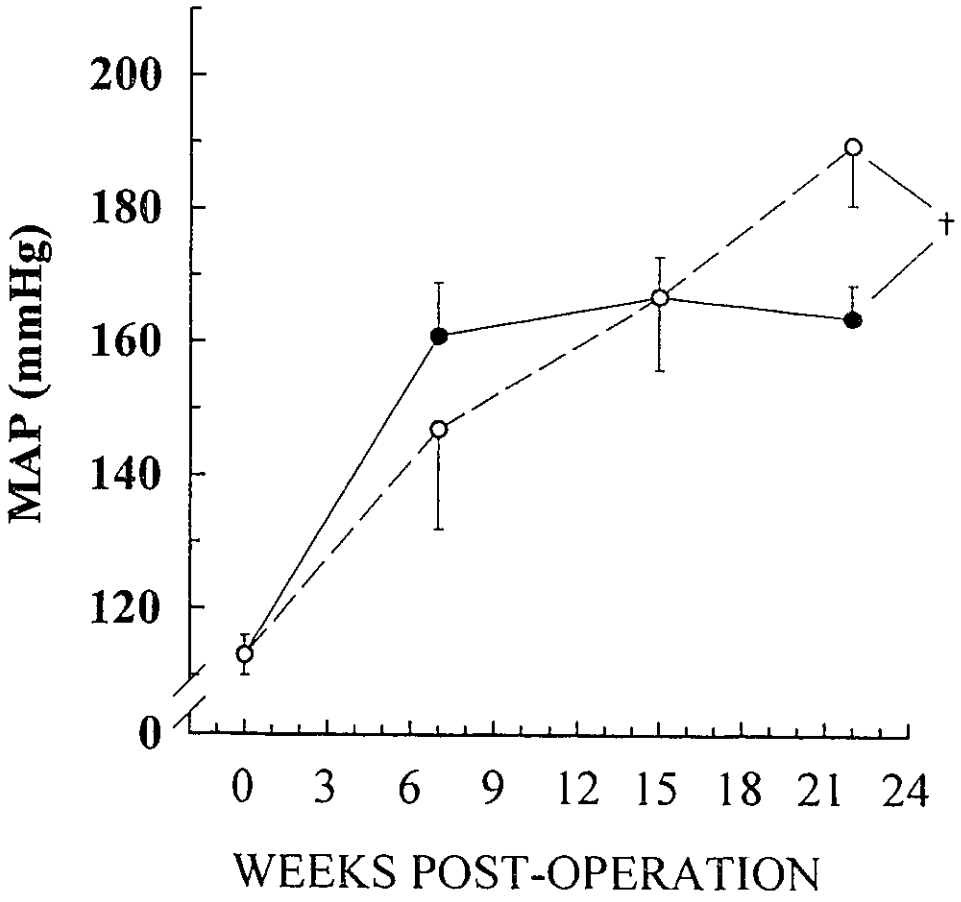


Fig. 8: Average mean arterial pressure (MAP) in Experiment 2 (pair-fed) pre-experimental, lesioned (black circle) and sham-lesioned (white circle) rats. † = $p<0.05$ vs. Sham-lesioned rats, Student's t-test. N = 5 lesioned, 6 sham-lesioned at 7 weeks; 6 lesioned, 7 sham-lesioned at 15 weeks; 5 lesioned, 7 sham-lesioned at 22 weeks. Error bars are S.E.

EXPERIMENT 2

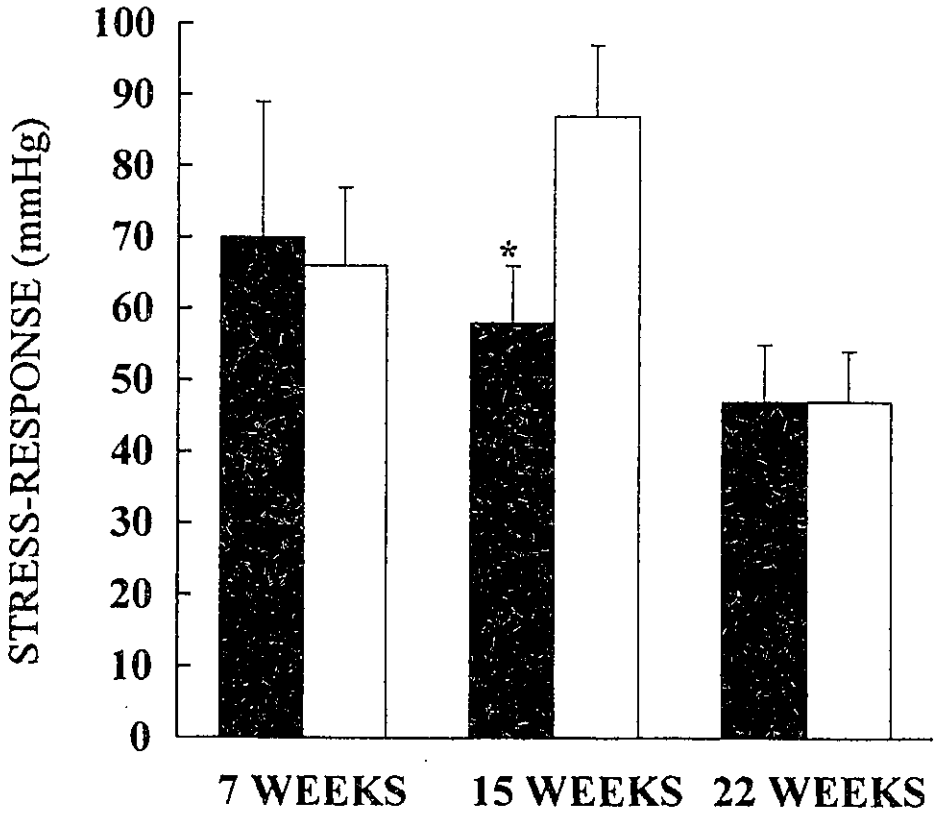


Fig. 9: Average peak MAP response to air-stress in Experiment 2 (pair-fed) lesioned (black bar) and sham-lesioned (white bar) rats. * = $p<0.05$ vs. Sham-lesioned, Student's t-test. N = 5 lesioned, 6 sham-lesioned at 7 weeks; 6 lesioned, 7 sham-lesioned at 15 weeks; 5 lesioned, 7 sham-lesioned at 22 weeks. Error bars are S.E.

EXPERIMENT 2

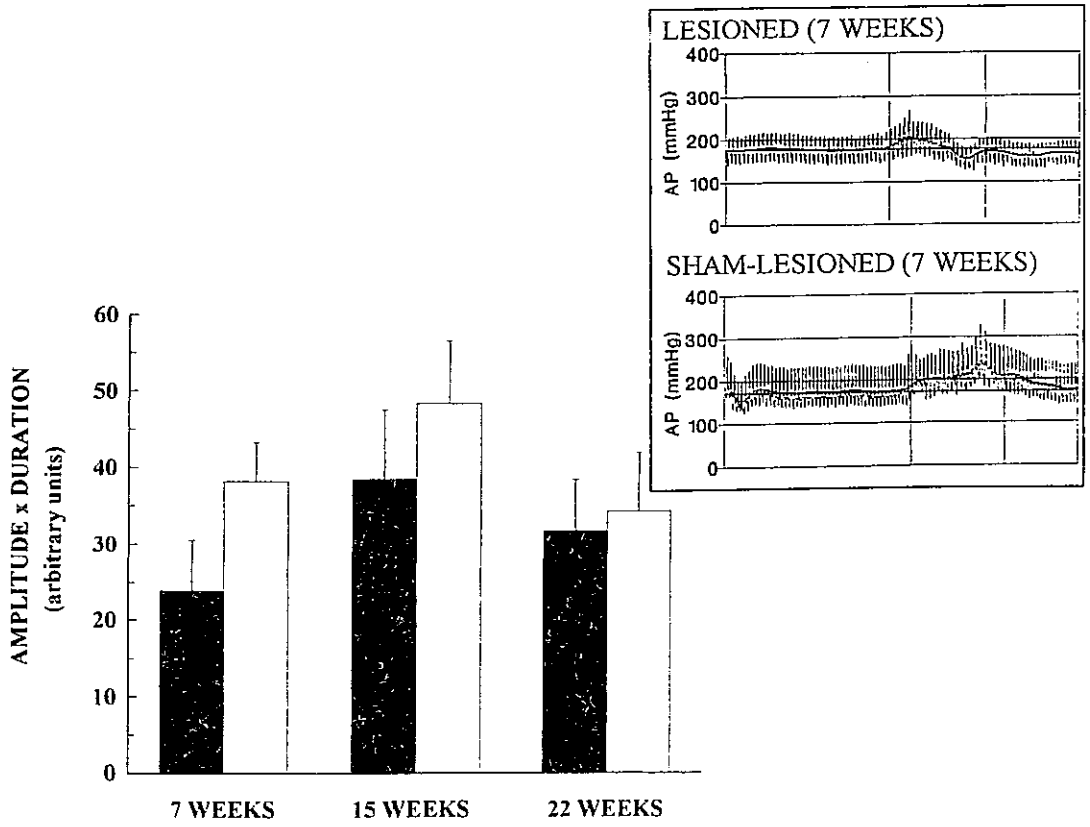


Fig. 10: Average integral (peak amplitude x duration) response to air-stress in Experiment 2 lesioned (black bar) and sham-lesioned (white bar) rats. Bars are S.E. Inset - example of pulsatile BP response to air-stress in 7 week post-operation lesioned (top) and sham-lesioned (bottom) rat. Shaded area indicates response integral. Black horizontal bar indicates activated air-jet.

Examination of cardiac anatomy measurements showed that no significant differences existed in average LV/BW, LV wall thickness, or LV wall thickness/LV i.d. between lesioned and sham-lesioned SHR at 7, 15 or 22 weeks post-operation.

3.5 Experiment 3

Three rats from the 4 week survival group died following ablation surgery. Three more rats in the 4 week survival group died during the cannulation procedure prior to BP measurement. From the 11 week survival group, two lesioned and one sham-lesioned rat became severely dehydrated and stopped gaining BW, and were removed from the study. Two more SHR, one lesioned and one sham-lesioned died during and following cannulation surgery, respectively.

There was no significant difference between lesioned (135 ±7 mmHg, N=6) and sham-lesioned (138 ±9 mmHg, N=4) MAP after 4 weeks on high NaCl diet. There was also no difference between groups in MAP (lesioned 167 ±9 mmHg, N=10; sham-lesioned 173 ±9 mmHg, N=11) after 11 weeks (see Fig. 11).

Due to the pair feeding, there were no weight differences between lesioned and sham-lesioned BWs throughout the duration of the experiment (see Fig. 12). There were also no significant differences in resting HRs after 4 weeks (lesioned 402 ±23, sham-lesioned 397 ±22) or 11 weeks (lesioned 363 ±9, sham-lesioned 319 ±21). BP responses to air-stress were not different between lesioned and sham-lesioned rats at 4 or 11 weeks (see Fig. 13).

Examination of cardiac anatomy results showed that no differences existed in average LV/BW, LV wall thickness, or LV wall thickness/LV i.d. between lesioned and sham-

EXPERIMENT 3

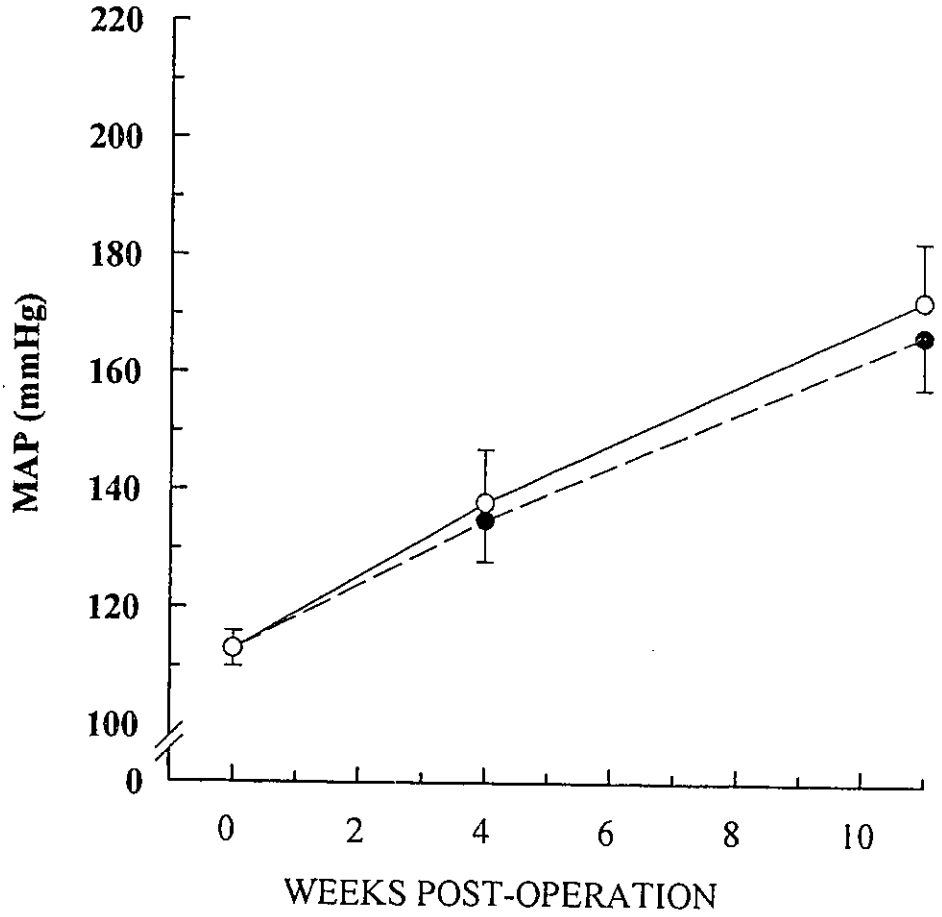


Fig. 11: Average mean arterial pressure (MAP) in pre-experimental, lesioned (black circle) and sham-lesioned (white circle) rats in Experiment 3 (pair-fed, high sodium diet). N = 6 lesioned, 4 sham-lesioned at 4 weeks; 10 lesioned, 11 sham-lesioned at 11 weeks. Error bars are S.E.

EXPERIMENT 3

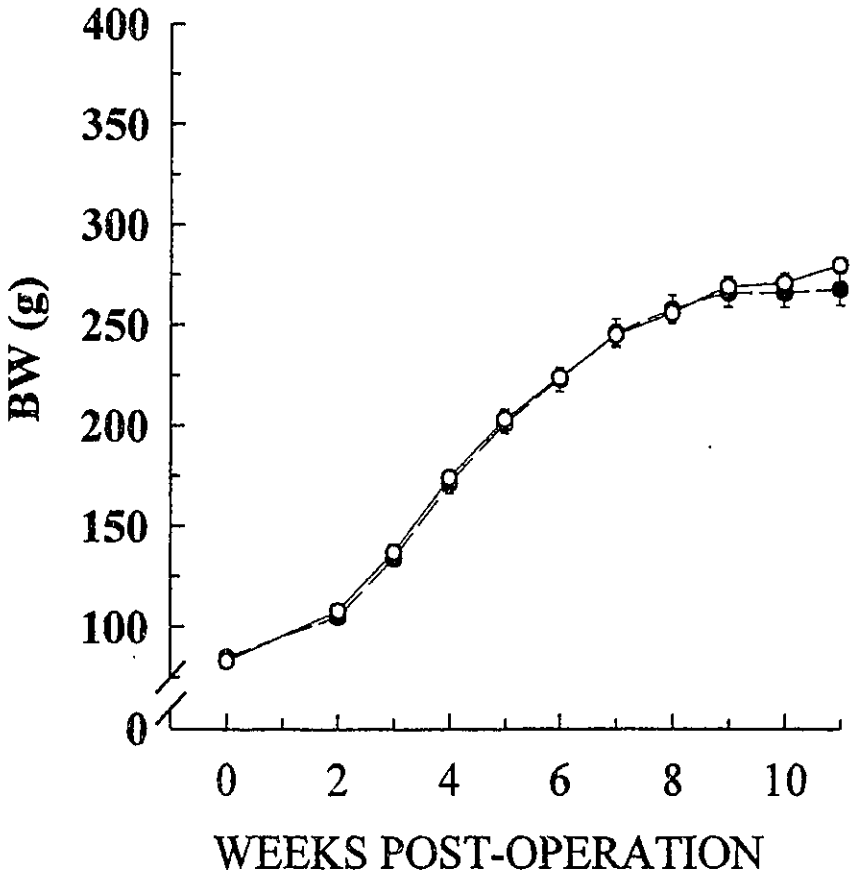


Fig. 12: Average body weight (BW) in Experiment 3 (pair-fed, high sodium diet) lesioned (black circle) and sham-lesioned (white circle) rats through 11 week experimental period. N = 16 lesioned, 15 sham-lesioned weeks 0 to 4; 10 lesioned, 11 sham-lesioned weeks 5 to 11. Error bars are S.E.

EXPERIMENT 3

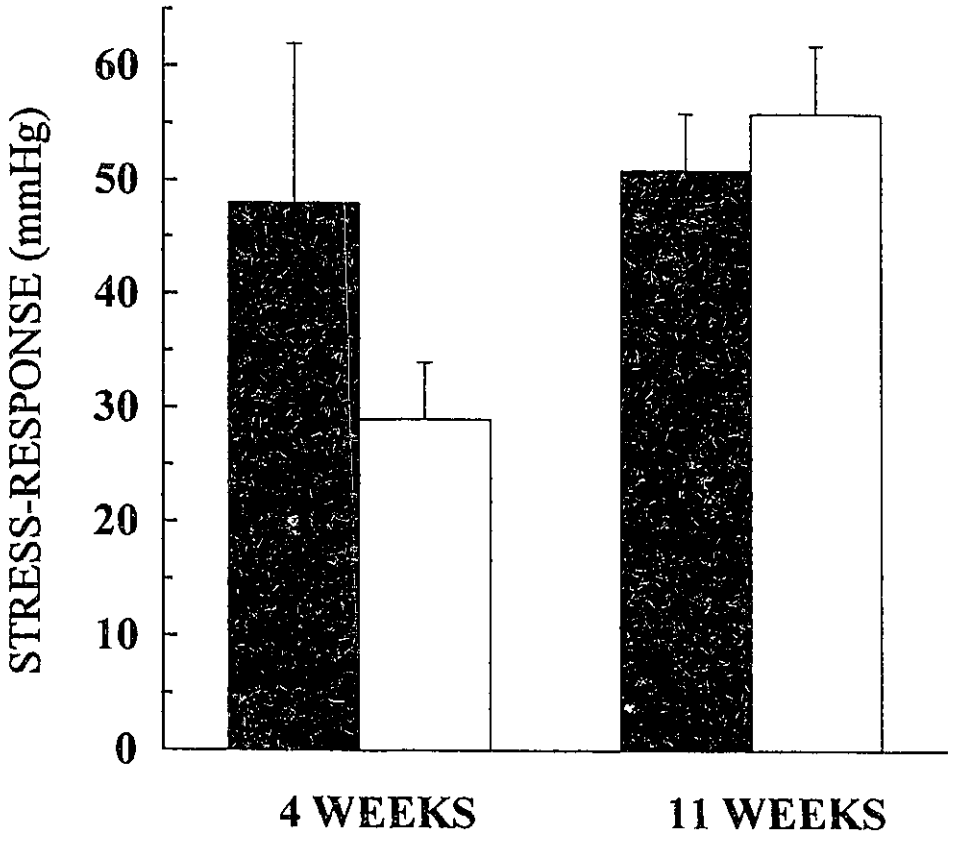


Fig. 13: Average peak MAP response to air-stress in Experiment 3 (pair-fed, high sodium diet) lesioned (black bar) and sham-lesioned (white bar) rats. N = 6 lesioned, 4 sham-lesioned at 4 weeks; 10 lesioned, 11 sham-lesioned at 11 weeks. Error bars are S.E.

lesioned SHR at 4 or 11 weeks post-operation.

3.6 Histology

Lesions in Experiment 1 were verified using cresyl violet (see Fig. 3 a,b for example of lesioned and sham-lesioned rats). Verification in Experiments 2 and 3 was done using both cresyl violet and CGRP immunohistochemical techniques.

Histological verification of the lesions after the different survival times showed that the area of the ACe was represented by a hole in a few rats only. There appeared to be a general shrinkage of the area of the ACe in lesioned vs. sham-lesioned rats. Measurement of distances between the boundaries of the ACe showed significantly shorter distances in lesioned rats. For example, the average dorsal-ventral diameter of the ACe in sham-lesioned SHR at 22 weeks was 1.75 ± 0.09 mm (8 ACe measured bilaterally), significantly longer than the average 1.38 ± 0.05 mm measured in lesioned animals at 22 weeks (10 ACe measured bilaterally). Significantly shorter values were also measured for the medial-lateral width of the ACe at its widest point and in the rostral-caudal extent of the ACe (measured by counting the number of sections collected with a visible part of the ACe), between sham-lesioned and lesioned SHR at 15 and 22 weeks. Tissue incubated with the anti-CGRP antibodies showed decreased amounts of immunoreactive terminals and/or terminals in the lesioned rats. Fig. 3 (c, d, e, f) shows examples sections with CGRP-labeled terminals of a lesioned and a sham-lesioned rat. The histological analysis of SHR in Experiment 2 confirmed the presence of complete ACe lesions in 5 rats in each of the 7 and 22 weeks survival subgroups, and in 6 rats in the 15 weeks survival subgroup. Four rats without a demonstrable lesion, and 3 rats with unclear lesions were removed from further analysis.

Histological verification of the lesions in Experiment 3 revealed a pattern of degeneration of neurons in the ACe similar to that seen in the other two Experiments. Lesions in the 4 week survival period were easily distinguishable from intact brain tissue with the cresyl violet preparation by means of the increased number of glia cells. Verification histology in the 11 week survival rats resulted in three rats being removed from further analysis due to incomplete lesions of the ACe.

4 DISCUSSION

Experiments 1 and 2 were performed in order to clarify the results of two previous studies that sought to determine the role of the ACe in the development of hypertension in young SHR [Galeno *et al.* 1982, Folkow *et al.* 1982]. Gelsema *et al.* [1987] questioned the role of the ACe in cardiovascular control after selective chemical stimulation of ACe neurons failed to elicit the pressor and behaviour responses elicited by electrical stimulation of the same area in conscious rats. Experiment 1 used a chemical ablation technique to destroy ACe neurons, in order to avoid the destruction of fibres of passage that would have resulted from using the electrolytic techniques of Galeno *et al.* [1982] and Folkow *et al.* [1982]. Experiment 2 methodology avoided the discrepancies in BW between lesioned and sham-lesioned groups that were observed in Experiment 1 and also reported in the study by Folkow and collaborators [1982]. This was necessary because of the well know strong positive correlation between BW and BP, and because studies have shown that lower BW leads to lower BP in SHR [Young *et al.* 1978; Wright *et al.* 1981]. With BP differences due to BW differences removed, the true effect of ACe ablation on the development of hypertension in young SHR could be observed in Experiment 2.

Beyond confirmation that destruction of the ACe attenuates the development of hypertension in young SHR, the results from Experiment 1 show for the first time that this effect is due to the ablation of neurons, and not to damage to fibres of passage in the ACe. The selectivity of the chemical ablation technique eliminated the possibility that disruption of fibres passing through the ACe may have affected the natural development of hypertension in these animals.

Although both Folkow *et al.* [1982] and Galeno *et al.* [1982] implicated the ACe in the development of high BP in young SHR, the electrolytic ablation technique used in their studies could not have confined damage to the ACe. Electrolytic ablation uses an electrical current to destroy neurons, axons and nerve terminals at the target site. The ACe contains fibres of passage whose destruction may affect remote areas involved in cardiovascular control. For example, the efferents of the BNST, an area suggested to be involved in cardiovascular regulation [Gelsema *et al.* 1993; Helix *et al.* 1990] form the stria terminalis, which projects through the ACe [Paxinos and Watson 1986; van der Kook *et al.* 1984]. The stria terminalis is a major fibre bundle that contains axons synapsing with many other sites in the brain, including cardiovascular areas such as the locus coeruleus, NTS, DMV [Holstege *et al.* 1985], PBN [Sawchenko and Swanson 1983] and LHA [Calaresu and Pearce 1965]. Therefore, the role of the ACe in the development of hypertension could not have been resolved by Folkow *et al.* [1982] or Galeno *et al.* [1982], because their ablation technique did not confine damage to the neurons of the ACe. This fact was briefly stated by Galeno *et al.* [1982], but neither study sought to clarify their results. This lead to the current re-investigation of the role of the ACe in the development of hypertension in SHR by using an excitatory amino acid to selectively destroy its neurons.

The scope and precision of lesions using excitatory amino acids can be well contained by controlling the volume of injection. In contrast to other excitatory amino acids, ibotenic acid does not cause lesions remote to its target [Schwarcz *et al.* 1979a; Guldin and Markowitsch 1981; Kohler and Schwarcz 1981]. It has been suggested that the precision of ibotenic acid lesions is due to rapid decarboxylation of the excitotoxin to muscimol, a non-

toxic GABA agonist [Schwarcz *et al.* 1979a,b; Kohler *et al.* 1979]. Among other excitotoxins, kainic acid is frequently used, but comparison between the two has shown that ibotenic acid is the superior experimental tool [Kohler and Schwarcz 1981; Schwarcz *et al.* 1979a], as ibotenic acid gives more restricted lesions [Schwarcz *et al.* 1979b; Guldin and Markowitsch 1981] and has been shown to ablate tissue resistant to kainic acid [Kohler and Schwarcz 1981]. Although ibotenic acid has been reported to cause prolongation of anaesthesia [Schwarcz *et al.* 1978] and sluggishness upon waking [Guldin and Markowitsch 1981] in rats after surgery, it does not produce the epileptiform activity seen in rats after kainic acid use [Guldin and Markowitsch 1981; Jellestad and Grahnstedt 1985]. Ibotenic acid, the excitotoxin used to place lesions in the present experiments, binds to receptors on neuronal soma, causing chronic depolarization of only those cells whose bodies reside within the target area [Olney *et al.* 1974]. Nerve fibres and terminals remain unaffected, so the use of ibotenic acid clarified that the attenuation of the development of hypertension in young SHR was truly due to the selective destruction of neurons of the ACe, and not to functional alterations in remote areas resulting from damage to fibers of passage.

Selective chemical ablation of ACe neurons resulted in significantly lower BP in the lesioned vs. sham-lesioned rats in Experiment 1, 15 weeks after placement of the lesion (see Figure 4). Although these results support those from two earlier studies in which the ACe of young SHR was electrolytically destroyed [Folkow *et al.* 1982; Galeno *et al.* 1982], there are also noticeable differences. The differences between lesioned and sham-lesioned rats in average MAP levels were larger in Experiment 1 than the differences reported in the previous studies. Lesioned rats in Experiment 1 developed a MAP 14 % lower than control

rats at 19 weeks of age, greater than the approximately 7 % lower MAP in lesioned rats reported by Folkow *et al.* [1982] at 27 week of age, and the approximately 8 % lower BP in lesioned vs. control SHR measured by Galeno *et al.* [1982] at 16 weeks of age. The larger BP differences measured between treatments in the current studies may be partially explained by the discrepancies in the MAP developed in the sham-lesioned rats in each study. SHR are genetically predisposed to develop high BP between the ages of 4 and 16 weeks [Okamoto 1969; Okamoto and Aoki 1963]. The sham-lesioned rats in Experiment 1 developed an average MAP of 201 ± 4 mmHg when they were 19 weeks old, a BP that is to be expected in SHR at this age [Okamoto and Aoki 1963; Okamoto 1969]. Neither the 27 week-old control rats of Folkow *et al.* [1982], nor the 16 week-old sham-operated SHR of Galeno *et al.* [1982], developed an average MAP over 180 mmHg - a fact not explained by either group.

Along with the differences in BP between lesioned and sham-lesioned SHR in Experiment 1, analysis of BW data revealed small (3.6 - 7.1 %) but statistically significant differences in BW between the lesioned and sham-lesioned rats 15 weeks after the operation (see Figure 5). Folkow *et al.* [1982] also reported an 8-10 % lower average BW in lesioned vs. control rats 21 weeks after operation. Galeno *et al.* [1982] reported no BW data. Neither group (Folkow *et al.* [1982] nor Galeno *et al.* [1982]), considered the possible implications of these BW differences on their experimental results, a clear oversight on their part. BW has repeatedly been shown to be positively correlated with BP, and even a small reduction in the rate of BW gain in the lesioned SHR in Experiment 1 and earlier studies ([Folkow *et al.* 1982; Galeno *et al.* 1982]) may have had a significant effect on the development of

hypertension. For example, Young *et al.* [1978] showed in 13-14 week-old SHR (i.e. in rats with established hypertension) significant reductions in BP upon a 4 day period of food restriction. More importantly, Wright *et al.* [1981] found that food restriction in young SHR resulted in a significant suppression of their natural rise in BP. They showed that a 35 % reduction in food intake, when started immediately after weaning and continued for 10 weeks, caused a significantly lower MAP (approximately 157 mmHg ± 3 mmHg) vs. age-matched SHR fed ad libitum (181 ± 1 mmHg). They also suggested that the most effective inhibition of rising BP may be expected from the earliest possible dietary restriction, i.e. immediately after weaning, at the onset of the genetically programmed rise in BP in this strain. These studies suggested that differences in BW may have affected the BP values in Experiment 1 of the present studies.

The validity of the use of an exictotoxin in the current studies was proven during histological verification of the lesions. Cresyl violet staining showed that the ACe in lesioned SHR contained few neuronal cell bodies but that they did contain an increased number of darkly staining glial cells (see Figure 3 b). Similar descriptions of the ACe were given by others following ibotenic acid ablation of the nucleus in rats [Riolobos and Garcia 1987; Jellestad *et al.* 1986; Van de Kar *et al.* 1991]. Cresyl violet histology also showed a general atrophy of the ACe. The rostral-caudal, medial-lateral and dorsal-ventral extent of the ACe was diminished in lesioned rats when compared to sham-lesioned rats. Areas surrounding the ACe were found to impinge upon the space normally occupied by the ACe. For example, the basolateral nucleus of the amygdala often appeared more medial in the brain of lesioned vs. control rats. The lateral ventricles, normally positioned caudal to the

ACe, were found to extend their boundary rostrally into lesioned brain tissue. This collapse of surrounding neurons upon the lesioned area was also noted by Zaczek *et al.* [1980] after excitotoxic ablation of brain tissue in rats. Cavities were found within the ACe of a few lesioned animals only, similar to holes described by Jellestad and Grahnstedt [1985] in the rat brain following ibotenic acid ablation of the amygdala. No significant neuronal degeneration was observed along the track made by the injection micropipette. This unwanted destruction of neurons was probably avoided as the micropipette was left in place for 10 minutes following the injection of ibotenic acid, as per the recommendation of Jarrard [1989].

To eliminate the discrepancies in BW between lesioned and sham-lesioned rats, each of the sham-lesioned SHR in Experiment 2 was paired with a lesioned rat. Equalization of food intake was meant to limit any BW differences and allow observation of the disparities (if any) in the development of hypertension between lesioned and control rats, without the confounding influence of differences in BW gain. This pair-feeding protocol successfully eliminated the differences in BW between lesioned and sham-lesioned groups (see Figures 7 and 12). However, this equalization of food intake and BW gain had a profound effect on the development of hypertension in the sham-lesioned rats. MAP in these rats was around 165 mmHg after the 22 weeks survival period of Experiment 2, representing a 35 mmHg attenuation of the normal development of hypertension in this strain [Okamoto 1969; Okamoto and Aoki 1963].

The need for a pair-feeding protocol in these Experiments was obvious. From the very start of the Experiment 2 it was obvious that the control rats had a greater appetite than

lesioned animals. The sham-lesioned animals were rarely found to have any food left in their hoppers after 24 hours, and had more often that not consumed all of their allocated food hours before the next allotment. An additional indicator of their unsatiated state was the observation that the sham-lesioned rats were usually highly agitated prior to feeding, and set upon their food the instant it was dispensed. Therefore, despite the fact that food intake was not measured directly in the lesioned and sham-lesioned SHR of Experiment 1, it was inferred from these observations that the appetite of lesioned rats was attenuated compared to that of sham-lesioned rats. These apparent differences in appetite justified the use of a pair-feeding protocol in Experiment 2 to minimize BW differences between ACe-lesioned and sham-lesioned rats, and supported the contention that previous studies were flawed in overlooking the effect of ACe ablation on BW (and BP).

As a result of the inhibitory effect of reduced BW gain on the development of hypertension in the control rats, the BP differences measured previously between lesioned and sham-lesioned rats were eliminated (see Figure 8). There was no longer any measurable difference in BP between ACe-lesioned and sham-lesioned SHR 7 or 15 weeks after operation on 4 week-old rats.

The BP results from Experiment 2 suggest that the attenuation of the development of hypertension in ACe-lesioned SHR may derive from a slight but significant reduction in BW gain in ACe-lesioned SHR. Numerous studies also point to a role for the ACe in food intake behaviour. Injection of muscimol, a $GABA_A$-selective receptor agonist, into the ACe of conscious, instrumented Wistar rats produced a dose-dependent decrease in food intake in both satiated and fasted adult rats [Miñano *et al.* 1992]. In another study, kainic acid

lesions in the ACe have been shown to cause a dose-dependent relative BW loss, hypo- or aphagia and hypo- or adipsia in adult rats [Hajnal *et al.* 1992]. Both of these studies demonstrated that inhibition of neuronal function in the ACe results in decreased food intake and/or BW gain in rats, and show that the effects of ACe ablation on BW reported Experiments 1 and 2 are not unprecedented.

Ablation of the neurons that form the connections between the ACe and these hypothalamic feeding centres could disrupt feeding patterns, and therefore BW gain in lesioned rats. Disruption of the connection between the ACe and hypothalamic feeding centres may have affected food intake behaviour in the lesioned SHR. Both the VMH and LHA have been implicated in food intake behaviour. Electrolytic lesions of the VMH produce obesity [Hetherington and Ranson 1939], while electrolytic ablation of the LHA produces aphagia and adipsia in rats [Anand and Brobeck 1951]. Both the VMH and LHA are connected to the ACe via the stria terminalis [Renaud and Martin 1975; Krettek and Price 1978], suggesting that ACe function may influence feeding behaviour.

As discussed, it has been shown that food restriction lowers BP in SHR, probably as a result of a decrease in sympathetic activity [Young and Landsberg 1977a; Wright et al. 1981]. Decreased sympathetic tone leads directly to decreased peripheral vasoconstriction and decreased cardiac contractility, thereby reducing both cardiac output and BP. Decreased sympathetic activity also results in diminished renin secretion and decreased tubular sodium reabsorption in the kidney, which decreases both blood volume and BP. Therefore, decreased cardiac output and blood volume through a reduction in sympathetic activity in the lesioned and sham-lesioned SHR up to 15 weeks post-operation in Experiment 2 may

have lead directly to the attenuation of the development of hypertension in these rats.

Decreased food intake in the animals of Experiment 2 may have had further impact on the development of hypertension in these animals, beyond the simple reduction in BW. Diminished food intake in these SHR would have lowered the intake of the essential carbohydrates, proteins, vitamins, minerals and ions necessary for the maintenance of normal SHR physiology (which includes an increased BP). For example, carbohydrates have been suggested to influence intravascular volume and vascular resistance through modulation of insulin and catecholamine synthesis and release [Young and Landsberg 1977a; Muirhead 1980]. Lower carbohydrate intake in the Experiment 2 animals could prevent this "normal" modulation of high BP, leading to an attenuation of the development of hypertension. Similarly, decreased food intake would mean decreased protein intake. L-tyrosine, an amino acid obtained through digestion of protein, is a precursor in the biosynthesis of catecholamines [Axelrod *et al.* 1972]. Therefore, lower food intake would lower protein, L-tyrosine and catecholamines in the SHR, leading to a decreased sympathetic outflow and BP. Potassium ion balance can modify the excitability of vascular tissue [Brunner *et al.* 1970]. Decreased potassium intake through lower food intake may decrease the excitability of vascular tissue, vasoconstriction and finally BP. An extensive review by McCarron *et al.* [1982] outlines the myriad ways malnutrition can effect BP.

Based on these theoretical considerations and on the present experimental results, it is proposed that the delaying effect of destruction of the ACe in young SHR on the subsequent development of hypertension may be explained, at least in part, through its suppressive effect on weight gain.

The data from the rats of Experiment 2 at 22 weeks provided additional information on the effects of ACe ablation on hypertension in SHR. The lesioned rats in Experiment 2 had, 22 weeks after the operation, an average MAP of 190 mmHg, which may be interpreted as fully hypertensive in this strain [Okamoto 1969; Okamoto and Aoki 1963]. This demonstrates that ACe lesions do not prevent the development of hypertension in young SHR, as was previously suggested [Folkow *et al.* 1982; Galeno *et al.* 1982], but only delay the development of full hypertension in these rats. At 22 weeks, not only were the lesioned SHR fully hypertensive, but their pressures were significantly higher than those in the sham-lesioned rats. Although no firm explanation can be offered for the differences in final MAP levels between the lesioned and sham-lesioned rats of Experiment 2 at 22 weeks, it is suggested that this effect may be related to the difference in daily satiety between the treatment groups. Whereas the lesioned animals were fully satiated based on the availability of food *ad libitum*, the control rats were never satiated. Control rats were almost always found to have consumed all their allocated food prior to refilling of their food hoppers, and were also observed to be more restless and showed increased motility compared to the lesioned (satiated) rats. The increased motility, as exercise, may have reduced the development of hypertension in the control SHR. The attenuation of hypertension in SHR by increasing their activity and exercise has been previously demonstrated [Evenwel and Struijker-Boudier 1979; Tipton *et al.* 1977].

Frequent elicitation of the defence response, with its acute pressor effects, has been hypothesized to contribute to the development of hypertension in SHR [Folkow 1982]. The ACe, with its possible role in the regulation of the cardiovascular component of the defence

reaction (see section 1.1), could be involved in this mechanism.

One of the chronic studies that examined the exaggerated BP and HR responses to acute stressors in SHR [Hällback and Folkow 1974] showed that the response to acute stress tends to decrease with age in SHR more than in WKY rats, implying that the differences between hypertensive and normotensive rats may fade with age. However, in another long-term study Yamamoto *et al.* [1987], MAP responses to acute stressors tended to decrease with age in both SHR and WKY, and actually did decrease in both SHR and WKY between the ages of 8 and 96 weeks. Note that the range of ages in that study was larger than that in the current experiments. In Experiment 2, the BP response to acute stressors was significantly smaller in the lesioned vs. sham-lesioned rats only at 15 weeks of age, but, similar to previous studies, the differences in amplitude-time integral of the lesioned vs. sham-lesioned rats tended to decrease with age (see Figure 10). This finding fits with the assumption of an excitatory role for ACe neurons in the elicitation of cardiovascular responses to stressors. Due to the specific objective and design of the current study, the present results do not further elucidate the actual process(es) responsible for the larger cardiovascular responses in SHR, nor those that cause their age-dependency.

In both Experiments 2 and 3, no significant differences in cardiac anatomy measures were noted between lesioned and sham-lesioned SHR. For the 7 and 15 week survival groups of Experiment 2, and the 4 and 11 week survival groups of Experiment 3, this was to be expected after noting no differences in BP between treatment groups. However, no differences in cardiac anatomy (the degree of cardiac hypertrophy) were noted in the 22 week survival group of Experiment 2, when there were significant BP differences between

groups. This finding may have been due to a number of reasons:

1) Equalization of food intake between groups, which equalized the growth rate of lesioned and sham-lesioned SHR may have precluded differences in cardiac tissue growth (hypertrophy).

2) The similar BP in both groups during the first 15 weeks of Experiment 2 may have dictated the similarity in the degree of hypertrophy between lesioned and sham-lesioned groups at 22 weeks.

3) Sympathetic activity has been shown to be positively correlated to food intake and BW in rats [Young and Landsberg 1977a, b]. Sympathetic activity is also positively correlated to the development of cardiac hypertrophy [Sen *et al.* 1974]. The pair-feeding protocol in Experiment 2 equalized the average BW in the lesioned and sham-lesioned groups, which may have equalized sympathetic outflow to the heart and therefore the rate of development of cardiac hypertrophy between groups, 22 weeks post-operation.

Immunohistochemistry using antibodies to CGRP provided an additional method of determining the success of ibotenic acid ablation of the ACe. Although gliosis was evident in rats up to 15 weeks after lesion placement, increased microglia in the ACe was not as evident 22 weeks post-operation through cresyl violet staining. It was also reported by Jarrard [1989] that the reliability of detecting gliosis as a marker for successful excitotoxic lesions diminished rapidly, 13 to 14 weeks after treatment. CGRP immunohistochemistry was especially useful for examination of brain tissue in the 22 week survival period SHR of Experiment 2. Anti-CGRP and CY3 incubated sections revealed distribution of CGRP throughout the ACe, but especially in the lateral subdivision of the ACe (see sham-lesioned

rats in Figure 3 c, e). This distribution of CGRP within the ACe is similar to that reported by others [Skofitsch and Jacobwitz 1985; Shimada *et al.* 1989; Yasui *et al.* 1991]. Examination of the ACe in sham-lesioned rats showed CGRP-containing terminals and presumptive boutons surrounding ACe neurons, in accord with previous histological studies [Shimada *et al.* 1989]. In lesioned SHR, immunofluorescence showed a re-arrangement of the normal appearance of CGRP-containing afferents to the ACe. Instead of the characteristic oval appearance of the ACe, lesioned SHR had afferent terminals laid out in smaller, irregularly shaped masses (see lesioned example in Figure 3 d, f). This irregular distribution of ACe afferents suggested that surrounding tissue impinged on the lesioned area. This type of rearrangement of surviving afferents to an area ablated using a neurotoxin was also reported by Zaczek *et al.* [1980]. In those sections in which a cavity was observed within the former boundaries of the ACe in lesioned animals, CGRP-containing afferents were identified along the perimeter of these holes. This suggested that the target of these afferents, the neurons of the ACe, were destroyed, leaving no termination point for incoming fibres. CGRP also appeared to be relatively depleted in ACe afferents, although the difference between lesioned and control animals was not quantified (compare brightness of immunofluorescence in Figure 3c to 3d, and 3e to 3f). CGRP immunohistochemistry supported the observation that the ACe was atrophied in lesioned vs. control animals.

Experiment 3 was done in order to determine how high sodium levels may affect the functioning of neurons in the ACe. If sodium-sensitive ACe-neurons increased their activity to increase sympathetic output (and thus increase peripheral vasoconstriction, cardiac output and HR), then it was expected that the sham-lesioned SHR would develop higher BP vs.

lesioned rats. These experiments were conducted based on the following reasons:

1) The rate of development of hypertension in SHR, and the final BP level reached is increased when these rats consume a high sodium diet, i.e. a diet with a higher than 0.8% (normal) NaCl content. Studies have shown that young SHR are especially sensitive to the level of sodium in their diet [Berk and Finkelstein 1982; Ely and Weigand 1983; Winternitz and Oparil 1982]. For example, Winternitz and Oparil [1982] demonstrated that 7 week-old SHR given a diet with 3.4 % sodium developed a systolic BP of approximately 214 ± 4 mmHg after 3 weeks, significantly higher than the 170 ± 2 mmHg measured in age-matched SHR that had received a diet with a normal amount of sodium in their food.

2) Neurogenic factors have been suggested to be involved in this sodium-aggravated hypertension in SHR. Along with the accelerated development of hypertension in their 7 week-old SHR, Winternitz and Oparil [1982] measured an increased peripheral resistance due to higher sympathetic activity (indicated by increased plasma norepinephrine levels and an exaggerated depressor response to ganglionic blockade), compared to age-matched control rats that had received a normal sodium diet. Other studies have also suggested that neurogenic factors may play a role in the salt-induced acceleration of the development of hypertension in SHR [Pawloski-Dahm and Gordon 1993; Ely and Weigand 1983].

3) The ACe has been implicated in the regulation of dietary salt intake in rats. Zardetto-Smith *et al.* [1994] measured cumulative 3 hour intakes of 2 % NaCl after sodium depletion using the diuretic furosemide, or subcutaneous injections of the α_2-adrenoreceptor antagonist yohimibine in Sprague-Dawley rats before and after electrolytic ablation of the ACe. While sham-lesioned rats maintained or increased their drinking of the 2 % NaCl in

response to furosemide or yohimbine treatment after operation, ACe-lesioned rats showed significant decreases in absolute 2 % NaCl intake. Zardetto-Smith *et al.* [1994] reported that both sham-lesioned and lesioned rats drank equivalent amounts of water in response to subcutaneous injection of Ang II or hypertonic saline, indicating the lesions specifically affected salt-appetite, and not drinking behaviour. Another study also found that the daily intake of 3 % NaCl was abolished in Sprague-Dawley rats after electrolytic destruction of the ACe, even in rats in which salt appetite should have been enhanced by systemic deoxycorticosterone acetate (DOCA) or renin injection, or by repeated sodium depletions through furosemide injection [Galaverna *et al.* 1992]. Galaverna *et al.* [1992] measured a significantly lower intake of 3 % NaCl in ACe-lesioned rats vs. sham-lesioned rats after DOCA or renin injection, although the lesioned animals did increase their water intake vs. pre-operative levels. Lesioned rats also consumed significantly less 3 % NaCl vs. sham-lesioned rats after sodium depletion with furosemide, and rejected all but the most dilute salt solutions (0.2 %) available to them after sodium depletion. Sham-lesioned animals, comparatively, showed a preference for higher concentrations of sodium in their drinking water. These results suggested that ACe lesions produce an impairment in salt intake behaviour.

4) It has been suggested that an increase in sodium intake may be positively correlated with an increase in aggression. Paterson and Vickers [1982] reported that male mice when given a 2 % NaCl solution as the only available source of drinking water 24 hours prior to testing were more aggressive (initiated more fights in pairs) than control mice that were given water prior to testing. Borderline hypertensive rats, the first filial cross

between an SHR and WKY, also show increasing aggression as the sodium content of their diet is increased (0.8, 2, 8 %) [Gelsema, unpublished observations]. These observations led to the suggestion that ACe neurons, which are reportedly participating in defensive behavior (including aggression, see section 1.2.3), may be chronically activated to higher levels by the higher sodium content of the diet, thus explaining an increase in both aggressiveness and BP.

The expected aggravation of the development of high BP in these SHR on a high sodium diet was not seen. Based on the four points outlined above, it was hypothesized that the ACe may be a key nucleus through which sympathetic control of the cardiovascular system is altered by a high sodium intake in salt-sensitive rats, leading to an aggravated development of hypertension in SHR on a high sodium diet. The pair-feeding protocol first applied in Experiment 2 was also used in the lesioned and sham-lesioned rats of Experiment 3 in order to eliminate possible differences in BP caused by discrepancies in BW.

The pair-feeding protocol in these experiments prevented the development of any differences in BW between ACe-lesioned and sham-lesioned rats. Four and eleven weeks after operation, there were no significant differences in the level of BP between lesioned and sham-lesioned SHR (see Figure 11). The BP responses to acute mental stress were also not significantly different among the treatment groups (see Fig. 13).

Thus, the ACe does not appear to be involved in the effects of high sodium on the development of hypertension in young SHR. There may be several reasons why ACe-ablation failed to have an effect on the resting or stressed BP in the lesioned SHR. The ACe may not have a role in salt appetite, as suggested by Galaverna *et al.* [1992] and

Zardetto-Smith *et al.* [1994]. The selective nature of the present ACe lesions with ibotenic acid left fibers of passage through the ACe intact. These fibers may have been destroyed in the earlier studies as a result of the use of electrolytic lesion techniques, and it is this latter effect which may have lead to the reported differences in sodium appetite. This would infer that other areas whose interconnections pass through the ACe are likely to be responsible for the reported differences in salt appetite between sham-lesioned and ACe-lesioned rats. Thus, disruption of ACe function without affecting other areas in the brain, as accomplished in the present experiments may provide more definitive evidence that the ACe is <u>not</u> involved in the regulation of sodium intake or sodium appetite. A similar amount of dietary sodium intake in both lesioned and sham-lesioned rats would have resulted in similar increases in BP in both groups (as observed, see Figure 11).

Also, as in Experiment 2, the animals in Experiment 3 were pair-fed, and any differences in BP due to sodium intake may have been masked by the effects of equalizing BW between lesioned and control SHR. The pair-feeding essentially equalized the amount of sodium consumed by each of the lesioned and sham-lesioned animals. This protocol must therefore have prevented the demonstration of a role of the ACe in the control of salt appetite. On the other hand, the hypothesis of an involvement of the ACe in the autonomic expression of an elevated salt intake is not supported by the present findings.

<u>Summary of conclusions</u>

It was concluded that selective destruction of only the <u>neurons</u> of the ACe with an excitotoxin (ibotenic acid) attenuated the development of hypertension in young SHR. This finding clarified the results from previous studies that implicated the ACe in the

development of high BP in this model of essential hypertension after non-selective electrolytic destruction of this CNS centre [Galeno *et al.* 1982; Folkow *et al.* 1982].

Results also showed that ACe ablation merely delays the development of hypertension in SHR, and that its effect was likely due to reduced BW gain in the lesioned animals. The development of high BP in SHR is clearly not prevented by placement of ACe lesions, as was previously thought [Galeno *et al.* 1982; Folkow *et al.* 1982].

The results of this study, combined with lack of behavioural and cardiovascular effects of selective activation of amygdaloid neurons by microinjection of an excitatory amino acid in conscious rats (Gelsema *et al.* [1987]), cast serious doubt on the widely held view that the ACe is involved in the development of hypertension induced by stressful stimuli (Folkow [1982]; Folkow *et al.* [1982]; Galeno *et al.* [1982]). The present results suggest that the ACe may affect the rate of development of hypertension through its interactions with hypothalamic control of food intake. The strong influence of the quality and quantity of food intake on the development of hypertension in animals and humans merits further experimental investigation into these central interactions.

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