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# A Study of Rat Chromosome 8 by Congenics:

# Mapping and Dissecting Quantitative Trait Loci into

# Opposite Blood Pressure Effects

par


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Biologie Moléculaire


Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de Maîtrise
en Biologie Moléculaire

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Mapping and Dissecting Quantitative Trait Loci into

Opposite Blood Pressure Effects

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# RÉSUMÉ ET MOTS CLÉS FRANÇAIS

L'hypertension essentielle est une maladie complexe affectée par des facteurs génétiques et environnementaux. Il est estimé que des facteurs génétiques contribuent entre 30% et 50% de la variation de la pression artérielle (PA) observée. L'identification des gènes peut apporter une meilleure compréhension sur la pathophysiologie de la maladie.

Les souches de rats consanguins sont utilisées dans cette étude sont le rat hypertendu Dahl sensibles au sel (S) et le rat normotendu Lewis (LEW). Mon étude se concentre sur le chromosome 8 (C8) du rat. Une étude antérieure des liaisons génétiques suggère qu'une région du C8 contient un locus pour le trait quantitatif (QTL) de la PA. L'objectif de cette étude consiste à vérifier si ce QTL existe et de le localiser à une région précise sur C8. Des lignées congéniques, obtenues par le croisement du rat S et du rat LEW, de façon à remplacer des régions du C8 du rat S, adjacentes et non chevauchants, par les régions homologues du rat LEW, permettent d'associer un changement potentiel de la PA à une région sur le C8. Les mesures du PA ont été faites par télémétrie. Des deux lignées congéniques produites, les allèles LEW d'une des souches baissent la PA ($p<0.001$). Par contre, les allèles LEW de la deuxième souche congénique augmentent la PA ($p<0.001$). Cette étude prouve qu'il existe sur la C8 deux QTLs de la PA, adjacents, mais séparés, avec des effets opposés ce qui n'a pas été observé dans les études antérieures de liaisons.

**Mots-clés :** Hypertension essentielle; rat Dahl sensibles au sel; locus pour trait quantitatif; modèle animal; lignées congéniques; facteurs génétiques; chromosome 8

# ENGLISH ABSTRACT AND KEYWORDS

Essential hypertension is a complex disease with genetic and environmental factors. It is estimated that 30 to 50 % of the variations observed in blood pressure (BP) is caused by genetic factors. The identification of the genes that affect BP is crucial for a full comprehension of the pathophysiology of the disease.

My study focuses on rat chromosome 8 (C8). According to previous linkage studies, C8 likely contains one quantitative trait locus (QTL) for BP. The purpose of my study is to determine whether such a QTL exists, and if so, to assess its effect on BP. Our basic strategy is to use congenic strains. Our congenic strains were constructed from inbred strains with contrasting effects on BP regulation, namely the hypertensive Dahl Salt-Sensitive (S) strain and the normotensive Lewis (LEW) strain. Non-overlapping, adjacent C8 regions of the S rat were replaced with homologous regions of the LEW rat. BP was measured by telemetry. Of the two congenic strains produced, LEW alleles of one congenic strain lower BP ($p<0.001$). In contrast, LEW alleles of the other increase BP (p< (0.001). Thus, two BP QTLs on C8, adjacent to each other, but with opposite effects exist on C8. The effect of the second, novel QTL has not been reported in previous linkage studies.

**Keywords:** essential hypertension; salt-sensitive Dahl rat; quantitative trait locus; animal model; congenic strain; genetic factors; chromosome 8

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# ABBREVIATIONS

| | |
|---|---|
| **ADH** | antidiuretic hormone |
| **ACE** | angiotensin converting enzyme |
| **ANP** | atrial natriuretic peptide |
| **ANS** | autonomic nervous system |
| **ATI** | angiotensin I |
| **ATII** | angiotensin II |
| **AVP** | vasopressin |
| **BNP** | brain natriuretic peptide |
| **BP** | blood pressure |
| **bp** | base pair (s) |
| **C8** | chromosome 8 |
| **C8S.L1** | chromosome 8 S-LEW 1 |
| **C8S.L2** | chromosome 8 S-LEW 2 |
| **cGMP** | cyclic guanosine monophosphate |
| **CHD** | coronary heart disease |
| **cM** | centimorgan |
| **cNOS** | constitutive nitric oxide synthase |
| **CNS** | central nervous system |
| **CO** | cardiac output |
| **cR** | centiRays |
| **CVC** | cardiovascular center |
| **CVD** | cardiovascular disease |
| **DAP** | diastolic arterial pressure |

| | |
|---|---|
| **DBP** | diastolic blood pressure |
| **DNA** | deoxyribonucleic acid (s) |
| **dNTP** | deoxyribonucleotide triphosphate |
| **D/R** | donor/recipient |
| **ET-1** | endothelin |
| **Gb** | gigabases |
| **GDB** | genome database |
| **HR** | heart rate |
| **iNOS** | inducible nitric oxide synthase |
| **KCl** | potassium chloride |
| **LEW** | Lewis strain |
| **LOD** | logarithm of odds |
| **MAP** | mean arterial pressure |
| **$MgCl_2$** | magnesium chloride |
| **mmHg** | millimeters of mercury |
| **NaCl** | sodium chloride |
| **NE** | norepinephrine |
| **NO** | nitric oxide |
| **NTS** | nucleus tractus solitarius |
| **PCR** | polymerase chain reaction |
| **PGI2** | prostacyclin |
| **PNS** | parasympathetic nervous system |
| **QTL** | quantitative trait locus/loci |
| **R** | Dahl salt-resistant strain |
| **RAAS** | renin-angiotensin-aldosterone system |

| | |
|---|---|
| **S** | Dahl salt-sensitive strain |
| **SAP** | systolic arterial pressure |
| **SBP** | systolic blood pressure |
| **SHR** | spontaneously hypertensive rat |
| **SNP** | single nucleotide polymorphism |
| **SNS** | sympathetic nervous system |
| **SV** | stroke volume |
| **SVR** | systemic vascular resistance |
| **U** | units |
| **WHO** | world health organization |

# DEDICATION

This work is dedicated to my parents, who have supported me throughout my life and have been pillars of strength when I really needed them. In loving memory of my grandmother whose favourite saying was 'Hitch your wagon to the highest stars' and in memory of Roselle Cossette, who was like an aunt to me, whose passing has left many lives with one less ray of sunshine.

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# REVIEW OF THE LITERATURE

# Chapter 1

# Hypertension: Clinical features of hypertension

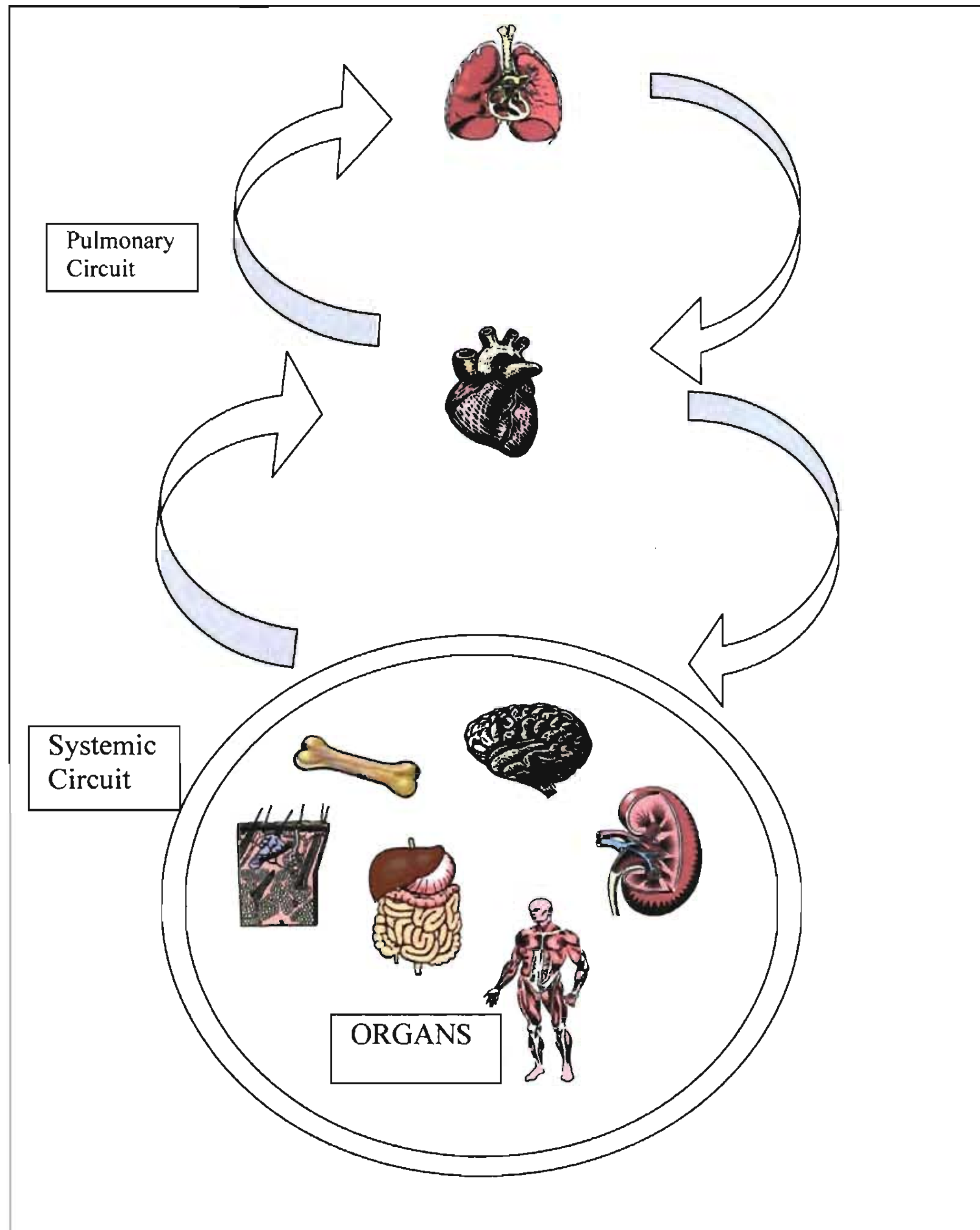
## 1.1 *The Cardiovascular System*

The cardiovascular system is an organ system whose function is to propel and transport blood and its constituents throughout the body. The components of the cardiovascular system are: the blood, the heart and blood vessels(67).

Blood consists of liquid plasma and cells. It is the medium by which substances such as nutrients, hormones, gases (oxygen, carbon dioxide) and metabolic wastes are transported to and removed from tissues. The vascular system is made up of arteries, veins, and capillaries (blood vessels), which carry blood to and from all tissues (Figure1).

The heart pumps blood at a high pressure through the vascular system. It sends blood to the lungs for oxygenation and via the aorta, distributes oxygenated blood to the tissues of the body. It is a four-chamber pump, with the right side receiving deoxygenated blood from the body at low pressure and pumping it to the lungs (the pulmonary circulation) and the left side receiving oxygenated blood from the lungs and pumping it at high pressure around the body (the systemic circulation)(20,95). It can also help stabilize body temperature, oxygen concentration, ionic composition, osmolarity and pH as part of homeostasis(119,124).

**Figure 1 - The Cardiovascular System**




The cardiovascular system is comprised of the heart, with its four chambers; arteries, in which blood moves away from the heart; veins, in which blood returns to the heart; and a system of capillaries, which transport blood between small arteries and small veins. The cardiovascular system is further divided into the pulmonary circuit, which delivers deoxygenated blood to the lungs, and the circulatory circuit, which delivers oxygenated blood to all of the organs and tissues.

### 1.1.1 Hemodynamics of Circulation

The primary determinants of systemic blood pressure (BP) are cardiac output (CO) and peripheral vascular resistance. CO is the amount of blood ejected per minute by the left ventricle into the aorta or by the right ventricle into the pulmonary trunk. It is determined by plasma volume, cardiac stroke volume, heart rate and myocardial contractility. It is calculated as CO (mL/min)= Stroke Volume (SV) (mL/beat) x Heart Rate (HR) (beats/min), where SV the amount of blood ejected by a ventricle during each systole. SV is related to the stretch of the heart before it contracts, the forcefulness of the contraction and the resulting pressure before ventricular ejection begins. Peripheral vascular resistance is a function of the balance of humoral vasoconstriction and vasodilation, adrenergic activity and arteriole smooth muscle tone. CO and peripheral vascular resistance are the primary determinants of systemic BP. Given these two factors, mean arterial BP is calculated as CO x peripheral vascular resistance.

Extremely complex physiological networks and other factors underlie BP regulation and hypertension susceptibility. Arterial BP is generated by the left ventricle ejecting blood into the systemic vasculature, which acts as a resistance to CO. With each ejection of blood (ventricular systole), the aortic blood volume increases, stretching the wall of the aorta. As the heart relaxes (ventricular diastole), blood flows from the aorta into distributing arteries that distribute the blood to the various organs. Within the organs, the arterial vasculature undergoes extensive branching and the vessel diameters decrease. The smaller arteries and arterioles serve as the chief resistance vessels. Through changes in their diameter, they serve to regulate systemic vascular resistance and organ blood flow. In hemodynamic terms, the mean arterial pressure (MAP) can be described by MAP = (CO x SVR) + CVP, where SVR = systemic vascular resistance, and CVP = central venous pressure. Therefore, increases in CO, SVR or CVP will lead to increases in MAP (95,119,124).

## 1.2 *Definition of hypertension*

The general term "blood pressure" was coined in the early 1700s by the man who first measured it, the Reverend Stephen Hales of England (75,112). The expression "blood pressure" (BP) refers to the arterial pressure in systemic circulation. The movement of

blood throughout the organism depends on the strength of the heartbeat and on the pressure gradient in the cardiovascular system. BP is dependent on cardiac pressure and vascular resistance. BP is highest in the aorta and progressively decreases as the blood flows further away from the heart. So the pressure gradient is such that there is a progressive lowering of BP as the blood travels from large arteries to small ones, then from the capillaries back through the veins, until it returns to the heart atria through the vena cava. BP is measured in millimetres of mercury (mmHg).

The cardiac cycle consists of two parts: systole (contraction of the heart muscle in the ventricles) and diastole (relaxation of the ventricular heart muscles). When the ventricles contract, they force the blood out of the heart into the arteries. The left ventricle empties into the aorta (systemic circuit) and the right ventricle into the pulmonary artery (pulmonary circuit). The heart valves open and close to limit flow to a single direction. During cardiac diastole BP fluctuates between a maximum value and a minimum value (diastolic blood pressure (DBP) or diastolic arterial pressure (DAP)). The systolic blood pressure (SBP) or systolic arterial pressure (SAP) is a measure of the BP within the artery at the moment when the heart contracts (ventricular contraction) and ejects blood towards the arteries. DBP corresponds to the BP when the heart relaxes and the aortic valve closes (BP between ventricular contraction). Mean arterial pressure (MAP) is the average between DBP and SBP. MAP is a measure that reflects the BP to which small blood vessels are continuously submitted to.

When the values for these pressures are chronically elevated, the condition is called hypertension. Hypertension is defined in general as a chronic elevation in BP in the arteries, when compared to values that are considered normal. In general, hypertension is defined as a BP systolic value $\geq$ 140 mmHg or diastolic value $\geq$ 90 mmHg (i.e. values exceeding 140/90 mmHg), or both. The official criteria on threshold values for hypertension vary, but the criteria set by the World Health Organization (WHO) are widely referred to (Table 1) (75).

Elevated BP is a common and powerful predisposing factor for stroke, coronary disease, cardiac failure and peripheral artery disease, imposing a 2-3 fold increased risk of one or more of these atherosclerotic sequelae. The risk ratio imposed by hypertension is greatest for cardiac failure and stroke, but in Western countries coronary disease is the most common and lethal hazard (88).

**Table 1 -Classification of hypertension**

| CATEGORY | Systolic (mm Hg) | Diastolic (mm Hg) |
|---|---|---|
| Optimal | < 120 | < 80 |
| Normal | < 130 | < 85 |
| High-normal | 130-139 | 85-89 |
| Grade 1 hypertension (mild) | 140-159 | 90-99 |
| Subgroup: borderline | 140-149 | 90-94 |
| Grade 2 hypertension (moderate) | 160-179 | 100-109 |
| Grade 3 hypertension (severe) | ≥180 | ≥110 |
| Isolated systolic hypertension | ≥140 | < 90 |
| Subgroup: borderline | 140-149 | <90 |

Table adapted from Hansson, L. Hypertension management in 2002: where have we been? where might we be going? *Am. J. Hypertens.* **15**, 101S-107S (2002). (75)

## 1.2.1 Types of Hypertension

Hypertension affects 15-30% of the human population, including 50 million Americans. The two main categories of hypertension are primary hypertension, also known as essential hypertension, and secondary hypertension. The vast majority of patients have essential hypertension, which is a form with no identifiable underlying cause(95). Unknown genetic and environmental factors are thought to play a role in the pathogenesis of essential hypertension. Secondary hypertension, which accounts for only 5-10% of cases is due to definable causes such as renal vascular disease, chronic renal failure, renal artery disease, thyroid disease pregnancy and endocrine abnormalities such as, primary hyperaldosteronism (95).

### 1.2.1.1 Primary or Essential Hypertension

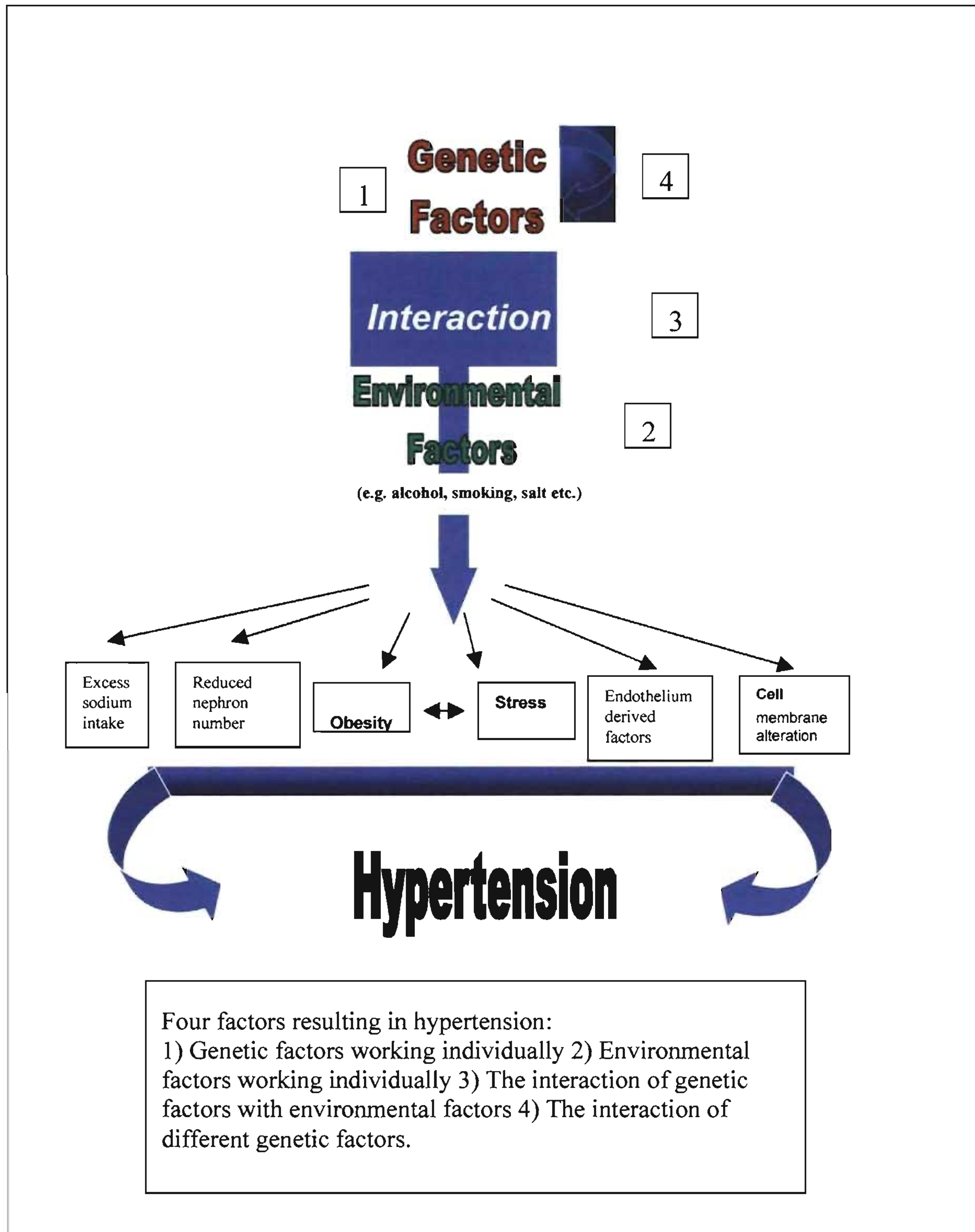
Despite many years of research, there is no unifying hypothesis to account for the pathogenesis of primary hypertension. There is a natural progression of this disease that suggests that early elevations in blood volume and CO might initiate increased resistance in the systemic vasculature. In hypertensive patients, the vascular endothelium produces less nitric oxide and the vascular smooth muscle is less sensitive to the actions of this powerful vasodilator. There is also an increase in endothelin production, which can enhance vasoconstrictor tone. Many mechanisms may operate to initiate and sustain hypertension.

Treatment of patients with primary hypertension generally involves pharmacological intervention (2) to modify factors such as angiotensin II, sympathetic activity and calcium entry into cells so as to reduce arterial pressure. These treatments do not target the underlying disease. Nevertheless, treatment of hypertension with antihypertensive drugs is important, because hypertension increases the risk for coronary artery disease, strokes, renal disease and other disorders. The three broad classes of drugs used to treat primary hypertension are diuretics (to reduce blood volume), vasodilators (to decrease systemic vascular resistance), and cardioinhibitory drugs (to decrease CO). Essential hypertension is further subdivided into a monogenic form and polygenic forms.

#### *1.2.1.1.1 Monogenic Form of Hypertension*

In the monogenic form of essential hypertension, the phenotype is due to lesions or mutations in a single gene, leading to eg., overproduction of mineralocorticoids. This form is also known as the Mendelian form of hypertension. The monogenic form of hypertension is only responsible for only 5% of cases (41,81). Mendelian forms of hypertension include Liddle's syndrome, glucocorticoid remediable aldosteronism, apparent mineralocorticoid excess and pseudohypoaldosteronism type II. The examples previously listed affect the homeostasis of salt and water reabsorption. Even though Mendelian forms of hypertension are more rare and severe than essential hypertension, there is a tremendous effort to better understand their etiology in the hope that it would lead to clues about the pathophysiology of essential hypertension (51,55,185,186,199).

**Figure 2 - Factors Contributing to Essential Hypertension**




Four factors resulting in hypertension:
1) Genetic factors working individually 2) Environmental factors working individually 3) The interaction of genetic factors with environmental factors 4) The interaction of different genetic factors.

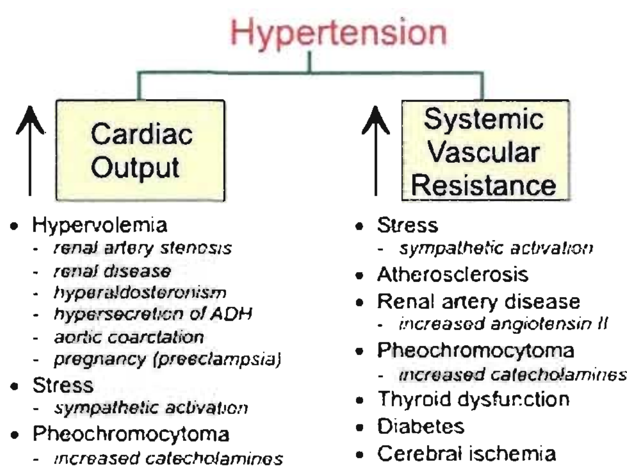
#### *1.2.1.1.2 Polygenic Form of Hypertension*

Polygenic hypertension is caused by the interaction of many genes, as opposed to the monogenic form, which is due to a single gene. It is also influenced by environment factors and the interaction between environmental factors and genetic factors (Figure 2). 90-98% of all patients with primary hypertension suffer from polygenic hypertension (178).

For example, it has long been known that hypertension aggregates in certain families. Familial clustering of high BP could be the consequence of inheritance of a BP gene or genes, household environment, or a combination of both. Familial aggregation studies have shown that clustering occurs early in life since siblings living apart do not become dissimilar and spouses do not become more similar even though they share the same environment. Phenotyping hypertension is remarkably complex because of the difficulty of accurately measuring BP and the vast variety of factors regulating BP. For example, BP is partly determined by the pumping action of the heart, so it is inherently pulsative in character. What is more, an individual's BP varies by time of day, season and other circumstances such as stress and emotions (137).

### 1.2.1.2 Secondary Hypertension

**Figure 3 - Various Ways Certain Conditions Cause Secondary Hypertension**




Disease conditions increase either cardiac output or systemic vascular resistance or both, and thereby increase blood pressure.
Adapted from Klabunde, R.E. Cardiovascular physiology concepts (2005) (95).

Secondary hypertension is the direct result of another disease or condition such as obesity, thyroid, or adrenal dysfunction. These conditions increase either SVR or CO or both, which in turn results in hypertension (Figure 3). The most common conditions resulting in secondary hypertension are as follows.

*Renal artery disease:* Renal artery disease causes stenosis, which is the narrowing of the vessel lumen. The reduced lumen diameter increases the pressure drop along the length of the diseased artery, which reduces the pressure at the afferent arteriole in the kidney. Reduced arteriolar pressure and reduced renal perfusion stimulate rennin release by the kidney. This increases circulating angiotensin II (ATII) and aldosterone. These hormones increase blood volume by enhancing renal reabsorption of sodium and water. Increased ATII causes systemic vasoconstriction and enhances sympathetic activity. Chronic elevation of ATII promotes cardiac and vascular hypertrophy. The net effect of these renal mechanisms is an increase in blood volume that augments CO. Therefore, hypertension caused by renal artery stenosis results in an increase in both SVR and in CO.

*Primary hyperaldosteronism*: This is the increased secretion of aldosterone, which generally results from adrenal adenoma or adrenal hyperplasia. Increased circulating aldosterone causes renal retention of sodium and water, so blood volume and arterial pressure increase. Furthermore, the adrenal medulla secretes more catecholamines (epinephrine and norepinephrine). Activation of the sympathetic nervous system also increases circulating ATII, aldosterone, and vasopressin, which can increase SVR. Prolonged elevation of ATII and catecholamines can lead to cardiac and vascular hypertrophy, both of which can contribute to a sustained increase in BP.

*Stress:* Physical or psychological stress leads to activation of the sympathetic nervous system, which causes increased release of norepinephrine from sympathetic nerves in the heart and blood vessels. This leads to increased CO and increased SVR.

*Sleep Apnea:* Sleep apnea is a disorder in which people repeatedly stop breathing for short periods of time (10-30 seconds) during their sleep. Individuals suffering from sleep apnea have a higher incidence of hypertension.

*Hyperthyroidism:* This results from an excess of thyroid hormone induces systemic vasoconstriction, an increase in blood volume, and increased cardiac activity, all of which can lead to hypertension.

*Pheochromocytoma:* This occurs when catecholamine secreting tumours in the adrenal medulla give rise to high levels of circulating catecholamines, both epinephrine and norepinephrine. Thus, the tumours themselves produce epinephrine and norepinephrine. The presence of these hormones leads to systemic vasoconstriction and cardiac stimulation, both of which contribute to significant elevations in BP.

*Aortic coarctation:* This is the narrowing of the aorta, typically just distal to the left subclavian artery, is a congenital defect that obstructs aortic outflow, leading to elevated BP proximal to the coarctation (i.e., elevated arterial pressures in the head and arms). Reduced systemic blood flow and reduced renal blood flow lead to an increase in the release of renin and an activation of the renin-angiotensin-aldosterone system (RAAS). This in turn elevates blood volume and BP. Although the aortic arch and carotid sinus baroreceptors are exposed to higher than normal pressures, the baroreceptor reflex in blunted due to structural changes in the walls of vessels where the baroreceptors are located. Also, baroreceptors become desensitized to chronic elevation in pressure and become "reset" to the higher pressure(95,124).

## 1.3 Traditional risk factors for hypertension

### 1.3.1 Salt sensitivity

"Hence, if too much salt is used, the pulse hardens" Huang Ti 2600 BCE. This ancient wisdom of Chinese doctors shows us for how long sodium has been associated with circulatory effects (130), and thus it is maybe the most studied risk factor predisposing for

hypertension. Large studies, like Intersalt (46-48,174-176), have linked sodium and BP convincingly. Moreover, primitive people with diets low in salt have no hypertension. However, the response of BP to variations in dietary salt intake is quite heterogeneous. In some hypertensive individuals, decreases in BP as a result of salt restriction are clinically significant, while in others little or no change in BP occurs, whereas in others, BP may actually increase with salt restriction(193). Salt-sensitive patients are also more likely than salt-resistant patients to exhibit left ventricular hypertrophy, microalbuminuria, and metabolic abnormalities. These effects naturally make salt-sensitive individuals attractive subjects for genetic studies(175,181,194,196-198).

An individual's sensitivity to dietary salt may also be related to ethnic background, gender, and other environmental factors. For example, the decline in the glomerular filtration rate with age is more marked in individuals of African origin. Also, in a study of more than 400 hypertensive subjects, seven missense mutations were found in the gene coding for the β-ENaC subunit of the amiloride-sensitive sodium channel, most of them in patients of African-American descent. Some of the ENaC polymorphisms might be associated with greater ENaC activity in vivo and contribute to ethnic differences in sodium retention and the risk of low-renin hypertension (180).

The identification of several molecular mechanisms linking dietary salt and hypertension confirm that salt sensitivity and BP are not determined by a single gene but rather by the combined action of a number of genes. Each of these genes may affect one or more channels, transporters, or enzymes associated with the neural, hormonal, vascular, and renal mechanisms affecting BP. For example, salt-sensitive hypertension may be associated with changes not only in vascular mechanisms but also in the renal Na+/Ca2+ exchanger protein (93). A high-salt diet may affect cellular mechanisms not only in vascular smooth muscle, but also in the endothelium. Some studies have shown that an increase in aortic basal tone in $\alpha_2$-$Na^+$-$K^+$-ATPase deficient mice that is dependent on the endothelium(93). Also, homozygous 11-HSD2-deficient mice that exhibit impaired renal sodium excretion may have an additional endothelial dysfunction, as demonstrated by enhanced norepinephrine-induced vascular contraction, which may contribute to hypertension(93).

It is thought that changes in BP normally serve as a physiological response whose purpose is to maintain sodium balance and extracellular volume within normal limits. Impairment of the mechanisms responsible for the pressure-natriuresis relationship will cause BP levels to rise in order to achieve the adjustments in urinary sodium excretion

required to maintain homeostasis. The BP regulatory system is thereby reset at a higher BP level (158).

### 1.3.2 Alcohol

Heavy alcohol intake has been found to associate with hypertension (120) .Up to now, no signalling mechanism has been discovered that could clearly explain this link. Among the male Caucasian population, SBP and DBP increase with the quantity of alcohol consumed. In male African-Americans on the other hand, there is a greater increase in diastolic pressure for comparable quantities of alcohol consumed. This tendency is also observed in women, but the increase in BP is less pronounced (5,116,120,140).

### 1.3.3 Obesity

Studies of the genetic epidemiology of obesity have suggested that the predisposition for development of obesity is partly genetically determined (5,11,77,171,174). Obesity is a leading risk factor for essential hypertension. Obese people have also more left ventricular hypertrophy than lean individuals. Those who weigh the most have, on the average, the highest BP of a given age group. Losing weight reduces BP and left ventricular hypertrophy. However, it has also been long known that not all obese individuals are hypertensive and vice versa. Additionally, in some populations, such as Mexican-Americans and African-Americans, obesity seems to have less effect on the development of hypertension than in Caucasians (193).

### 1.3.4 Insulin Resistance

Insulin resistance syndrome, also known as metabolic syndrome or "syndrome X", is a condition which is characterized by a combination of pathologies including hypertension, diabetes, abdominal obesity, and dyslipidemia, among others. This syndrome is very significant when considering the genetic makeup of the hypertensive population (13,149-155,164).

Insulin is a polypeptide hormone secreted by the pancreas. Its main purpose is to regulate the levels of glucose in the body antagonistically with glucagon through negative feedback loops. It also exhibits vasodilatory properties.

Insulin resistance is characterized by a blunting of the body's normal reaction to the regulating influence of insulin, resulting in an over-production of insultin to compensate. It

is one of the causes of diabetes and is the main cause of obese individuals being diabetic. In the hypertensive patients with insulin resistance, the reduction in MAP, after a glucose load, was blunted, when compared to insulin-sensitive hypertensive patients and normotensive patients (172). In normotensive and insulin-sensitive hypertensive individuals, insulin may stimulate sympathetic activity without elevating MAP (117).Though it is known that diabetes often appears simultaneously with hypertension, the mechanism or mechanisms, causing syndrome X have yet to be discovered.

### 1.3.5 Gender, Age and BP

Men generally have a higher mean BP than women, regardless of age. As for SBP, men also have higher levels than women during early adulthood. Above the age of sixty, the opposite becomes true (138). DBP, on the other hand, tends to be slightly higher in men than in women regardless of age.

The prevalence of hypertension increases progressively with age in both men and women. However, for BP there is a sexual dimorphism (14,160). For women, although long-term studies such as the Framingham(78,90) have not documented a rise in BP with menopause, other studies have found significantly higher SBP and DBP in postmenopausal compared to pre-menopausal women, even after adjustment for age and body mass index (160). According to these studies, postmenopausal women are twice as likely to have hypertension than pre-menopausal women. This menopause-related increase in BP has been attributed to a variety of factors, including estrogen withdrawal, over-production of pituitary hormones, weight gain, or perhaps a combination of these and other yet undefined neurohumoral influences.

Androgen is thought to play a role in the sex differences in BP, though its effects are more pronounced in men. One possible mechanism may be the blunting of the pressure-natriuresis relation (138). Female sex hormones and their receptors may also be implicated in BP differences between men and women. Though no significant associations have been detected between estrogen receptor genes and BP among women, a genetic association study by the Victorian Family Heart Study investigators found that men inheriting the "α" allele on the estrogen receptor a gene had significantly higher SBP levels (5 mm Hg) than men with other genotypes (49,138).

Increased age is an important risk factor for higher BP (160), since it may reflect the accumulation of different factors predisposing for hypertension as well as factors related to normal aging, such as pre-programmed senescence or just general wear and tear (167,170).

### *1.4 High BP as a Risk Factor for Morbidity and End-Organ Damage*

Hypertension is one of the most important risk factors for cardiovascular diseases (66). The risk of coronary heart disease and stroke increases with increased BP. Patients who do not control high BP face a reduced life span, because hypertension can cause certain organs to deteriorate over time. Some of the most common complications of hypertension are listed in Table 2.

For the heart, for example, high BP contributes to 75% of all strokes and heart attacks, according to the National Heart, Lung, and Blood Institute. Compared with normal individuals, hypertensive people can have as high as ten times the risk of stroke and five times the risk of a heart attack, depending on the severity of the hypertension. The risk for developing congestive heart failure is also significantly higher with increased BP. People whose high BP has led to enlargement of the left side of the heart (left ventricular hypertrophy) remain at risk for strokes, heart attacks, sudden death, and heart failure even after their BP is under medical control.

Hypertension is also implicated in kidney dysfunction and in calcium retention problems. High BP causes 30% of all cases of kidney failure, a rate that is second only to diabetes. Animal studies have indicated that when heart cells enlarge in response to high BP, they undergo molecular changes that cause an abnormal release of calcium, a mineral crucial for healthy heart contractions. This defect appears to be irreversible. Hypertension also increases the elimination of calcium in urine, which in turn may lead to loss of bone mineral density (136,188). This is a significant risk factor for fractures, particularly in elderly women.

Cardiovascular disease, (CVD) shares most of the same risk factors as hypertension, such as age, smoking, dyslipidemia, particular personality characteristics (aggressive or type A personality), diabetes and obesity. For example, excess alcohol consumption predisposes to acute cardiac events, whereas it has been suggested that moderate alcohol consumption protects against CVD morbidity (89). On the other hand, left ventricular hypertrophy, a form of CVD, is a common complication of hypertension. It is a powerful

risk factor for coronary artery disease and sudden death, independent of alcohol consumption(191).

**Table 2-Hypertension and end organ damage**

| **Cardiac** | |
|---|---|
| Coronary heart disease | Left ventricular hypertrophy |
| Myocardial infarction (heart attacks) | Diastolic dysfunction |
| Sudden death | Congestive heart failure |
| **Cerebrovascular** | |
| Cerebral thrombosis / hemorrhage | Dementia and reduced cognitive function |
| Hypertensive encephelopathy | Stroke |
| **Renal** | |
| Microalbuminuria | |
| Chronic renal insufficiency | |
| **Other** | |
| Carotid artery stiffening | Retinopathy |
| Arterial aneurysms | |

From L. Hasson article "Assessment of hypertensive organ damage" (118)

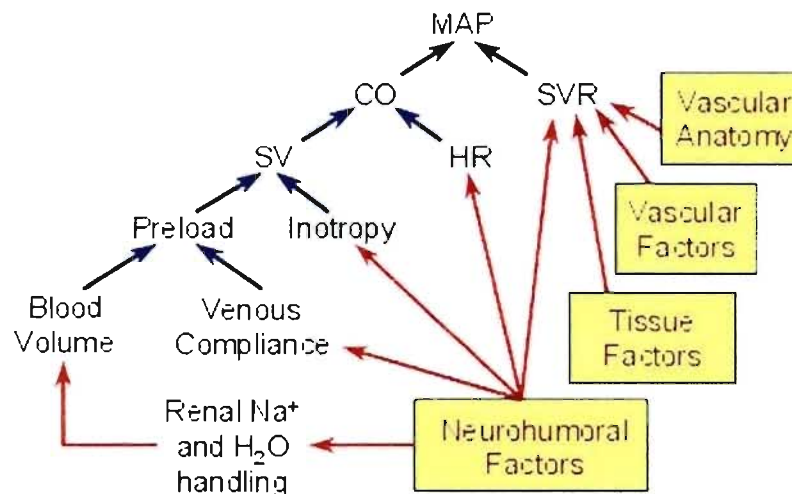
## *1.5 Factors Regulating Arterial BP*

BP and blood flow is part of the homeostatic system regulated by feedback mechanisms. Information on BP and blood flow is picked up by sensory receptors, mainly baroreceptors and chemoreceptors(16), and transmitted to higher brain regions such as the cerebral cortex, the limbic system and the hypothalamus (123,200). These regions transmit regulatory responses along sympathetic nerves (cardiac accelerator nerves and vasomotor

nerves) and opposing parasympathetic nerves (vagus nerves). The endocrine system is also implicated in the complex homeostasis of BP.

The following scheme (Figure 4) summarizes the general factors that regulate CO and SVR.

**Figure 4 - Factors Affecting Mean BP**




Mean arterial pressure is regulated by changes in cardiac output and systemic vascular resistance. See text for details.
Adapted from Klabunde, 2005 (95).

MAP is regulated by changes in SVR and CO. The SVR depends on the vascular anatomy, in vascular, tissue and neurohumoral factors. CO, on the other hand is the product of stroke volume (SV) and heart rate (HR), while SV is a function of ventricular preload (defined as the initial stretching of the cardiac myocytes prior to contraction) and inotropy. Ventricular preload is dependent on venous compliance and blood volume, which itself relies on sodium and water handling by the kidneys. Neurohumoral factors control CO indirectly, by directly affecting HR, inotropy, venous compliance and sodium and water regulation in the kidneys. Genes that regulate any component of this scheme will modify MAP.

SVR is determined by the anatomy of the vascular network. Generally, vascular structure remains relatively constant; however, certain pathological conditions, such as vascular thrombosis can affect the number of perfused blood vessels. Furthermore, other pathological conditions can result in changes in the relative number of parallel and series resistance elements of the vascular network.

The most important mechanism for changing SVR involves changes in vessel lumen diameter. In chronic hypertension, vessel radius is often reduced due to thickening of the vessel wall leading to a reduction in lumen size. Vascular factors such as nitric oxide, endothelin, and prostacyclin also influence vessel diameter. Furthermore, myogenic mechanisms intrinsic to the vascular smooth muscle can also alter vessel diameter. Tissue factors (e.g., adenosine, potassium ion, hydrogen ion, histamine) are chemicals released by parenchymal cells surrounding blood vessels and can significantly alter vessel diameter. In general, tissue factors are involved with regulating organ blood flow more so than systemic arterial pressure; however, any change in vessel tone will affect both organ blood flow and systemic arterial pressure. Finally, neurohumoral mechanisms play a very important role in regulating SVR and BP (2,95).

## 1.5.1 Neural Regulation of BP

### 1.5.1.1 The Nervous System

The nervous system is the system of cells, tissues, and organs that regulates the body's responses to internal and external stimuli. The nervous system is divided into two parts, the central nervous system (CNS) and the peripheral nervous system (PNS).

The central nervous system (CNS) represents the largest part of the nervous system, including the brain and the spinal cord. The peripheral nervous system consists of the nerves and neurons that extend outside the CNS , such as the nerves in the limbs and organs.

The peripheral nervous system is divided into the somatic nervous system and the autonomic nervous system (ANS). The somatic nervous system includes all the neurons connected with the muscles, sense organs and skin. It consists of afferent fibers that receive information from external sources and efferent fibers that are responsible for muscle contraction. It is responsible for receiving external stimuli and for the voluntary control of body movements through the action of skeletal muscles.

The ANS is the part of the nervous system that controls homeostasis. It does so mostly by controlling cardiovascular, digestive and respiratory functions. The ANS is further divided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS), which typically function in opposition, complementing each other.The SNS is involved in actions requiring quick responses, while the PNS is involved

in actions that do not require immediate reaction. The SNS acts via adrenergic receptors, a class of guanosine nucleotide-binding regulatory protein -coupled receptors that are targets of catecholamines. Adrenergic receptors specifically bind their endogenous ligands, the catecholamines epinephrine and norepinephrine and are activated by these. The PNS acts via muscarinic receptors. Receptors that are membrane-bound acetylcholine receptors are more sensitive to muscarine than to nicotine. Those, for which the contrary is true, are known as nicotinic acetylcholine receptors.

#### *1.5.1.1.1 Innervation of the Vasculature*

The ANS is controlled from the medulla, located in the brainstem above the spinal cord. The medulla receives sensory input from various systemic and central receptors (e.g., baroreceptors and chemoreceptors) as well as signals from other brain regions (e.g., the hypothalamus). Autonomic outflow from the brainstem is divided principally into sympathetic and parasympathetic (vagal) branches. Efferent fibers of these autonomic nerves travel to the heart and blood vessels where they modulate the activity of these organs.

Cardiac function is altered by neural activation. Sympathetic stimulation increases heart rate, inotropy (contractility), and conduction velocity, whereas parasympathetic stimulation of the heart has opposite effects. Sympathetic and parasympathetic effects on heart function are mediated by beta-adrenoceptors and muscarinic receptors, respectively.

Blood vessels and nerves travel primarily along the same pathways in the body. Sympathetic adrenergic nerves travel along arteries and are found in the adventitia (outer walls of a blood vessels). Varicosities, which are small enlargements along the nerve fibers, are the site of neurotransmitter release. Capillaries receive no innervation. Activation of vascular sympathetic nerves causes vasoconstriction of arteries and veins mediated by alpha-adrenoceptors. The release of acetylcholine (ACh) from parasympathetic nerves has a direct vasodilatory action. ACh release is often coupled to nitric oxide formation and guanylyl cyclase activation.

The SNS, the fast-acting component of the ANS, plays a major role in the regulation of arterial pressure. The norepinephrine-releasing, sympathetic adrenergic nerves that innervate the heart and blood vessels are postganglionic efferent nerves. Within the medulla are located sympathetic excitatory neurons that have significant basal activity, which generates a level of sympathetic tone to the heart and vasculature even under basal

conditions. The sympathetic neurons within the medulla receive input from other neurons within the medulla (e.g., vagal neurons), from the nucleus tractus solitarius (which receives input from peripheral baroreceptors and chemoreceptors), and from neurons located in the hypothalamus. Together, these neuronal systems, with the PNS, regulate outflow to the heart and vasculature(2,91,95,123,124,183).

#### *1.5.1.1.2 Arterial Baroreceptors*

Baroreceptors are pressure sensors that furnish the most immediate input to the negative feedback systems that regulate arterial BP. Arterial baroreceptors are located in the carotid sinus at the bifurcation of external and internal carotids and in the aortic arch. The aortic arch baroreceptors are innervated by the aortic nerve, which then combines with the vagus nerve, traveling to the brainstem. Arterial baroreceptors are sensitive to stretching of the walls of the vessels in which the nerve endings lie. Increased stretching augments the firing rate of the receptors and nerves, and recruits additional afferent nerves.

The carotid sinus receptors respond to pressures ranging from 60-180 mmHg. The receptors within the aortic arch have a higher threshold pressure and are less sensitive than the carotid sinus receptors. Therefore, the carotid sinus receptors are the dominant type of arterial baroreceptor. Maximal carotid sinus sensitivity occurs near the normal mean arterial pressure. This "set point" changes during hypertension, heart failure, and other disease states. Receptors are sensitive to the rate of pressure change as well as to the steady or mean pressure. Therefore, at a given mean arterial pressure, decreasing the pulse pressure (SBP minus DBP) decreases the baroreceptor firing rate. This is important during conditions such as hemorrhagic shock in which both pulse pressure and mean pressure decrease. The combination of reduced mean pressure and reduced pulse pressure reinforces the baroreceptor reflex (2,16,91).

##### *1.5.1.1.2.1 How Baroreceptors Respond to a Sudden Change in BP*

Baroreceptors function as “sampling areas” for many homeostatic mechanisms involved in maintaining BP. A decrease in arterial pressure (mean, pulse or both) results in decreased baroreceptor firing. The "cardiovascular center" within the medulla responds by increasing sympathetic outflow and decreasing parasympathetic outflow. Under normal physiological conditions, baroreceptor firing exerts a tonic inhibitory influence on

sympathetic outflow from the medulla. Therefore, hypotension results in a disinhibition of the medullary centers. These autonomic changes cause vasoconstriction (increased SVR), tachycardia and positive inotropy. The latter two changes increase CO. The increases in CO and SVR then lead to a partial restoration of BP.

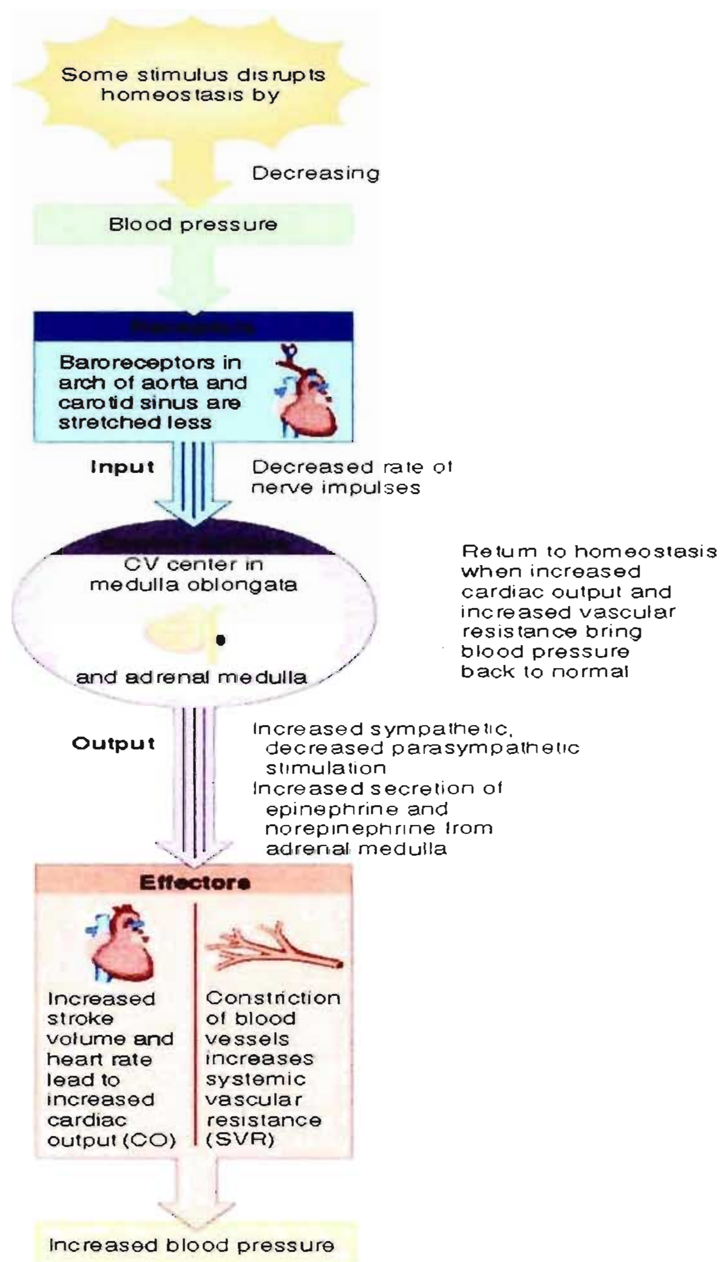
#### *1.5.1.1.3 The Cardiovascular Center*

The cardiovascular center (CVC)(119) is located in the medulla oblongata. It controls the neural and hormonal negative feedback systems that regulate BP and blood flow. Groups of neurons within the CVC regulate HR, contractility of ventricles and blood vessel diameter. The CVC receives information from the cerebral cortex, limbic system, and hypothalamus, as well as from sensory receptors: proprioceptors, baroreceptors, and chemoreceptors. The response signals flows along sympathetic and parasympathetic neurons of the ANS.

Neural control of BP occurs via negative feedback loops (Figure 5) that occur as two types of reflexes: baroreceptors and chemoreceptors. The two most important baroreceptors are the carotid sinus reflex and the aortic reflex. The carotid sinus reflex helps regulate BP in the brain, while the aortic reflex helps regulate systemic BP.

The chemoreceptors monitor the chemical composition of the blood. They consist of the carotid and aortic bodies, which are located close to the carotid sinus reflex and the aortic reflex, respectively. They detect changes in blood level of oxygen, carbon dioxide, and hydrogen ions

**Figure 5 - How the Cardiovascular Center Regulates Blood Pressure**




Negative feedback regulation of blood pressure via baroreceptor reflexes

## 1.5.2 Neurohumoral Mechanisms - The Endocrine System

The heart and vasculature are regulated, in part, by neural (autonomic) and humoral (circulating) factors. Neural mechanisms primarily involve sympathetic adrenergic and parasympathetic cholinergic branches of the autonomic nervous system. In general, the sympathetic system stimulates the heart and constricts blood vessels, resulting in a rise in arterial BP. The parasympathetic system depresses cardiac function and dilates selected vascular beds. Humoral mechanisms, on the other hand directly or indirectly alter cardiac function, vascular function, and arterial pressure. Important humoral systems include circulating catecholamines, the renin-angiotensin system, vasopressin (antidiuretic hormone), atrial natriuretic peptide, and endothelin.

### 1.5.2.1 Hormones

A hormone is a substance, usually a peptide or steroid, conveyed by the bloodstream to effect physiological activity, such as growth or metabolism. They are primarily derived from vascular tissues, but are produced also in the adrenal gland, kidney and brain. Hormones play important roles in the physiology and pathophysiology of sodium and water balance.

#### *1.5.2.1.1 Renin-Angiotensin-Aldosterone System*

The renin-angiotensin-aldosterone system (RAAS) (Figure 6) plays an important role in regulating blood volume, BP, and cardiac and vascular function. Pathways for the RAAS have been found in a number of tissues. The main output of the RAAS is angiotensin II (ATII), a vasoconstrictor involved in salt excretion and control of baroreceptor functions, among others. ATII is derived from angiotensinogen, a circulating protein present throughout the body, in a two-step process.

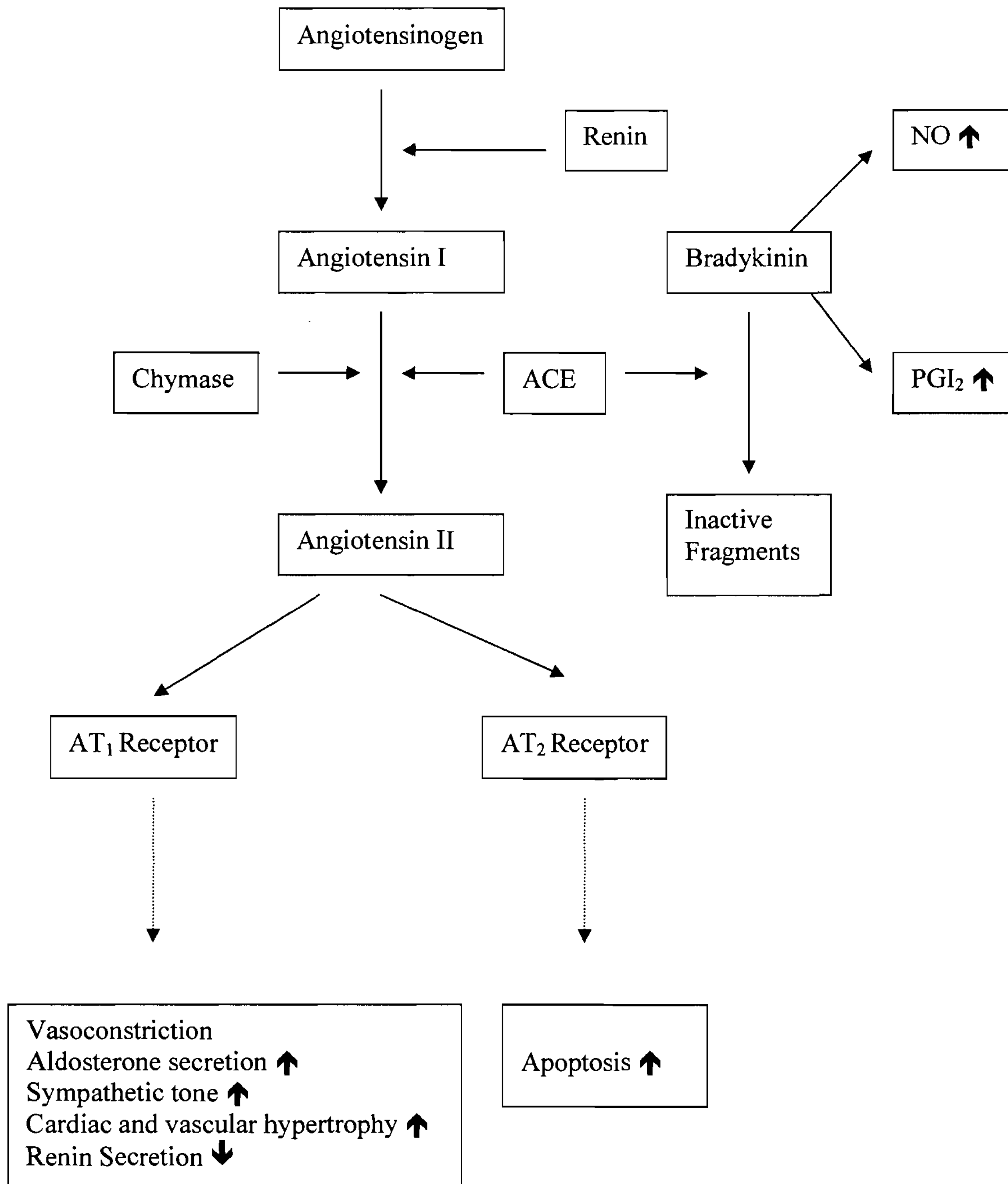
In the first step, angiotensinogen undergoes proteolytic cleavage by renin to form the decapeptide angiotensin I (ATI). The most important site for renin release is the kidney. Sympathetic stimulation, renal artery hypotension, and decreased sodium delivery to the distal tubules stimulate the release of renin by the kidney. The second step involves further cleavage of two amino acids from ATI, producing the octapeptide ATII. One mechanism for this cleavage is angiotensin-converting enzyme (ACE), which is almost entirely

localized within the vascular endothelium, particularly in the lungs. In addition to ACE-dependent ATII generation, non-ACE pathways for ATII generation have also been identified, such as the chymotrypsin-like serine protease, chymase.(113) Chymases are found in mast cells in many tissues and species. Chymase is thought to be responsible for >80% of tissue ATII formation in the human heart and >60% of that in the arteries.(54,131)

ATII has several very important functions (19)(Table 3), including vasoconstriction, dipsogenesis, increased cardiac contractility and the release of catecholamines from nerve endings, aldosterone from the adrenal gland and vasopressin from the posterior pituitary resulting in renal sodium and water absorption. It constricts resistance vessels (via ATII receptors) thereby increasing SVR and BP. It acts upon the adrenal cortex to release aldosterone, which in turn acts upon the kidneys to increase sodium and fluid retention. It stimulates the release of vasopressin (antidiuretic hormone, ADH) from the posterior pituitary, which acts upon the kidneys to increase fluid retention. It stimulates thirst centers within the brain. It facilitates norepinephrine (NE) release from sympathetic nerve endings and inhibits NE re-uptake by nerve endings, thereby enhancing sympathetic adrenergic function. It stimulates cardiac hypertrophy and vascular hypertrophy.

Due to the far-reaching effects of the RAAS pathway, therapeutic manipulation of this pathway is very important in treating hypertension and heart failure. ACE inhibitors and ATII receptor blockers are used to decrease arterial pressure, ventricular after-load, blood volume and hence ventricular preload, as well as to inhibit or reverse cardiac and vascular hypertrophy.

**Figure 6 - Components of the RAAS system and their main effects on the cardiovascular system**




Renin catalyzes the cleavage of angiotensinogen into the inactive angiotensin I, which is subsequently converted to the active hormone angiotensin II by both ACE and chymase. ACE also degrades bradykinin into inactive fragments, preventing bradykinin induced increases in the vasodilators NO and Prostacyclin. Most known effects of Angiotensin II are due to the activation of two of the Angiotensin II receptors, namely $AT_1$ and $AT_2$ (19).

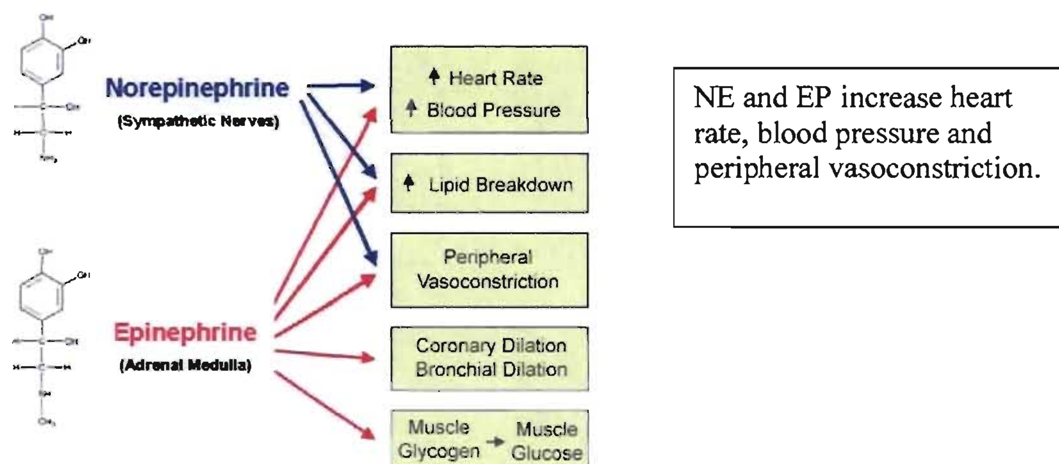
| Table 3- Physiologic actions of angiotensin II | |
|---|---|
| **Vascular** | *Vasoconstriction* - increased total peripheral resistance by direct actions on the contractile elements via enhancement of norepinephrine release from nerve terminals innervating the blood vessels, increased sympathetic discharge, increased medullary epinephrine release<br><br>*Remodeling* - enhanced migration; proliferation and hypertrophy of vascular smooth muscle cells, cardiomyocytes and fibroblasts; increased matrix formation; indirect stimulation of cardiomyocyte proliferation and hypertrophy by theincreased cardiac afterload due to volume expansion secondary to sodium retention |
| **Renal/Adrenal** | *Antinatriuresis* – prevention of the excretion of excessive amounts of sodium in the urine; direct proximal tubule effect to stimulate sodium reabsorption and indirect effect via stimulation of aldosterone release and subsequent sodium reabsorption in distal tubule.<br><br>*Hemodynamic* - renal vasoconstriction; increased renal sympathetic tone |
| **Brain** | *Sympathostimulation and attentuation of the baroreceptor reflex*<br><br>*Dipsogenesis - Thirst Stimulation*<br><br>*Stimulation of salt appetite*<br><br>*Stimulation of vasopressin and oxytocin secretion* |
| **Pituitary Gland** | *Synergism with corticotropin-releasing hormone adrenocorticotropin secretion* |

### *1.5.2.1.2 Adrenergic and Dopaminaerigic Receptors and Actions*

NE and epinephrine (EP) are endogenous catecholamines released by the postganglionic sympathetic nerve terminals and the adrenal gland. They interact with cell surface receptor molecules in various target organs. This interaction starts a cascade of membrane and intracellular events, which result in altered cellular activity (Figure 7). Adrenergic receptors and dopaminergic receptors are coupled to G proteins and activate cells through alterations in intercellular calcium, cyclic nucleotides, inositol phosphates, and protein phosphorylation. The activation of adrenergic receptors increases HR and the strength of cardiac contraction, and causes cardiac and vascular hypertrophy, bronchodilation, vasoconstriction, sedation, and analgesia. Adrenergic receptor inhibition results in vasodilation, decreased heart rate, and strength of contraction and relaxation of prostate smooth muscle. All these actions are important in the treatment of diseases such as hypertension, congestive heart failure, and angina.

The three endogenous catecholamines in humans, NE, EP and dopamine act as the chemical effectors of the SNS, adrenomedullary hormonal system and DOPA-dopamine autocrine / paracrine system. All three systems play important roles in tonic and phasic cardiovascular regulation.

**Figure 7 - Physiological Effects of Norepinephrine and Epinephrine**




Sympathetic adrenergic nerves are found in the heart where they innervate the sinoatrial and atrioventricular nodes, conduction pathways, and myocytes. Sympathetic adrenergic fibers are also found innervating arteries and veins in the peripheral vasculature. These adrenergic nerves release NE, which binds to specific receptors in the target tissue. Parasympathetic cholinergic nerves derived from the vagus nerves also innervate the heart. Acetylcholine (ACh) released by these fibers binds to muscarinic receptors in the target tissue. The vasculature in some organs of the body is innervated by either parasympathetic cholinergic fibers or by sympathetic cholinergic fibers. These nerves release ACh, which binds to muscarinic receptors on the smooth muscle and/or endothelium.

In the heart, NE released by sympathetic nerves preferentially binds to adrenoceptors causing positive inotropy, chronotropy, and dromotropy. In blood vessels, NE binds adrenoceptors to cause smooth muscle contraction and vasoconstriction. (Figure 7) NE can also bind to adrenoceptors, which causes vasodilation (this can be observed during alpha adrenoceptor blockade). Circulating EP binds to the adrenoceptors to cause vasodilation in some organs. NE regulates its own release by stimulating adrenoceptors, which inhibit its release, or inhibiting other adrenoceptors, which facilitate its release.

In the heart, ACh is released by cholinergic nerves, which bind to a cholinergic receptor (M2 muscarinic receptor). This produces negative inotropy, chronotropy, and dromotropy in the heart. Prejunctional M2 receptor activation inhibits NE release and is one mechanism by which vagal stimulation overrides sympathetic stimulation in the heart. In blood vessels, M2 receptors on the vascular endothelium are coupled to the formation of nitric oxide (NO), which causes vasodilation; however, ACh causes smooth muscle contraction through a smooth muscle M3 receptor when formation of NO is blocked. Sympathetic cholinergic nerves that release ACh and cause vasodilation innervate some arterial blood vessels, for example in skeletal muscle. Neurotransmitter binding to the adrenergic and cholinergic receptors activates signal transduction pathways that cause the observed changes in cardiac and vascular function. Drugs are available for blocking adrenergic and cholinergic receptors. For example, beta-blockers are used in the treatment of angina, hypertension, arrhythmias, and heart failure. Alpha-blockers are used in treating hypertension(19).

#### *1.5.3.1.3 Vasopressin (Antidiuretic Hormone)*

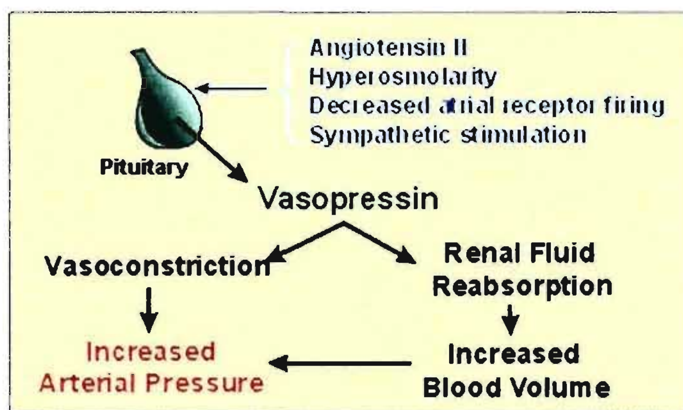



**Figure 8 - Vasopressin release and physiological effects**

Vasopressin is released from the pituitary due to stimulation by ATII, hyperosmolarity, decreased atrial receptor firing and sympathetic stimulation. Vasopressin release results in vasoconstriction, which increases BP and renal fluid reabsorption, increasing blood volume, which in turn increases BP.
Diagram from Klabunde, R.E. Cardiovascular physiology concepts (2005) (95)

Vasopressin (AVP), also known as antidiuretic hormone, is a peptide hormone formed in the hypothalamus, then transported via axons to the posterior pituitary and released from there. AVP has two principal sites of action: the kidney and the blood

vessels. The primary function of AVP in the body is to regulate extracellular fluid volume by affecting renal handling of water. It is also a vasoconstrictor and vasopressor agent. AVP acts on renal collecting ducts to increase water permeability, which leads to decreased urine formation. This increases blood volume, CO and arterial pressure. A secondary function of AVP is vasoconstriction. AVP binds to receptors on vascular smooth muscle to cause vasoconstriction via the inositol phosphate-3 signal transduction pathway, which increases arterial pressure. The normal physiological concentrations of AVP are below its vasoactive range. In severe hypovolemic shock, when AVP release is very high, AVP contributes to the compensatory increase in SVR (Figure 8).

There are several mechanisms regulating the release of AVP. Hypovolemia, during hemorrhage and dehydration, results in a decrease in atrial pressure. Cardiopulmonary baroreceptors within the atrial walls and large veins entering the atria decrease their firing rate when there is a fall in atrial pressure. Afferent nerve fibers from these receptors synapse within the nucleus tractus solitarius (NTS) of the medulla, which sends fibers to the hypothalamus, a region of the brain that controls AVP release by the pituitary. Atrial receptor firing normally inhibits the release of AVP by the posterior pituitary. With hypovolemia or decreased central venous pressure, the decreased firing of atrial stretch receptors leads to an increase in AVP release. Hypotension, which decreases arterial baroreceptor firing and enhances sympathetic activity, increases AVP release. In dehydration, hypothalamic osmoreceptors sense extracellular osmolarity and stimulate AVP release when osmolarity rises. ATII receptors located in a region of the hypothalamus regulate AVP release, so an increase in ATII simulates AVP release.(30)

#### *1.5.2.1.4 Nitric Oxide*

Though nitric oxide (NO) is not itself a hormone,we include it here because it acts similarly to a hormone in BP regulation.

Nitric oxide (NO) is produced by vascular endothelium, smooth muscle, cardiac muscle and many other cell types. The substrate for NO is L-arginine that is transported into the cell. When acted upon by nitric oxide synthase (NOS), NO and citrulline are formed. There are two general forms of NOS: constitutive NOS (cNOS) and inducible NOS (iNOS). NO is continuously produced by cNOS. The cNOS found in endothelial cells is also referred as eNOS. The activity of cNOS is modulated by calcium that is released from subsarcolemmal storage sites in response to the binding of certain ligands to their receptors.

Substances such as acetylcholine, bradykinin, histamine, insulin, and substance P stimulate NO production by this mechanism. Another important mechanism regulating the release of NO is shearing forces acting on the luminal surface of the vascular endothelium. By this mechanism, increased flow velocity stimulates calcium release and increased cNOS activity. The inducible form of NOS (iNOS) is stimulated by the actions of cytokines (e.g., tumour necrosis factor, interleukins) and bacterial endotoxins (e.g., lipopolysaccharide). Induction of iNOS occurs over several hours and results in NO production that may be more than a thousand-fold greater than that produced by cNOS. The induction of iNOS is an important mechanism in the pathogenesis of inflammation.

Nitric oxide serves many important functions in the cardiovascular system . It is implicated in vasodilation (ligand mediated and flow dependent), in the inhibition of vasoconstrictor influences (e.g., inhibits ATII and sympathetic vasoconstriction), in the inhibition of platelet adhesion to the vascular endothelium (anti-thrombotic), and in the inhibition of leukocyte adhesion to vascular endothelium (anti-inflammatory). It is antiproliferative (e.g., it inhibits smooth muscle hyperplasia following vascular injury) and it is a scavenging superoxide anion (anti-inflammatory).

The mechanism of many of these actions of NO involves the formation of cGMP. When NO is formed by an endothelial cell, it readily diffuses out of the cell and into adjacent smooth muscle cells where it binds to a heme moiety on guanylyl cyclase and activates this enzyme to produce cyclic guanosine monophosphate (cGMP) from guanosine triphosphate. Increased cGMP activates a kinase that subsequently leads to the inhibition of calcium influx into the smooth muscle cell, and decreased calcium-calmodulin stimulation of myosin light chain kinase. This in turn decreases the phosphorylation of myosin light chains, thereby decreasing smooth muscle tension development and causing vasodilation. The anti-platelet aggregatory effects of NO are also related to the increase in cGMP. Drugs that inhibit the breakdown of cGMP (inhibitors of cGMP-dependent phosphodiesterase such as sildenafil or Viagra) potentiate the effects of NO-mediated actions on the target cell.

Damage to the vascular endothelium or dysfunctional vasocontriction result in impaired NO production. This decrease in NO production leads to platelet aggregation and and adhesion leading to thrombosis, as well as upregulation of leukocyte and endothelial adhesion molecules. All these conditions lead to enhanced inflammation.

Hypertension, obesity, dyslipidemias (particularly hypercholesterolemia and hypertriglyceridemia), diabetes (both type I and II), heart failure, atherosclerosis, cigarette smoking, aging, and vascular injury are associated with endothelial dysfunction and reduced NO production and availability(2,32,95,124,161).

### *1.5.2.1.5 Atrial and Brain Natriuretic Peptides*

Atrial natriuretic peptide (ANP) is a 28-amino acid peptide that is synthesized, stored, and released by atrial myocytes in response to atrial distension, angiotensin II stimulation, endothelin, and beta-adrenoceptor mediated sympathetic stimulation. Therefore, elevated levels of ANP are found during hypervolemic states (elevated blood volume) and congestive heart failure. ANP is first synthesized and stored in cardiac myocytes as prepro-ANP, which is then cleaved to pro-ANP and finally to ANP, the biologically active peptide.

**Figure 9 - Atrial and Brain Natriuretic Peptides release and physiological effects**

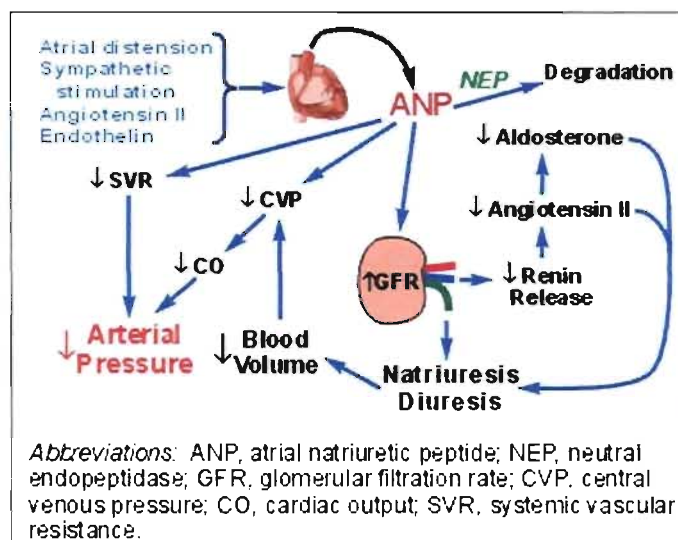



Diagram from Klabunde, R.E. Cardiovascular physiology concepts (2005) (95)

A second natriuretic peptide (brain natriuretic peptide; BNP) is a 32-amino acid peptide that is synthesized within the ventricles as well as in the brain (where it was first identified). BNP is first synthesized as prepro-BNP, which is then cleaved to pro-BNP and finally to BNP. BNP is released by the same mechanisms that release ANP, and it has similar physiological actions. Proteolysis of pro-BNP (108 amino acids) results in BNP (32 amino acids) together with the N-terminal piece of pro-BNP (76 amino acids).

Natriuretic peptides are involved in the long-term regulation of sodium and water balance, blood volume and BP. ANP and BNP decrease aldosterone release in the adrenal cortex. They increase glomerular filtration rate (GFR), produces natriuresis and diuresis (potassium sparing). They also decrease renin release, thereby decreasing the level of ATII. These actions contribute to reductions in blood volume and therefore central venous pressure, CO, and arterial BP. Chronic elevation of natriuretic peptides appears to decrease arterial BP primarily by decreasing SVR. Natriuretic peptides released by the heart serve as a counter-regulatory system for the RAAS (Figure 9)(2,95,124).

#### *1.5.2.1.6 Endothelial Mechanisms and Endothelin*

Various blood-borne agents in contact with vascular endothelial cells cause the production and release of endothelial factors that elicit contraction or relaxation of vascular smooth muscle. Capillary endothelial cells possess the capacity to deactivate substances such as prostaglandin, serotonin and bradykinin(67). They can also convert ATI to ATII(67). Endothelial factors therefore modulate the effects of NE released by sympathetic nerves as well as the effects of tissue metabolites and humoral factors. The three most important endothelial-derived substances are: NO, endothelin (ET-1), and prostacyclin. NO and prostacyclin act as vasodilators, whereas ET-1 serves as a vasoconstrictor.

ET-1 activation causes hypertrophy in small arteries and promotes vascular fibrosis. ATII is a stimulant of ET-1 production. ET-1 production is enhanced in part because of endothelial damage, hypertension, oxidized low-density lipoprotein cholesterol and increased oxidative stress. All of these processes contribute to the progression of vascular disease and atherosclerosis. Damage to the vascular endothelium due to atherosclerotic processes or following ischemia and reperfusion alters the formation and release of endothelial factors. When endothelial damage occurs, the endothelium produces less NO and prostacyclin, which causes the adrenergic vasoconstrictor tone to be unopposed. This can lead to increased vascular tone and vasospasm. Furthermore, decreased production of

both of these endothelial factors can lead to increased platelet adhesion and aggregation, leading to enhanced thrombogenesis. The role of ET-1 in the pathophysiology of hypertension is still unclear. ET-1 may play either a vasorelaxant role or a vasoconstrictor role in different vascular beds in normotension and in mild hypertension. In moderate to severe hypertension, enhanced expression of ET-1 produces a predominant vasoconstrictor effect associated with enhanced growth of the vascular wall, which contributes to further elevation of BP and to complications of hypertension.

ET-1 release is stimulated by ATII, ADH, thrombin, cytokines, reactive oxygen species, and shearing forces which act upon the vascular endothelium. ET-1 release is inhibited by prostacyclin and by ANP, as well as by NO. ET-1 stimulates aldosterone secretion, produces positive inotropy and chronotropy in the heart, decreases renal blood flow and GFR, and releases ANP. ET-1 has been implicated in the pathogenesis of hypertension, vasospasm, and heart failure. ET-1, which is synthesized by vascular endothelium, is a powerful vasoconstrictor substance. Vascular smooth muscle contraction is observed at concentrations as low as 10-11 M. An endothelin precursor (big ET-1 or pro-ET-1; 39 amino acids) is cleaved to ET-1 (21 amino acids) by an endothelin-converting enzyme found on the endothelial cell membrane

ET-1 can then bind to ETA receptors found on adjacent vascular smooth muscle cells leading to calcium mobilization and smooth muscle contraction. The ETA receptor is coupled to a G-protein linked to phospholipase-C and the formation of inositol phosphate 3. There are also ETB receptors located on the vascular smooth muscle, which also produce contraction. Furthermore, ET-1 can bind to ETB receptors located on the vascular endothelium, which stimulate the formation NO by the endothelial nitric oxide synthase. This ET-1-stimulated release of NO appears to modulate the ETA receptor-mediated contraction of the vascular smooth muscle. When ET-1 is administered intravenously, there is a transient hypotensive response due to NO release, followed by a prolonged hypertensive response due to ET-1 acting on smooth muscle ETA and ETB receptors. NO also inhibits the formation of ET-1.(2,27,95)

#### *1.5.2.1.7 Arachidonic Acid Metabolites*

Endothelium, smooth muscle, leukocytes, platelets and parenchymal cells are all capable of producing a variety of vasoactive substances that are products of arachidonic acid metabolism. Among these are prostaglandins, prostacyclin, leukotrienes, and

thromboxanes. These substances are either vasodilators or vasoconstrictors, among their many other biological activities.

Membrane phospholipids, acted upon by phospholipase A2 or C, form arachidonic acid, which serves as the precursor for prostaglandins, prostacyclin, and thromboxanes. These are derived from the cyclooxygenase (COX) pathway. COX is an important enzyme that is inhibited by aspirin and non-steroidal anti-inflammatory drugs. There are two important isoforms of COX: COX-1 and COX-2. The COX cyclo-endoperoxoide products, PGG2 and PGH2, are acted upon by thromboxane synthase within platelets or prostacyclin synthase within the endothelium to form thromboxanes or prostacyclin, respectively.

The enzyme, 5-lipoxygenase also acts upon arachidonic acid. This occurs primarily in leukocytes, to form leukotrienes. Prostacyclin plays an important role in vascular function because, like NO, it inhibits platelet adhesion to the vascular endothelium and is a strong vasodilator. Damaged endothelial cells do not produce prostacyclin, thereby making the vessel more susceptible to thrombosis and vasospasm. Thromboxanes and leukotrienes produce vasoconstriction and are important modulators of vascular function during tissue injury and inflammation. Prostaglandins have a vascular role during inflammation, and also play a subtler role in normal flow regulation, most notably as modulators of other control mechanisms. Prostaglandins have both vasoconstrictor and vasodilator activities. Leukotrienes and prostaglandins can also make the vascular endothelium more "leaky" thereby promoting edema during inflammation(2).

#### *1.5.2.1.8 Kinins*

Kinins are blood plasma proteins that influence smooth muscle contractions. They affect BP, especially during hypotension. They increase blood flow throughout the body by increasing the permeability of small capillaries, and by stimulating pain receptors. Kinins, bradykinin in particular, are produced by the action of kallikrein upon kininogenm itself released from glandular tissue by Ach. Kinins cause increased capillary permeability and venous constriction, along with arterial vasodilation in specific organs. The vasodilator effects of bradykinin are thought to be particularly potent because it is one of the rare stimuli that elicits the activation of the three most important endothelium-derived vasodilator autacoids, NO, prostacyclin, and the endothelium-derived hyperpolarizing factor(54) .ACE degrades bradykinin into inactive fragments, thereby preventing bradykinin-induced increases in the vasodilators.

## *1.6. Anti-hypertensive drugs*

Sympatholytic drugs can block the sympathetic adrenergic system at three different levels. First, peripheral sympatholytic drugs such as alpha- and beta-receptor antagonists block the influence of norepinephrine at the effector organ (heart or blood vessel). Second, there are ganglionic blockers that block impulse transmission at the sympathetic ganglia. Third, there are drugs that block sympathetic activity within the brain. These latter are called centrally acting sympatholytic drugs(19).

Neurotransmission within the sympathetic and parasympathetic ganglia involves the release of acetylcholine from preganglionic efferent nerves, which binds to nicotinic receptors on the cell bodies of postganglionic efferent nerves. Ganglionic blockers inhibit autonomic activity by interfering with neurotransmission within autonomic ganglia. This reduces sympathetic outflow to the heart thereby decreasing CO by decreasing heart rate and contractility. Reduced sympathetic output to the vasculature decreases sympathetic vascular tone, which causes vasodilation and reduced systemic vascular resistance, which decreases arterial pressure. Ganglionic blockers also reduce parasympathetic outflow(27).

# Chapter 2

# The Search for Genes Causing Hypertension

## 2.1 Quantitative Traits

A phenotype can be classified into two different types of traits: qualitative and quantitative traits. A qualitative trait has a phenotype that falls into discrete categories, which are not necessarily ordered. Blood type is a good example of a qualitative trait. The environment has very little influence on the phenotype of these traits.

A quantitative trait is polygenetic: it is the sum of several small effects caused by several genes. An example is an animal's metabolism, which is under the influence of many different genes. There are three types of quantitative traits: threshold, meristic and continuous.

Threshold traits have only two possible phenotypes, but are multifactorial. It is inherited quantitatively, but is expressed qualitatively. When a certain threshold is crossed, the phenotype will jump onto another level, passing from one category to another. The inheritance of such a trait is polygenetic, but the only thing that can be recorded is whether the given phenotype appears. There are no in between phenotypes in a threshold trait: it is inherited quantitatively, but expressed qualitatively. An example of a threshold trait is schizophrenia.

Meristic traits are discrete: their expression involves a variation in some characteristic whole number. Examples include fish scale number and hair number. Rare in humans, they are often studied in plants and animals, such as fish and flies.

Continuous quantitative traits vary continuously along a measurement scale, and are often well described by a normal (bell-shaped) curve. In some cases, the phenotype values do not follow a normal distribution, even though the trait has a polygenetic inheritance. For a continous quantitative trait, the phenotype is measured on a linear or quantitative scale. Multiple genes control such a trait. The environment, to a degree, can also affect these traits. They show a continuous spectrum of variation from low to high values in the general population. Many human phenotypes such as IQ, learning ability, height, weight, skin color and BP are continous quantitative traits.

A quantitative trait locus (QTL) is a location on a chromosome or a gene that is thought to regulate an organism's phenotype for a quantitative trait. QTL analysis is a way of searching for the answers to questions such as: Which genes influence the expression of a given quantitative trait? Where are they located on the chromosomes? What is the function of each gene and what is its level of influence on the trait? The investigation of QTLs requires accurate phenotypic, pedigree and genotypic data from a large number of individuals.

### 2.1.1 BP as a Quantitative Trait

BP is a quantitative trait since it varies continuously. Like most quantitative traits, BP differences result from the contributions of many genes, including direct contributions and interactions of different genes, and environmental effects.

### 2.1.2 Studying Quantitative Traits

Many common diseases and traits such as BP aggregate in families but do not segregate as Mendelian traits. This indicates a complex pattern of inheritance, with a significant influence from environmental factors. Familial aggregation can be due to shared genes, shared environments or both. Studies among twins are methods used for assessing diseases that aggreagate in families.

In humans, twin studies are a common way of providing a quantitative assessment of the genetic component of a trait. Monozygotic twins share identical genomes while dizygotic twins share, on average, half of their genomes, greater similarity of monozygotic compared to dizygotic twins is evidence for a possible genetic component for a trait. Estimates of heritability can be derived from other kinds of relationships too. Heritability is strictly population-and phenotype-specific. By selecting individuals with rare phenotypes, for example, one can " enhance" heritability for greater genetic control. Further resources for studying the genetic contribution of a trait are, adoptees, immigrants, genetically isolated populations, and native cultures, which may totally lack some environmental risk factors such as a low salt diet. Another way of studying the genetic contribution to a trait is to use animal models, including inbred strains.

The prevalence of essential hypertension is 3.8 times greater in individuals with a positive family history of hypertension, suggesting that hypertension has a genetic component (180). Studies using twins show that the BP correlation is higher in

monozygotic twins than in dizygotic twins(7,111). However, even in monozygotic twins, BP variation is sufficiently large that a substantial "environment" effect has been implicated. The environmental factors, such as diet and stress, significantly affect BP variation among individuals (74). A differential response of BP to dietary salt intake has been demonstrated in the pathogenesis of hypertension. Some individuals respond to an increase in dietary sodium intake with an increase in BP and manifest a decrease in BP with salt restriction while other individuals show little or no change in BP, suggesting that a genetic heterogeneity may account for the difference in the etiology of essential hypertension (193-195).

## *2.2 Strategies for Searching for Genes of Complex Traits*

A straightforward way to analyze whether a gene predisposes an individual to a disease would be to analyze its sequence, from several affected individuals and look for variants associated with the disease. Given the limitations of current sequencing methods, the large number of genes and the high cost involved, this method is not practical. There are four common types of methods for this analysis: linkage analysis, allele-sharing methods, association analyses and studies using model organisms (105). Mapping methods, such as linkage analysis often take advantage of cell division processes such as meiosis.

### 2.2.1 Mapping

Two types of maps can be used to describe a genome: genetic maps, which are derived from recombination frequencies between genetic markers; and physical maps, which are constructed from information about the physical location of genes on chromosomes. Genetic mapping takes advantage of genetic markers. A genetic marker is a segment of DNA with an identifiable physical location on a chromosome whose inheritance can be followed. It may be possible to align the two types of maps if they share markers.

#### 2.2.1.1 Genetic Maps

Linkage is the tendency of two markers on the same parental chromosome to be passed on together, in contrast to markers on different chromosomes, or far apart on the same chromosome, behaving independently at meiosis. The amount of recombination

between two gene loci vary widely, and the recombination percentage between two loci is related to how far apart they are physically.

The technique for genetic mapping is therefore to mate parents differing in two or more traits (that is, having different alleles at two or more loci) and then to score the patterns of segregation of parental alleles among the offspring. Two genes on different chromosomes will segregate randomly at meiosis, so that a quarter of the offspring will show each of four combinations of alleles at the two loci. However, if the genes are close together on the same chromosome, parental combinations of alleles will pass to the offspring together, unless they are separated by recombination. The probability of recombination increases as the physical distance between genes increases, so the recombinant percentage of the progeny provides a measure of the genes' relative distance apart.

If the parents differ at three or more loci, recombination between them is additive, allowing for multiple recombination. This allows the three loci to be placed in a "linkage group," a linear array in which recombination percentages represent the relative distances between the loci. A map can be built up in stages from different crosses.The linkage group describes the gene order along a whole chromosome.

Linkage maps depend entirely on the availability of polymorphisms--the existence of two or more alleles at a locus. This has traditionally been the great limitation of mapping, but the availability of highly polymorphic DNA markers has revolutionized genetic mapping in many animal species.

### *2.2.1.1.1 Homologous Recombination*

In many cases, genes on the same chromosome that are inherited together produce offspring with unexpected allele combinations. This results from a process called crossing over or homologous recombination. Sometimes at the beginning of meiosis, a chromosome pair (one chromosome from the mother and one from the father) may intertwine and exchange sections or fragments of a chromosome. The pair then breaks apart to form two chromosomes with a new combination of genes that differs from the combination supplied by the parents. Through this process of recombining genes, organisms can produce offspring with new combinations of maternal and paternal traits that may contribute to or enhance survival.

Homologous recombination is the process by which two chromosomes, paired up during prophase 1 of meiosis, exchange a distal portion of their DNA. Crossover occurs when two chromosomes, normally two homologous pairs of the same chromosome, break and then reconnect, each to the end piece of the other. If they break at the same place or locus in the sequence of base pairs, the result is an exchange of genes, called genetic recombination. This outcome is the normal way for crossover to occur. Any pair of homologous chromosomes may be expected to cross over several times during meiosis, depending on the species and on the length of the chromosome. The recombination is actively assisted in the cell by machinery that has been well conserved through evolution. This process reduces the genetic linkage between alleles on the same chromosome and increases the genetic variation within a population. Because there is some crossing over of DNA when the chromosomes segregate, it can happen that alleles on the same chromosome are separated and go to different cells. There is a greater probability of this happening if the alleles are far apart on the chromosome, as in this case it is more likely that a crossover will occur between them. The relative distance between two genes can be calculated using the offspring of an organism showing two linked genetic traits or markers, and finding the percentage of the offspring for which the two traits are not together. The higher the percentage of descendants not exhibiting both traits, the further apart on the chromosome the two traits will be.

### 2.2.1.2 Genetic Markers

A genetic marker is defined as a segment of DNA whose physical location on a chromosome is known and whose inheritance can be followed. A genetic marker can have a function and thus be a gene or it can be a section of DNA with no known function. Eukaryotic DNA contains very short simple sequence repeats, called microsatellites throughout the genome that can be used as markers. Microsatellite markers are inherited following a codominant pattern and a variation in the number of repeats between individuals is frequent (179). This variation in repeat number makes them useful for genotyping. These markers can be amplified by polymerase chain reaction (PCR) using primers specific to their unique flanking DNA and typed on polyacrlyamide sequencing gels or agarose gel electrophoresis.

To align genetic markers along the chromosomes and to determine the distance between them, different mapping techniques are available. These include linkage, radiation hybrid (RH) and physical mapping.

### 2.2.1.3 Linkage Analysis

Among individuals of an experimental population or species, some phenotypes or traits occur randomly with respect to one another. This situation is known as independent assortment. An exception to independent assortment, called linkage develops when genes appear the same chromosome. Such genes tend to be inherited together in genetic crosses, because they are part of a single chromosome that is passed along as a unit. Thus they are said to be linked genes. Linkage analysis is the study of linkage between genes. It is valuable in gene-hunting and genetic testing.

Because DNA segments that lie near each other on a chromosome tend to be inherited together, markers are often used as tools for tracking the inheritance pattern of a gene that has not yet been identified but whose approximate location is known. The statistical estimate of whether two loci are likely to lie near each other on a chromosome and are therefore likely to be inherited together is called the LOD score. A LOD score of 3 or more is generally taken to indicate that the two loci are linked and are close to one another, since it indicates a likelihood of less than 1 in 1000 of observing the result if the two loci are not linked.

The rate of crossing over between linked genes differs. The crossover frequency indicates the distance separating genes on the chromosome. Greater the distance between linked genes, greater the chance will be, for the non-sister chromatids to crossover or recombine, in the region between the genes. By working out the number of recombinants it is possible to obtain a measure for the distance between the genes. This distance is called a genetic map unit, or a centimorgan (cM), and is defined as the distance between genes for which one product of meiosis in 100 is recombinant. A linkage map is created by finding the map distances between various traits or loci that are present on the same chromosome. Ideally one avoids having significant gaps between traits to reduce the possibility of multiple recombination events, which would decrease the accuracy of the map.

Linkage mapping is critical for identifying the location of genes that cause genetic diseases. In a normal population, genetic traits and markers will occur in all possible combinations, with the frequencies of combinations determined by the frequencies of the

individual genes. A linkage map is a chromosome map of a species or experimental population that shows the position of its known genes and/or markers relative to each other, rather than as specific physical points on each chromosome. It is not a physical map of the chromosome.

A genetic map is a map based on the frequencies of recombination between markers during crossover of homologous chromosomes.

Linkage analysis determines the position of a gene in relation to the known positions of particular genetic landmarks. It establishes links between known genetic markers and unknown genes. Linkage mapping of QTL in organisms amenable to inbreeding begins by choosing parental inbred strains that are genetically variable for the trait of interest, with filial generations, F1 and F2. Usually the parent strains will have different mean values for the trait, but this is not necessary, as two strains with the same mean phenotypic value can vary genetically owing to complementary patterns of positive and negative QTL allelic effects. A mapping population is then derived by back-crossing the F1 progeny to one or both parents, mating the F1 together to create an F2 population, or constructing recombinant inbred lines by breeding several generations of F2 sublines up to homozygosity. These methods are very efficient for detecting marker-trait associations. By successively reducing the size of the region exhibiting linkage with the QTL ultimately to a single gene, by the process described above, one obtains a hight resolution map. Given a high-density polymorphic molecular marker map spanning the interval in which the QTL is located and a population of recombinant genotypes with breakpoints between adjacent markers, it is a simple matter in principle to define the QTL position relative to a pair of flanking markers. The challenge posed for high-resolution QTL mapping is that individual QTLs for BP are expected to have small effects on phenotype that are sensitive to the environment.

### 2.2.1.4 Statistics: The LOD Score

The LOD score is an efficient statistic for evaluating pedigrees for linkage. Given a certain pedigree including recombinants, it is possible to calculate the overall likelihood of the pedigree on the alternate assumptions that the loci are linked (recombination fraction = 0) or not linked (recombination fraction = 0.5). The ratio of these two likelihoods gives the odds of linkage and the logarithm of the odds is the LOD score.

The LOD score is usually calculated by computer programs such as MAPMAKER(114). A LOD scores of + 3 is generally taken as the threshold between linkage and exclusion. A positive LOD gives evidence in favor of linkage, while a negative LOD is evidence against linkage. LOD score analysis is a powerful method for scanning the genome to locate a disease gene. Its weaknesses include vulnerability to error, problems with locus heterogeneity and limits on the ultimate resolution achievable, together with the need to specify a precise genetic model, detailing the mode of inheritance, gene frequencies and penetrance of each genotype.

It has been proposed (105,167)that a series of threshold criteria be used when attempting to find linkage for complex diseases. The different thresholds are described as suggestive linkage, significant linkage, highly suggestive linkage, and confirmed linkage.

### 2.2.2 Physical Maps

Genes may be assigned to physical positions within chromosomes or chromosome regions by somatic cell genetics and radiation hybrid mapping.

Somatic cell genetics was developed in the 1970s. This method does not require a breeding colony, or even a live animal, as long as cell samples are available. Nor does it require polymorphic markers, since somatic cell genetics, uses interspecific variation. However, it has low-resolution. Somatic cell genetics simply establishes synteny groups and assigns them to a chromosome, but does not specify position or order.

Somatic cell genetic analysis uses viable hybrid cells derived from the fusion of somatic cells from different species. It depends on the observation that chromosomes are lost from only one of the two parental sets. For example, rat-hamster hybrids segregate rat chromosomes, so that it is possible to derive a hybrid panel uniquely representing each rat chromosome. Hybrids all retain and express the full set of hamster genes; however, a hybrid will retain and express only the rat genes on the particular rat chromosomes retained in that hybrid. Thus, by detecting patterns of presence and absence of rat markers in a set of hybrids and correlating these with the patterns of the presence or absence of particular chromosomes, it is possible to assign a rat gene to a particular rat chromosome. Some regional mapping is possible using hybrids that retain only portions of a chromosome.

### 2.2.2.1 Radiation Hybrid Mapping

The use of radiation hybrids (RH) and RH maps has revolutionized physical mapping of disease loci in humans. It is a simple approach: in a single 96-well PCR plate one can map a candidate gene with relatively high resolution. RH mapping is an extension of regional, physical mapping. Hybrids are constructed from donor cells (cells from the species to be mapped or cell hybrids bearing a single chromosome of the species to be mapped) that have been lethally irradiated to cause chromosome fragmentation. The hybrids therefore contain only small regions of the irradiated donor genome incorporated into chromosomes of the unirradiated parent. These radiation hybrids are more likely to bear two genes if the genes question are physically close together on a chromosome, so the frequency of concordance of markers may be used as a measure of their physical proximity.

The construction of a rat RH map is of great significance because the rat has been, and continues to be, utilized as a powerful model system for investigating human physiological and genetic diseases, particularly multifactorial diseases. Rat cells carrying a selectable marker are irradiated with a dose of X-rays to fragment their chromosomes; the irradiated cells are fused with hamster cells, and a panel of fusants is selected using the rat-derived marker. These cell lines carry random fragments of rat DNA integrated into the hamster chromosomes. Any particular fusion line carries only a small fraction of the entire rat genome. As a result, genes, which lie relatively close to each other in the rat genome, tend to be found simultaneously in the same fusion cell line, much more often than expected by chance. The frequency of co-occurrence is a measure of the physical distance between the two genes. Relative frequencies of co-occurrence can be used to order sets of three genes as well.

RH mapping can be simply done by performing PCR. Radiation hybrids, which have marker-related sequences, are revealed by the hybridization of DNA molecular markers to an array of DNAs from the panel of fusants. Molecular markers for hybridization may also be cosmid, BAC, or similar clones, rather than specific genes. In this case, radiation hybrid mapping may be used to determine non-overlapping regions called contigs. Markers need not be polymorphic in the rat, as long as they are distinguishable from hamster homologs.

#### 2.2.2.2 Physical Mapping Using Genome Sequences

The draft sequences for human, mouse and rat genomes have been published. This has facilitated the mapping of markers. It is possible to locate the genetic markers by blasting their sequence on each genomic sequence. The maps obtained provide the physical position of a marker and the distances are measured in base pairs (bp).

Physical maps using genome sequences are not based on a pattern of inheritance like the linkage maps. They include polymorphic and non-polymorphic markers. Since the distances are given in base pairs, it provides a more accurate estimate of the relative position of the markers and the distance separating them.

### 2.2.3 Co-segregation analysis

Co-segregation analysis is used to identify loci related to the phenotype, in this case, BP regulation. It is based on the principle that the frequency of recombination between two given loci is dependent on the physical distance that separates them. In order to perform a co-segregation analysis, first, there must be a linkage map of the genome of the species being studied. Second, there must be a good method available to measure how BP varies among animals. A breeding experiment is then conducted mating animals, whose BPs have been measured and genotyped. Using a computer program, the variation in BP is correlated with each marker. Considering one family in a breeding experiment (a male, female and their offspring), if the BP is always high with a certain sequence of a marker and always low with another sequence, then the gene for BP regulation is close to the first marker on the chromosome. If the variation in the BP is more or less random in relation to the marker, then there is little or no linkage between BP and that chromosomal location. The level of association between BP and chromosomal location is called an "LOD" score. The higher the LOD score, the more "important" a gene may be in regulating BP. These chromosomal locations probably do not code directly for BP, but instead for factors which shape the BP. Co-segregation analyses provide a statistical assessment of the probability that a marker is inherited in conjunction with the BP phenotype because the two loci are physically close or by chance only. BP is not the only example to which this analysis can apply.As long as there is a definite phenotype that can be observed, co-segregation analysis can be applied to locate the chromosomal locations of the genes that regulate that phenotype.

Knowing which chromosomal locations are important enables genetic manipulations of the key genes affecting the regulation of the trait to be tracked. The other possible outcome of locating a QTL is being able to identify the gene influencing the BP and the regulation of its expression.

### 2.2.4 Candidate gene approach

Genes considered to affect the risk of contracting a given disorder due to their function, expression or presumed interaction with a disease-causing gene, are called candidate genes. The majority of studies on the genetics of complex diseases are carried out by analyzing candidate genes. At present, we know only of a partial list of genes associated with the cardiovascular system, to say nothing of their variations. It has been estimated that there are 15000-30000 genes that are expressed in any given cell of the cardiovascular system(72). Also, the large variation of genes in the population is an obstacle to the design and interpretation of disease-association studies. Although candidate gene studies are potentially useful in the determination of predisposing genes, at present they face many problems. The use of candidate gene method does not allow for the discovery of novel genes.

### 2.2.5 Animal models

Using animal models allows the knowledge of systems to be extrapolated to other species. New animal models are continually being identified and developed to evaluate the pathogenic mechanisms (28,79), diagnostic (156) and therapeutic procedures (73,106), nutrition (52), metabolic disease, and the efficacy of novel drug development (26).

To be successful, a genetic animal model should be inbred (homozygous throughout the genome) so as to reduce heterogeneity in terms of genetics and etiology. It should be physiologically and pathologically well characterized. It should display some characteristics of the clinical picture. Finally it should also be amenable to biochemical, physiological, pharmacological, and genetic studies.

In order for the results obtained to be significant and valid, so that conclusions about the disease being studied can be drawn are accurate, it is also critical to select a suitable animal.

Small animals provide significant advantages in modeling human diseases. Environmental factors of laboratory animals can be controlled. The litters are large,

inbreeding is a possible technique, and relevant tissue samples are available more easily than from humans. Also, since the time of development and lifespan is much shorter for such animals, the animal models allow for longitudinal measurements of variables and multi-generation pedigrees are easily produced.

Usually, one uses various crosses of inbred strains expressing the phenotype differently, and the progeny are then genotyped to find the loci linked to the trait. Once the predisposing genes have been identified, transgenic rats or mice will provide the final proof for the biological significance of these genes. However, the farther apart evolutionally, the two species compared are, the more caution must be exercised in generalizing from one to the other.

Thus it is important to consider whether animal data is useful for understanding corresponding conditions in humans. A good animal model 1) shares a homologous region of the genome with at least a subset of human patients, 2) enables the analysis of a complex multifactorial disease into single gene regions and 3) enables the rapid assessment of physiology, biochemistry and pharmacology.

With reference to hypertension in particular, experimental nongenetic approaches have resulted in development of extremely useful models that allow studying the effects of induced hypertension on end-organ damage in different species. These approaches include surgically induced hypertension, as shown in the Goldblatt experiment, which introduced the first animal model of hypertension in dogs evoked by unilateral constriction of the renal artery (29,109,118). Surgical induction of hypertension has been studied in rats, rabbits, dogs, pigs(108,109,205), monkeys(118) and mice. In the phenotype-driven experimental approach, takes advantage of the natural variation among inbred strains and crosses to find quantitative traits and determine which genes are responsible for them (182). In contrast, in a genotype-driven approach, a known gene is studied with genetically based interventions (over-expressions or ablation) and is characterized.

Naturally occurring animal models of a disease can be extremely useful in providing new biological insights into a given phenotype. There are many advantages in the use of animal models in studies.

To use animal models in the study of hypertension, one must understand: (102)

1. The manner in which the hypertensive strains and their normotensive controls have been derived.
2. The number of major genes that may be contributing to the hypertension.

3. The genetic relationship between the hypertensive strains and their normotensive counterparts and the limitations of inter-strain comparisons for investigating mechanisms of hypertension (173).
4. The genetic homogeneity of the experimental strains.
5. The principles behind the development and use of specialized congenic strains.

For hypertension, the most widely used animal for studies is the rat, not mouse as in most other diseases. The reason for this is simple. It is much harder to measure the BP of a mouse. For atheroscerosis, mice, rabbits, and dogs are often used.

### 2.2.5.1 Development of the Rat Model

Historically the laboratory rat, *Rattus norvegicus* was the first mammalian species to be domesticated for scientific research(69). The first genetic studies focused on the inheritance of coat colour.

For mammalian geneticists, the mouse eventually became the model of choice because of its smaller size, which simplified housing requirements, also because it is smaller and quicker to breed, and thus easier to manipulate genetically. There are also many mouse strains with varying coat colours, together with other mouse mutants with Mendelian patterns of inheritance that had been collected by mouse fanciers(132,133).

Unfortunately for the present research, a good inbred mouse model of hypertension has not yet been developed. Furthermore, the gene knockout and overexpression approaches for which the mouse is very useful cannot be applied to the discovery of naturally occurring genes that produce hypertension. Although mice are of great use in identifying the action of given genes and for validating the importance of a candidate gene, these approaches cannot be used for finding genetic variants or pathways that can lead to hypertension(25).

Rat strains were developed primarily by physiologists and other biomedical researchers in their own laboratories, and were selected and bred to have traits of biomedical interest. Many of theses strains were inbred. They are generated by systematic inbreeding, which fixes certain alleles in a strain so that they replace all other alleles present in an outbred population. Rat research has had a different focus to that of mouse research, being more physiological in nature: the rat provides unique opportunities for disease research that can be integrated with the resources generated by the Rat and Human Genome Projects. Hence, researchers have created more than 234 inbred strains of rat by

selective breeding. This has'fixed' disease alleles in particular strains, for disorders that range from hypertension to urological defects(69). Hence, the rat is used in biomedical research such as physiology, experimental medicine and drug development.

The rat became the third mammal to have its genome sequenced(69), after mice and humans. This genome sequence enables researchers to view the genomic evolution of the rat compared to that of the mouse and the human (69). The rat genome, being 2.75 gigabases (Gb) is smaller than the human genome, which is 2.9 Gb, but appears to be larger than the mouse, which is variously evaluated to be between 2.5 and 2.6 Gb. Most of the human genes associated with disease have orthologues in the rat genome(69).

The knowledge of the rat genome sequence allows for the direct comparison of rat and human orthologues. The effects of inheritance on disease phenotypes can be easily pursued in both species and the identification of QTLs and complex trait differences that are involved in disease processes can be pursued accurately(69).

Combined physiological and genomic information should lead to the development of better pre-clinical models of human disease, which will aid in the development of new therapeutics. The rat was chosen as the animal model due to the wealth of physiological and pharmacological data for the rat. The knowledge of the genome sequence of the rat should facilitate the discovery of mammalian genes that are involved in disease.(69)

### 2.2.5.2 The Rat as an Animal Model in the Study of Hypertension

Considering the tendency for essential hypertension to run in families, it is logical to create a model using selection to obtain an experimental model resembling as closely as possible the human hypertensive patient. This approach consists of identifying among randomly bred animals, the ones displaying higher arterial pressure values, and breeding them over subsequent generations until the BP of the descendant population is found to be consistently elevated. At the same time, descendants of from the original parents showing lower BP values are also bred to develop normotensive progeny. This strategy has been widely used in "traditional" models of genetically determined experimental hypertension.

Inbreeding models of hypertension are frequently used to study mechanisms of BP control, to test and develop anti-hypertensive drugs and to investigate the pathogenesis of spontaneous hypertension. (102) The formal definition of an inbred strain is: a set of animals that is produced by at least 20 consecutive generations of parent crossed with offspring mating that can be traced to a single ancestral pair in the 20th subsequent

generation. Animals of an inbred strain are nearly fully homozygous, thus providing a well-defined and consistent genotype for analysis.

The most commonly used method to develop the inbred experimental models is as follows. The BP of a large number of non-inbred animals is measured. Using this information, one selectively breeds those having the highest BP. In each successive generation, the offspring with the highest BP are interbred (i.e. brother crossed with sister) to produce an inbred strain. After 20 generations of brother-sister mating, the offspring should be homozygous at >99% of loci, and therefore all animals within the strain should be highly inbred and near isogenic (genetically identical).

The most commonly used traditional inbred genetic models of hypertension include the Milan hypertensive rat, the Lyon hypertensive rat, the New Zealand genetically hypertensive rat, the Sabra hypertensive rat, the Dahl Salt-Sensitive hypertensive rat (S) and the Spontaneously hypertensive rat. With these models, substantial progress has been made in identifying physiological, biochemical and environmental factors that can affect BP. Very less progress has been made in identifying primary genetic determinants of hypertension.

Any hypertensive strain, in order to be studied, must have normotensive 'control' strains for comparison. Most normotensive or control strains' progenitors have been derived from the same colony of animals as the model hypertensive strain, in the hope that the control rats would be genetically very similar to the hypertensive rats. Selection of the hypertensive strain is established by the selective inbreeding of offspring with the highest BP. The normotensive strain is established by the selective inbreeding of offspring with relatively normal or even low BP. Although a hypertensive strain and its traditional corresponding normotensive control strain may be traced back to the same colony, the animals in the original colony were genetically diverse, and differed with respect to alleles at multiple loci, not just those affecting BP. Thus during the process of selection and inbreeding of the hypertensive and normotensive strains, alleles that were unrelated to BP could randomly be fixed in the homozygous state. Depending on the variety of alleles present in the original breeding colony, the hypertensive strain and the normotensive control strain could fix different alleles at many loci simply by chance. As a consequence of genetic drift, the hypertensive strain and the normotensive strains end up differing with respect to multiple loci throughout the entire genome, not just in those affecting BP.

Therefore, one can detect differences between the hypertensive strains and their corresponding normotensive controls with respect to a large number of molecular, physiological and biochemical characteristics. The presence of a given trait or polymorphism in a normotensive strain does not exclude the possible involvement of that trait or polymorphism in the pathogenesis of hypertension. In a normotensive strain the capacity of certain alleles to promote hypertension may be counterbalanced by other alleles that tend to lower BP. It is evident that there are limitations to traditional models due to this approach, due to potentially incomplete genetic homogeneity and the effect of genetic drift. Generally, in most phenotype driven models, hypertension is associated with high BP, cardiac hypertrophy, endothelial dysfunction and renal functional impairment (proteinuria, decreased creatinine clearance). The outcomes seem to depend on the underlying origin, genetic background, and possibly species differences, as well as on the degree of hypertension. The phenotypic characteristics and pathophysiology of rats carrying spontaneous or genetically engineered mutations are most often attributed to alterations in the modified gene. But the 'genetic background' defined as a collection of all genes present in an organism that influence a trait or traits and the surrounding environment are factors that can significantly affect the observed phenotype. So it is important to consider these epigenetic and extragenetic factors when using rats to study complex diseases. The use of strains with a uniform genetic background greatly facilitates the interpretation of experimental results(50).

While most of the inbred strains used in hypertension research share a common origin, each strain has its own unique set of characteristics or background lesions. Strain characteristics are the result of sequence differences in genes (allelic variance) among inbred strains. Strain attributes can be the result of single genes or a combination of genes. The phenotype of the rat, carrying a modified gene will vary, depending on the genetic background.because of the presence of genetic modifiers (allelic variants at loci other than the one being genetically modified) in the inbred strain genome. Genetic modifiers may function through a number of mechanisms: (1) they can suppress or enhance the expression of genes involved in physiological or pathological pathways; (2) they can alter DNA transcription rates or mRNA stability; (3) they can have epigenetic effects causing changes in DNA methylation or chromatin structure; and (4) they can result from a variation of gene copy (103,115,125). Phenotypic differences among different strains carrying the same

mutation may therefore be the direct result of genetic modifiers that are unlinked to the modified gene or allelic variants.

#### *2.2.5.2.1 The New Zealand Genetically Hypertensive Rat (GH)*

The GH strain was the first selected strain to consistently develop high BP(109). The rise in BP in GH compared to its normotensive control starts early in life, since it shows a higher BP at 2 days of age. In this model the RAAS, fluid and sodium retention and salt-sensitivity do not seem to play a major role in the mechanism resulting in hypertension(29).

#### *2.2.5.2.2 The Spontaneously Hypertensive Rat (SHR)*

The SHR was bred for high BP, without any provocative dietary or environmental stimuli(144), and is a strictly inbred strain. In terms of end-organ damage, many SHR rats develop cardiac hypertrophy and progress to heart failure between the age of eighteen and twenty-four months. Despite the uniformity of the model, not all the rats exhibit signs of heart failure after twenty-four months, showing that there may exist individual differences seen amongst the rats in the model(109,139). The model allows for a wide variety of genes to cosegregate and may mimic a subtype of the human primary hypertension that is inherited in a Mendelian fashion. The genetic mechanisms implicated in the hypertension of SHR have been attributed to both neural and vascular alterations(118).

#### *2.2.5.2.3 The Dahl Salt-Sensitive Hypertensive Rat (S)*

In 1960 Lewis K Dahl and coworkers(4,143,145)selectively bred rats for susceptibility (S rats) or resistance (R rats) to the hypertensive effect of high- salt diet (8% Sodium Chloride (NaCl)). This model was developed after the observation that consumption of salt resulted in hypertension in some individuals whereas others remained normotensive despite a high salt diet. He started with Sprague-Dawley stock, and after three generations of selective breeding, using 7.3% (NaCl) and L-tridothyronine, an agent that accelerates the appearance of salt-induced hypertension, he had successfully separated distinct S and R lines. In the R line, the level of dietary salt had very little effect on the BP, but in the S line, increased dietary salt caused a markedly elevated BP. Thus, the two lines provided an interesting model for the interaction of an environmental factor (salt) with genotype(29).

Generally, S rats on a high-salt diet develop a fulminant hypertension and are dead by 8 weeks of treatment, while those fed on a low-salt diet (0.3% NaCl) diet survive well, even though they also develop marked hypertension. It is a common misconception that S rats develop high BP only when fed excess salt. The BP rise is much slower in S rats on a low salt diet than the S rats fed a high salt diet. BP in R rats remains at a low level and is unaffected by dietary salt. S rats on a low-salt diet did consistently have pressures significantly higher than R rats fed either high or low salt diets. Inbred S rats are 'salt-sensitive' in the sense that they develop marked hypertension on low (0.3-4% NaCl) or normal (1%NaCl) salt diets(143).

The age at which S rats start on a high-salt diet influences BP response. If rats are fed a high salt diet at weaning, the rise is BP is rapid (4,31,143). If salt feeding is delayed until 3 months of age, the rise in BP is less rapid. BP in S rats is influenced by dietary potassium as well as sodium. If dietary sodium is kept constant, BP of S rats is inversely related to dietary potassium(59,143) . On diets with different absolute concentrations of NaCl and KCl, but with identical Na/K molar ratios, S rats on the higher absolute NaCl intake have higher BP, Thus both the absolute amount of sodium and the Na/K molar ratios are important in determining the final BP. It has been shown that in the S strain, the suppression of the sodium–potassium pump is not the cause of hypertension(135).

The S rat is more sensitive than the R to hypertension induced by experimental procedures other than high dietary salt. These include treatment with deoxycorticosterone, cortisone, or adrenal regeneration hypertension, renal artery clipping, injection of cadmium and psychological stress. Thus, the selective breeding of rats for one form of hypertension has apparently influenced their sensitivity to many forms of experimentally induced hypertension(29).

Inheritance of BP characteristics in S and R rats is polygenic. This means that genes at several genetic loci influence BP. Cross-transplantation of kidneys between S and R rats has a significant effect on the BP of the recipient animal. An S kidney transplanted into an R rat increases BP, whereas a R kidney transplanted into an S rat decreases BP. Thus, the kidney mediates part of the genetic predisposition to hypertension. It has been reported that S rats have 15% fewer glomeruli than R rats. Following 20-30 days of high-salt feeding the S rats compared to R rats, showed increased single nephron blood flow, increased single nephron glomerular filtration rate and decreased resistance at the afferent and afferent arteriolar segments(29).

In terms of the difference in nervous systems, the S rat has been observed to possess reduced baroreceptor sensitivity, due to a neural component of the baroreceptor reflex. Basal sympathetic activity and pressor responsiveness to hypothalamic stimulation are enhanced in S over R rats. Renal $\alpha 1$ and $\alpha 2$-receptor concentrations were higher in S than in R rats, and high-salt feeding caused an increase in renal $\alpha 2$-receptors in S but not in R rats.

In terms of hemodynamic parameters, when rats were placed on a high salt-diet (8% NaCl), after 3 days R rats showed an 18% increase in CO, a 14% decrease in peripheral resistance and no change in BP. At 7 days of salt feeding, the CO and peripheral resistance in R rats were back to the levels seen in control rats on a 0.3% NaCl diet, while BP remained unchanged. S rats responded with a 10% increase in CO, a 10% increase in peripheral resistance and a 20% increase in BP after 3 days on a high salt diet. At 7 days on high salt, CO had returned to the control value, but BP and peripheral resistance remained elevated.

Salt feeding significantly increased aortic prostaglandin I2 synthesis in S but not in R rats. Exercise has a pronounced effect on the BP response of S rats to salt feeding. If exercise, such as running, is started soon after weaning, salt-induced hypertension is delayed and mortality is reduced.

In S rats, hypertension may be related to decreased availability of vasodilatory NO leading to increased vasoconstrictive response to norepinephrine and ATII. The high-salt-induced decrease in NO availability and vascular relaxation could be due to inhibition of NO synthase or increased superoxide production. This is supported by reports (87)that arginase inhibition restores arterial endothelial function in S rats with salt-sensitive hypertension (29).

In essence the S rat share many of the phenotypic traits commonly found in hypertensive humans. Like many hypertensive humans, the S rats are salt sensitive, are hyperlipidemic, are insulin resistant, have reduced renal function, have blunted pressure natriuresis and exhibit low renin hypertension.

#### *2.2.5.2.4 The Milan Hypertenive Rat (MHS)*

The MHS rat is mainly characterized by an increase in renal sodium reabsoption, related to mutations in the gene that codes for adducin, a skeletal protein involved in transepithelial sodium transport. It is of interest mainly because of the genetic link between

adducin polymorphisms and primary hypertension as seen in some hypertensive patients(10).

#### *2.2.5.2.5 The Lyon Hypertensive Rat (LH)*

In this inbred strain, low-renin hypertension has been implicated, because it shows hypersensitivity of preglomerular vessels to ATII(166).

#### *2.2.5.2.6 The Sabra Hypertensive Rat (SBH)*

The SBH strain exhibits gender specific quantitative trait loci for salt susceptibility on chromosome 1(201).

### 2.2.5.3 Advantages of using the Rat as a Genetic Model in Hypertension

Human essential hypertension is a complex disease, so researchers have selectively bred rats for hig BP to provide animal models for the disease. Table 4 summarizes the reasons for the use of the rat as a model for human hypertension by pointing out the limitations and difficulties of studying the genetic factors controlling hypertension in the human population(92,104).

**Table 4- Advantages of the rat as a model for genetic hypertension**

| Characteristics | Inbred rats | Humans |
|---|---|---|
| **Heritability of hypertension** | ~60% | 30–40% |
| **Environmental factors** | Controllable | Uncontrollable |
| **Onset of hypertension** | Early age | Advanced age |
| **Genetic homogeneity** | High (inbred) | Low (outbred) |
| **Reproducibility of BP measurements** | Relatively good | Relatively poor |
| **Analytical approaches** | QTL mapping in experimental crosses<br>Development of congenic strains | Linkage analysis (e.g., affected sib-pair)<br>Association study (unrelated subjects) |

### 2.2.5.4 Rat Chromosomes of S Implicated in BP Control

Previously published studies that looked for linkage to BP using genetically hypertensive S rats and normotensive rats have yielded many putative quantitative trait loci (QTL) for BP on all rat chromosomes except chromosomes 4, 11, 14, 19, 20, X and Y (144). Table 5 describes and lists the various QTLs found on the S rat chromosomes.

**Table 5- BP QTLs localized in the Dahl salt sensitive rat**

| Rat Chromosome | Contrasting Normotensive Strain | Number of QTLs | Confirmed by Congenic Strains | Reference | Features |
|---|---|---|---|---|---|
| **1** | LEW | 3 | Yes | (59,86,162) | |
| **2** | WKY<br>MNS<br>LEW | 3<br>2<br>1 | Yes<br>Yes<br>No | (35,36,39,61)<br>(35,39,44,45)<br>(59) | Interacting with Chr. 10 QTLs(147) |
| **3** | R<br>LEW | 2<br>2 | Yes<br>Yes | (21,107)<br>(59,134) | |
| **5** | LEW | 2 | Yes | (34,59,62,159) | |
| **6** | BN | 10 | No | (126) | Linkage study |
| **7** | R | 1 | Yes | (22,24,63) | 11 β-hydroxylase might be the QTL |
| **8** | LEW | 2 | Yes | (6) | Current study |
| **9** | R | 1 | Yes | (60,122,146) | |
| **10** | MNS<br>LEW | 2<br>4 | Yes<br>Yes | (37,39,43,65) | 3 QTLs are epistatic to one another, while 4th QTL is additive (18) |
| **12** | WKY | 1 | No | (92) | |
| **13** | R<br>BN | 1<br>1 | Yes<br>Yes | (85,204)<br>(177) | |
| **15** | WKY | 1 | No | (33,34) | |
| **16** | LEW<br>BN | 1<br>1 | Yes<br>Yes | (59,127,128)<br>(121) | |
| **17** | LEW | 1 | In dispute | (34,59) | |
| **18** | LEW<br>BN | 1<br>1 | No<br>Yes | (59)<br>(121,177) | |

Table 5 is based on Deng 2005 (38).

## *2.3 Previous Studies on Rat Chromosome 8*

In a linkage study in 1995, Schork (168)and coworkers conducted a linkage study in a F2 population originating from a cross between an SHR and a Brown Norway rat. They mapped a BP QTL where the SHR alleles increased BP. It had a significant LOD score of 5.1 at chromosome 8.

Independently in 1995, Takami and colleagues found a locus for the peroxisomal 3 keto-acyl CoA thiolase on chromosome 8 cosegregated with BP in crosses involving SHR and Dahl/Iwai salt-sensitive rats (184). Peroxisomal 3 keto-acyl CoA thiolase is involved in the last reaction of the β-oxidation cycle. It is involved in the thiolytic cleavage of 3-ketoacyl-CoA to acetyl-CoA, resulting in an acyl-CoA shortened by two carbon atoms. In Takami's study, it was found that the locus located on rat chromosome 8 had a hypotensive effect.

In 1996, Kren et al. established a congenic strain in which the gene for polydactyly-luxate syndrome (Lx) was used to move a region on chromosome 8 from Brown-Norway rats (BN) to the SHR background (97-100). The resulting congenic strain had a BP that was 20 mmHg lower than that of SHR(99). This demonstrated the existence of a QTL on chromosome 8.

In a linkage study in 1998, using F2 generations derived from LEW and S, Garrett et al. (59) mapped a BP QTL to rat chromosome 8 (Figure 10). The statistical LOD score was two, which meant that it barely reached suggestive statistical significance.

**Figure 10 - LOD score and QTL location-Garrett 1998**

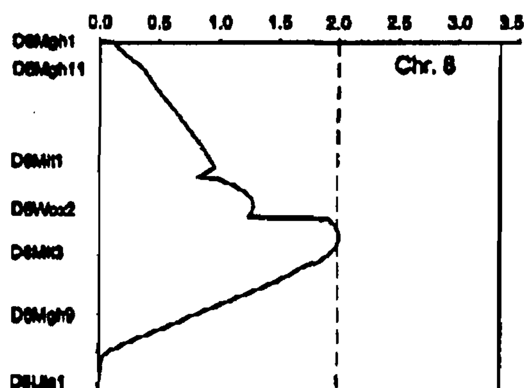
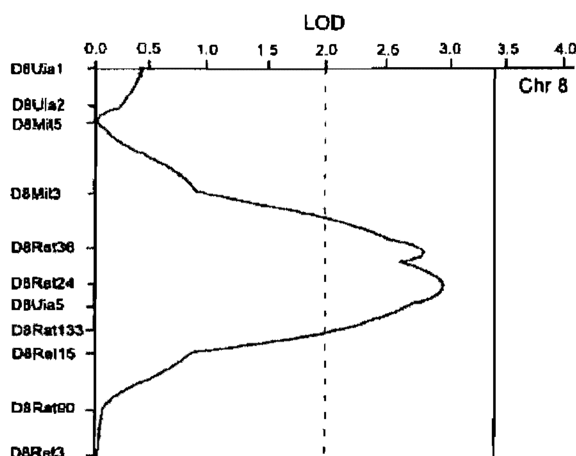


**Figure 11 - LOD score and QTL location-Garrett 2000**




Later in 2000, Garret et al. (64) in another linkage study using F2 generations derived from the two hypertensive strains, the S and the SHR, in an attempt to find QTLs that differentiate these two hypertensive strains. They localized a QTL on chromosome 8, which also attained LOD scores that are considered suggestive for linkage to BP (Figure 11). In this case, the S alleles increased BP.

In 2002, Khan et al. (94), using the congenic strain established by Kren, found a nicotinic receptor gene cluster on rat chromosome 8 implicated in nociceptive and BP hyper-responsiveness.

Nociception is the reception of a noxious stimulus. Pain is a potential outcome of nociception. Nociception occurs in the receptor portion of afferents and the action potentials are conveyed to the spinal cord via afferent axons. Nociception is relayed to the brainstem, hypothalamus and thalamus via tract cells or neurons. In the case of pain, nociception is relayed to the thalamus via neospinothalamic and paleospinothalamic tract cells or neurons. From the thalamus, certain thalamacortical fibers project to the limbic system, where the feeling of hurt or pain is generated. Nociception can also, activate generalized autonomic responses independently of the relay of pain to conscious levels causing pallor, sweating, bradycardia, a drop in BP, subjective faintness, nausea and syncope.

All nociceptors are free nerve endings that have their cell bodies outside the spinal column in the dorsal root ganglion and are named based upon their appearance at their sensory ends. There are mechanical, thermal, and chemical nociceptors. They are found in

skin and on internal surfaces such as periosteum and joint surfaces. Deep internal surfaces are only weakly supplied with pain receptors and will propagate sensations of chronic, aching pain if tissue damage in these areas occurs.

There is a relationship between BP and acute pain sensitivity. Research on the hypoalgesia associated with hypertension has highlighted the importance of inter-relationships between the cardiovascular and pain regulatory systems(203). Even in the absence of clinical hypertension, familial risk for hypertension in healthy individuals appears to be associated with diminished responsiveness to acute pain, apart from the influence of actual BP levels. There is suggestive evidence that these effects are mediated in part by elevated central descending pain inhibitory activity. Although these studies might suggest that BP-related hypoalgesia is specifically associated with central mechanisms contributing to hypertension risk, other studies have demonstrated that regardless of hypertension status, elevated of the resting BP level is associated with decreased pain sensitivity in healthy normotensive individuals.

The relationship between resting BP and pain sensitivity arises from the "central autonomic network", a group of integrated brain regions that coordinate responses to environmental stimuli. The brain regions underlying control of the cardiovascular system are known to overlap substantially with those contributing to antinociception. In particular, the nucleus tractus solitarius (NTS), which serves as the interface between autonomic and sensory systems, participates in pain regulatory pathways, as evidenced by the fact that stimulation of the NTS induces antinociception.

The NTS is located at the first synapse in the baroreceptor reflex pathway. It plays an important role in the processing of visceral information, receiving major afferent input from both the vagus nerve (subserving the baroreflex) and spinal laminae involved in nociceptive processing. Antinociception elicited by activation of the NTS may derive in part from its direct and indirect efferent projections to the periaqueductal gray and other brain structures, such as the nucleus raphe magnus and rostral ventrolateral medulla, that are known to be involved in modulation of pain pathways. In addition to the brain regions mentioned, efferent projections from the NTS to the A5 nuclei and A6 nuclei, also known as the locus coeruleus of the medulla, may be important contributors to BP-related antinociception, given that direct stimulation of these regions elicits analgesia. Interconnections between the NTS and the locus coeruleus may be particularly important in mediating nonopioid analgesia, given that the locus coeruleus is a primary source of

noradrenergic neurons in the neuraxis. Pathways from the NTS to spinal cord nuclei modulating cardiovascular tone, including sympathetic and parasympathetic preganglionic nuclei, also interact with descending pain modulation pathways. Thus, through a baroreceptor feedback loop, descending pain inhibitory pathways may be able to self-regulate their activity through actions in autonomic centers of the spinal cord modulating cardiovascular function(12).

A model of the relationship between BP and pain sensitivity has been proposed. According to this model, baroreceptor activation has a role in the relationship between resting BP and acute pain sensitivity. According to the model, pain, through a somatosensory reflex, increases sympathetic arousal thereby increasing BP. This increase in BP leads to increased baroreceptor stimulation, which in turn triggers decreasing pain inhibitory activity, thereby helping to return arousal levels to a state of homeostasis. Persistent baroreceptor stimulation related to BP elevation lasting from a few minutes to a few days may result in a resetting of baroreceptors. It is believed that this situation increases the BP set point around which the baroreflex is centered and thus elevating the BP threshold required to trigger baroreceptor activation (12) .

In 2002, Ueno et al. (190) carried out an acute pharmacogenetic analysis in an F2 generation, derived from Prague hypertensive-hypertriglyceridemic rats and LEW rats. They found a BP QTL on chromosome 8, with a significant LOD score of 7.0 after the blockade of the sympathetic nervous system using pentolinium. Sympathetic blockade of blood vessels causes vasodilation and hypotension. Pentolinium acts as a ganglionic blocking agent and inhibits the release of adrenaline and noradrenaline from adrenergic nerves. In addition, pentolinium binds to the nicotinic acetylcholine receptor. This receptor is permeable to a range of divalent cations including calcium. The influx of calcium may activate a potassium ion current, which hyperpolarizes the cell membrane. Blockage of the receptor leads to smooth muscle relaxation and vasodilaton.

# Chapter 3

# Experimental Methods Used to Study Rat Chromosome 8

## *3. 1 Objective of My Study*

According to previous studies, rat chromosome 8 (C8) likely contains a BP QTL(59). The purpose of the present study is to provide strong evidence that such a QTL exists. Previously, linkage analysis of a population of rats, in which $F_2$ (SxL) were bred together had provided statistically suggested evidence of the presence of least one QTL in chromosome 8. Of course, linkage analysis is prone to false positives since it is based on probability, not on certainty. So to demonstrate conclusively that the chromosome region truly contains a BP QTL, a more stringent genetic test must be applied. If a QTL does in fact exist, we also want to map it to a precise chromosomal region. The final goal of this study is to identify the effect of this QTL on BP.

## *3.2 Materials and Methods*

### 3.2.1.Rat Strains Used-Dahl Salt -Sensitive vs. Salt-Resistant Rats

The Dahl S strain is used as the salt sensitive hypertensive strain in this study. Although the R strain is usually compared and contrasted with the S strain, using the R strain generally impedes genetic mapping. Since both S and R strains are derived from the same stock, their genomes are quite similar. So polymorphisms among chromosome markers capable of differentiating S from R are rare (about 29%). So we used a normotensive strain derived from a different originator stock to facilitate genetic mapping.

### 3.2.2 Lewis Strain

Bred by Dr. Margaret Lewis, this strain originated from Wistar stock. The Lewis strain (LEW) has low BP whether it is fed a high salt diet or a low salt diet. It is a normotensive strain. The rate of polymorphisms among chromosome markers capable of differentiating S from LEW is about 50%.

### 3.2.3 Strategies used in the Study of the Rat Model

To prove that a chromosome region truly contains a BP QTL, it is useful to breed congenic strains. A congenic strain provides a definite genetic test. We bred congenic strains for chromosome 8 by crossing the S strain with the Lewis strain. The effect of this cross is to replace a region of chromosome 8 on the S strain rat with the homologous region of the L strain rat. Thus, congenic strains permit the association of changes in BP with a specific region of the chromosome.

### 3.2.4 Congenic Strains

A major limitation of existing animal models of hypertension is that hypertensive strains differ from normotensive control strains in multiple regions of the genome, not just in regions that contain genes regulating BP(102). With the availability of simple methods to detect DNA polymorphisms, it has become possible to create hypertensive strains that are genetically identical to comparable normotensive strains except in selected chromosome regions.

The development of congenic strains is a very powerful analytical tool whose purpose is to transfer a mutation or a chromosomal segment from rats of one inbred genetic background to another (Figure 12).

Strains that are genetically identical except for a single chromosomal segment are said to be congenic. To produce congenic animal models, one first backcrosses the donor inbred strain with the recipient inbred strain at least 8 to 10 times. At every generation, one selects for the locus or chromosomal region to be transferred. After 8 to 10 backcrosses, the only region of the genome that originates from the donor strain is the single segment containing the selected locus. At this stage, heterozygous rats are intercrossed to produce rats homozygous for the introgressed region. Although this process is long, it yields closely related inbred strains that differ in only one small region of their genomes. Later we will

discuss a more sophisticated protocol, called "speed congenics," that shortens the generation time by counterselecting the alleles from the donor strain(31,129).

If congenic strains exhibit a difference in BP, one may draw the conclusions that a locus affecting BP exists within the differential chromosome segment. Systematic studies can then be performed to investigate further the physiological, biochemical and molecular mechanisms that contribute to the strain differences in BP. The size of the differential chromosome segment can be reduced by molecular selection techniques and by continued backcross breeding.

It may be possible to have multiple congenic 'controls' for a single hypertensive strain, where each congenic control differs from the hypertensive parental strain with respect to a specific region of the genome. In addition to providing evidence for the existence of BP regulatory loci in restricted chromosome regions, the congenic strains can be used to study how different portions of the genome interact to affect BP, permitting for example, the differentiation between individual effects of two interacting chromosomes and their combined effect.

It is expected that each congenic strain, will have lower BP compared with the S strain, because the regions introgressed should all contain the minus BP QTL allele derived from the normotensive strain.

Figure 12 - The Congenic

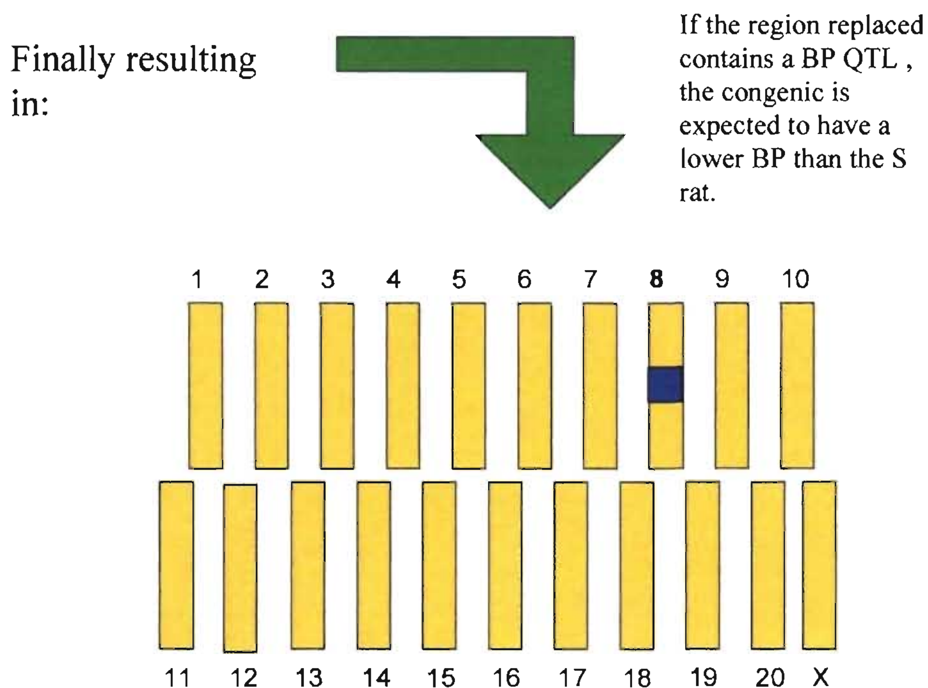
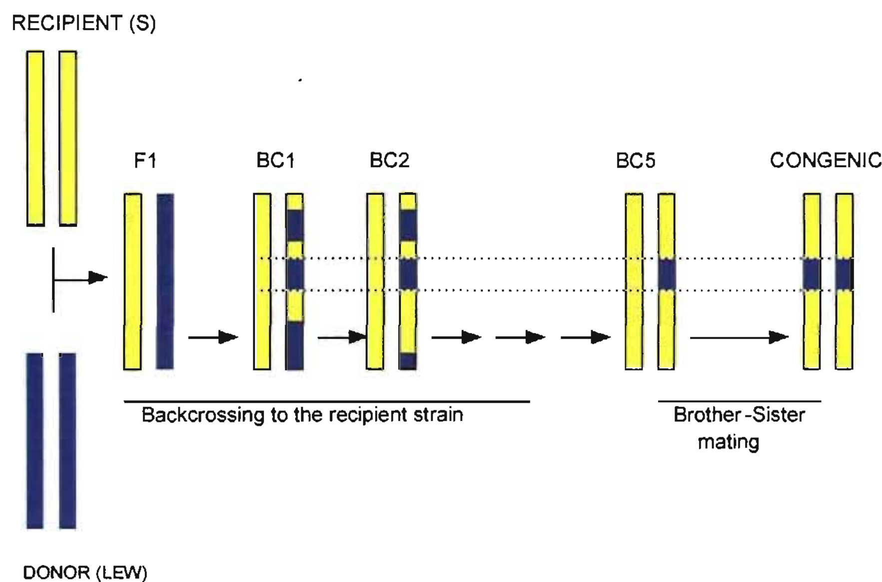


**Figure 13 - Speed Congenic-**

Strategy to Construct a Congenic




### 3.2.4.1 Backcrossing and Speed Congenics

A process of whole genome marker assisted selection called "speed congenics" can accelerate the development of congenic strains. Assuming one has genotyped the whole genome, one selects the breeders that, besides containing the target region of the donor, have a greater proportion of alleles from the recipient strain throughout the genome (Figure 13). The rats chosen as backcrossing breeders should preferably be male, since the male can be paired with many females of the receiver genome. One screens the polymorphic genetic markers covering the entire background of the genome to select male offspring with the fewest donor alleles in their background. This produces the congenic strain more efficienctly. Using the 'best' male in the next round of breeding, in this way, dramatically reduces the time taken to clear the background of the recipient strain. If 60 background markers spaced on average 25 cM, are used to screen males at each generation, it is calculated that by four backcross generations the donor genome contamination would be less than 1%. With this method, developing a congenic strain is reduced from 4-5 years to 2-3 years, reducing the breeding time by a factor of 2 since only 5 backcross generations are needed for the genetic composition of the congenic rat to be more than 99% from the

recipient and less than 1% from the donor strain (Figure 14). The only drawback of the method is the cost of typing the many markers needed.

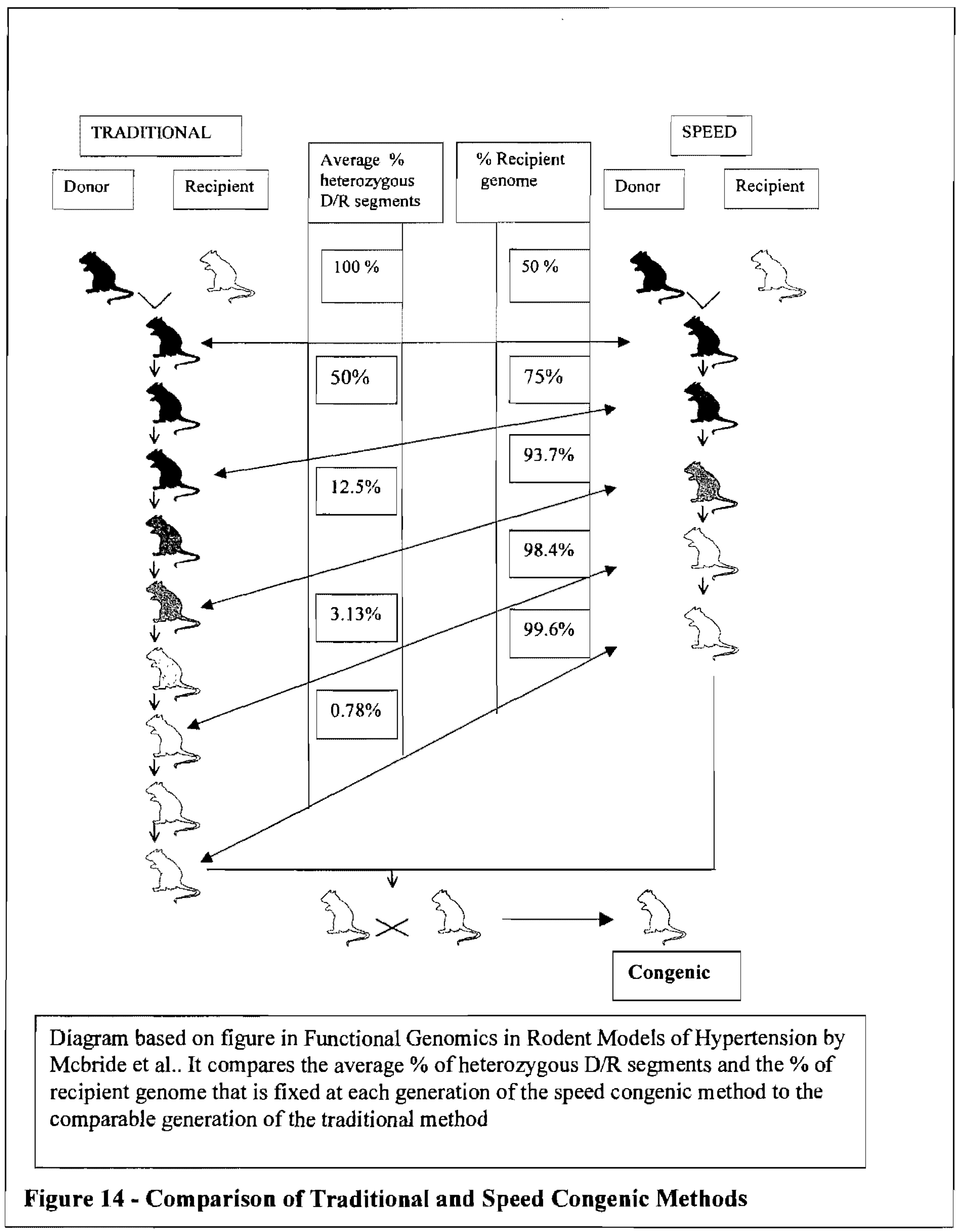



Diagram based on figure in Functional Genomics in Rodent Models of Hypertension by Mcbride et al.. It compares the average % of heterozygous D/R segments and the % of recipient genome that is fixed at each generation of the speed congenic method to the comparable generation of the traditional method

**Figure 14 - Comparison of Traditional and Speed Congenic Methods**

### *3.2.4.1.1 How Chromosome 8 Congenics Strains in this Study were Selected.*

The basic scheme for our studies was to put a specific region of chromosome 8 of the normotensive strain LEW onto the genetic background of the Dahl S strain, using a standard breeding protocol. Dahl S rats were initially crossed to rats of LEW. F1 progeny were backcrossed to S to produce the first backcross generation (BC1). BC1 rats were genotyped for 92 markers, which were evenly spaced throughout the rat genome with an average of about 18 cM (see Table 6). The ideal breeder selected and designated for a subsequent breeding with an S rat to produce BC2, amongst the BC1 generation was heterozygous, SL, for chromosome 8, but possessed the maximum SS homozygosity for the rest of the irrelevant genome. BC2 rats were genotyped and screened exactly as for the BC1 rats, to derive an ideal breeder amongst the BC2. This process was repeated for a total of five backcrosses, each time choosing rats heterozygous throughout the region of interest as breeders for the next backcross. At this point, it was found that only the markers delineating a chromosome 8 region of interest were heterozygous SL, whereas the markers for the rest of the genome including those flanking the chromosome 8 region of interest were homozygous SS. At BC5, two heterozygotes were intercrossed to generate homozygotes. This fixed the region of interest of chromosome 8 from the normotensive strain onto the background of the S strain. The markers found on Tables 6 and 7, and on Figure 19 were used in selecting rats on the basis of their genotypes.

**Table 6 Chromosome Markers used to make congenic Strains**

| Chromosome | Chromosome Markers Tested |
|---|---|
| 1 | *D1Wox25–D1Rat304–D1Mco27–Sa–D1Uia12–D1Arb33–D1Rat19–D1Mco4* |
| 2 | *Camk2d–Gca–D2Rat302–Cpb–D2Mco13–D2Rat199–D2Uia5* |
| 3 | *D3Rat107–D3Rat17–D3Rat24–D3Rat66–D3Wox3–D3Rat52* |
| 4 | *D4Mgh1–D4Mgh16–D4Uia1–D4Mit17–D4Uia4* |
| 5 | *D5Rat130–D5Mit5–D5Uia2–D5Mco34–D5Uia8–D5 Rat95–D5Mco2–Ela2* |
| 6 | *D6Rat105–D6Mit1–D6Mgh3* |
| 7 | *D7Mgh1–D7Rat152–D7Rat44–D7Rat110–D7Rat18–D7Rat128–D7Rat115* |
| 8 | *D8Mgh11–D8Rat134–D8Rat43–D8Uia2–D8Rat55* |
| 9 | *D9Rat64–D9Uia9–D9Wox23* |
| 10 | *D10Mco10–D10Mco17–D10Mgh6–D10Wox6–D10M11Mit58–D10Mco15–D10Rat11–D10Mgh1* |
| 11 | *D11Rat50–D11Mit1–D11Uia1* |
| 12 | *D12Mit6–D12Rat32–D12Mit4* |
| 13 | *D13Mgh4–D13Uia3–D13Uia8* |
| 14 | *D14Wox10–D14Uia2–D14Uia1* |
| 15 | *D15Uia8–D15Mgh2–D15Rat126* |
| 16 | *D16Rat14–D16Rat67–D16Uia2–D16Rat21–D16Mit2–D16Rat12* |
| 17 | *D17Wox7–D17Mit5–D17Mgh5–Agtr1a–Edn1* |
| 18 | *D18Uia6–D18Mit8–D18Mco6–D18Wox7* |
| 19 | *D19Rat82–D19Rat25–D19Rat57* |
| 20 | *D20Wox3–D20Wox1–D20Mgh1* |
| X | *DXUia2–DXMco1–DXRat93* |

All the markers are polymorphic between S and LEW strains. Table taken from Deng et al. 2002 (127). The rest of the markers are mostly anonymous.

## 3.2.5 Genome-wide scanning

A genome-wide scan is the genotyping of hundreds of polymorphic markers, preferably evenly through the genome, in a selected sample of individuals with or without a qualitative trait or with a measurable quantitative trait. It is used in situations where certain alleles for a marker segregate with a phenotype. Through genome-wide scanning, it is possible to find novel loci associated with a trait when the research is not biased by previous hypotheses of molecular pathogenesis. Usually highly polymorphic repeat markers are used, although new DNA-array technology, monitoring thousands of densely spaced single nucleotide polymorphic markers, have drawn much attention lately. In order to ensure that the strains were not genetically contaminated, a rigorous and strict quality

control procedure was instituted, consisting of genetic monitoring with genome-wide markers (Table 6) together with physical discrimination of the rats.

### 3.2.6 Choice of Markers:

It is essential to maintain the target region of heterozygous throughout the process of producing congenics. The greater the distance between the markers used to monitor the genotype, the greater the chance of a double recombination event occuring between the markers. The development of congenic lines does not help in constructing genetic maps, but it does render them more accurate.

### 3.2.7 Microsatellite markers

The advent of the polymerase chain reaction (PCR) has made mapping relatively quick and easy. A microsatellite is a DNA sequence containing short sequences repeated in tandem arrays. They are usually mono-, di-, tri- and tetranucleotide repeats, repeating on the order of 10 to 100 times. These euchromatic repeated sequences are scattered throughout the non-coding DNA of eukaryotes. They are highly polymorphic in terms of length and are useful in PCR genotyping. Figure 15 is an example of a microsatellite.

```
..TCCAGACAAGGTGGTGTGTGTGTGTGTGTG
TGTGTGTGTGTGTTTCTCCAGTGAGATTTA...
```

**Figure 15 - Microsatellite example**

In order to make a marker, the region of interest is screened for microsatellites in the rat genome sequence given at the NCBI database. The microsatellite and 50 nucleotides around it are inputted into a primer selection program such as the Primer3 program(3). Using the output from the program, the best set of oligonucleotides is selected based on the statistical output for each set of derived primers that the program has devised. The new marker should be "blasted" in the NCBI database in order to make sure that it is unique to the region of interest. If it is unique, then the marker is tested at different temperatures

(45°C, 50°C, 55°C, and 60°C) to find the optimal temperature. If the marker is polymorphic and specific then it can be used to genotype congenic strains and to place the marker on the genetic map based on the genotype that results. Key factors in PCR primer design, in order to produce successful amplification of markers are:

-Seventeen to thirty nucleotides in length

-GC content ideally about 50%

-If the GC content is low, a longer primer is preferable in order to avoid low melting points.

-One should avoid primer sequences with long runs of a single nucleotide, with significant secondary structure or those that have complementarity between the two primers.

### 3.3 Sample preparation and DNA analysis

Polymorphic markers were genotyped by PCR. The PCR reaction mixture consisted of 1 X PCR buffer containing 1.5mM $MgCl_2$, 200µM of each of the dNTPs, 1µM of each primer, 0.12U Taq DNA Polymerase and 10-200 ng of rat genomic DNA, which was extracted by tail biopsy from each rat using Qiagen Genome kit. The genotype of each rat was determined by PCR. PCR was initiated at 95 °C for 5 minutes, followed by 30 cycles of 94 °C for 40 cycles, 45°C or 50°C or 55°C or 60°C for 40 seconds depending on the marker, 72°C for 1.5 minutes and ended by 5 minutes of extension at 72°C. The PCR products were analyzed either on a 4% agarose gel or on a 6% nondenaturing polyacrylamide gel; depending on the size differences of the products among rat strains of interest (please refer section 3.4).

To obtain PCR products that can be analyzed on a polyacrylamide gel, a PCR reaction of 10µL containing the ingredients that are essentially the same as above, except that 1µCi of alpha- Phosphorus-32 label dCTP was added to each PCR reaction sample, while reducing the unlabeled dCTP to one tenth of that normally used. After PCR, samples were loaded on 6% nondenaturing polyacrylamide gel containing 5% glycerol. This was run at twenty to thirty watts, at room temperature, until the bromophenol blue dye reached the bottom of the gel. PCR products were then scored by autoradiography.

## *3.4 Molecular separations used in this study*

### 3.4.1 DNA Gel Electrophoresis

Electrophoresis is a technique used to separate or purify macromolecules, especially proteins and nucleic acids, that differ in size, charge or conformation (Figure 16) (192).

In this technique, one makes a gel with slots in it. The gel itself is composed of either agarose or polyacrylamide. Agarose is a polysaccharide extracted from seaweed. It is typically used at concentrations of 0.5% to 4%. Higher concentrations of agarose facilitate separation of small DNA fragments, while low agarose concentrations allow resolution of larger fragments. Agarose gels are easy to prepare; and are also non-toxic. Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50, 000 bp can be separated using standard electrophoretic techniques.

Finally, the DNA is stained with a fluorescent dye, such as ethidium bromide and the gel is examined under ultraviolet illumination. Ethidium bromide is an intercalating agent commonly used as a nucleic acid stain for techniques such as this one. When exposed to ultraviolet light, it will fluoresce with a red-orange color, which intensifies almost 20-fold after binding to DNA(192).An unknown DNA fragment can be electrophoresed in parallel with commercially bought standard fragments, and its size can be estimated if it falls within the range of the standards.

Polyacrylamide, the other common reagent, is a cross-linked polymer of acrylamide. It is toxic. The length of the polymer chains is dictated by the concentration of acrylamide used, typically between 3.5% and 20%. Polyacrylamide gels are more difficult to prepare than agarose gels. Because oxygen inhibits the polymerization process, the gel must be poured between glass plates or cylinders. Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved(192). The gel is immersed within an electrophoresis buffer. A small amount of DNA is added in a slot and an electric current is run through the gel. The DNA is negatively charged because of the phosphates in its backbone, and it migrates towards the positive pole at the end of the gel. The gel separates DNA fragments of different sizes due to friction: small DNA molecules experience little frictional drag from solvent and gel

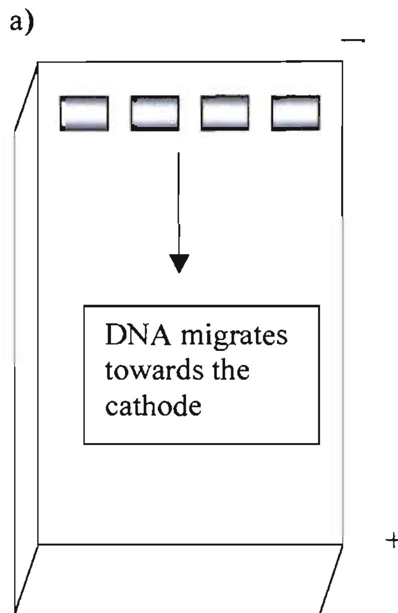
molecules, so they migrate more rapidly than larger fragments. The result is that the electric current will distribute the DNA fragments according to their sizes, the largest near the top, and the smallest near the bottom.

### 3.4.2 Autoradiography

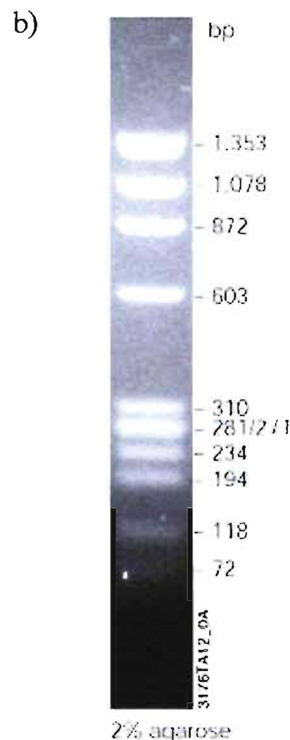
To detect the DNA in polyacrylamide gels one uses autoradiography (Figure 17). Autoradiography is a means of detecting radioactive compounds with a photographic emulsion. The form of emulsion favoured is a piece of X-ray film. Radioactive tracers allow small quantities of substances to be detected, enabling small differences in labeled PCR products to be seen. Direct measurement of the minute differences using ultraviolet light absorption or by staining with dyes is not possible because these methods have limited. Once radioactive DNA fragments are electrophoresed on a gel, the gel is placed in contact with the X-ray film and is left in the dark for a few days. The radioactive emissions from the bands of DNA expose the film. When the film is developed, dark bands appear, corresponding to the DNA bands on the gel. To enhance the sensitivity of autoradiography an intensifying screen is often used. This screen is coated with a compound that fluoresces when it is excited by β-rays at low temperature. In this study, the β-rays were produced from the radioactive isotope $^{32}P$. A radioactive gel is placed on one side of a photographic film and the intensifying screen on the other. Some β-rays expose the film directly, but others pass through the film and would be lost without the screen. When the high-energy electrons strike the screen they cause fluorescence, which is detected by the film(192).

**Figure 16 - DNA gel electrophoresis**

a)



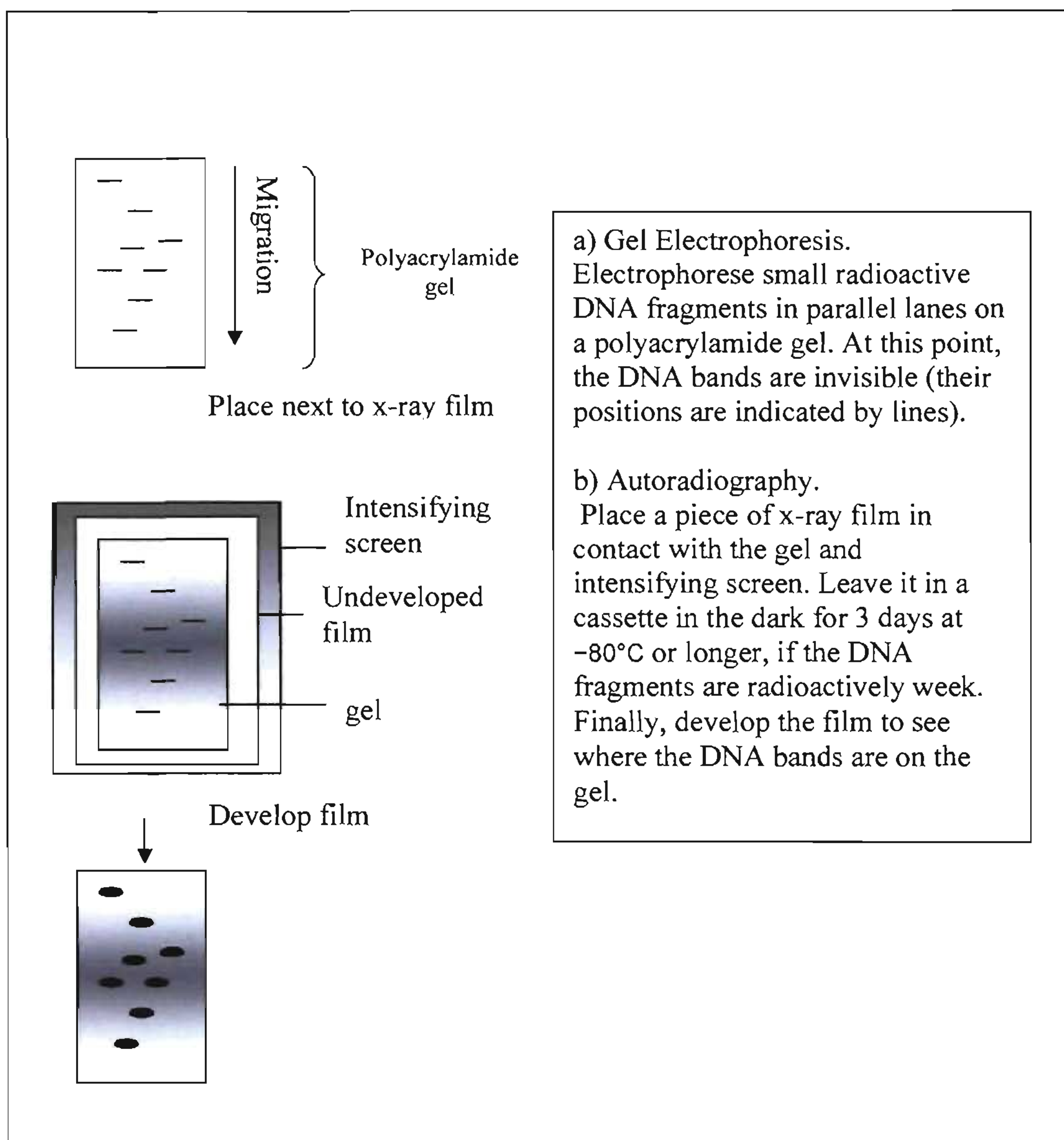

b)




**a)** Scheme of the method: A horizontal gel made of agarose ( a substance derived from seaweed, and the main component of agar). The agarose melts at high temperature, then gels as it cools. A "comb" is inserted in the molten agarose; after the gel cools , the comb is removed, leaving slots, or wells/ lanes(shaded regions). The DNA is then placed in the wells and an electric current is run through the gel. Because the DNA is an acid, it is negatively charged at neutral pH and electrophoreses or migrates, towards the positive pole This results in that the DNA fragments will be distributed according to their sizes; the largest near the top, the smallest near the bottom. The gel is then soaked in a fluorescent dye, such as ethidium bormide (a fluorescent dye which stains the DNA) and the gel is examined under ultraviolet illumination. When exposed to ultraviolet light, the dye will fluoresce with a red-orange color, intensifying almost 20-fold after binding to DNA.

**b)** An unknown DNA can be electrophoresed in parallel with commercially bought standard fragments, and its size can be estimated if it falls within the range of the standards. A commercially bought molecular standard ΦX174 DNA/*Hae* III which is loaded 5μl/lane is pictured here.

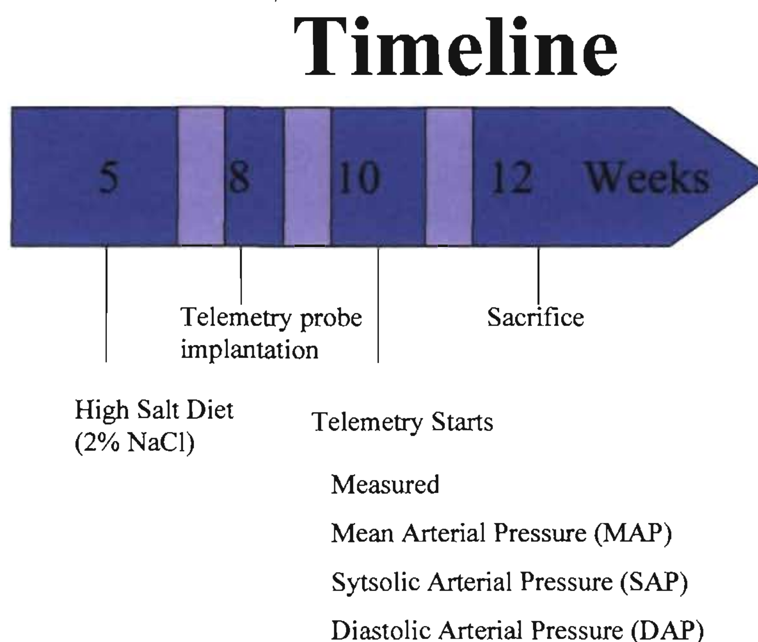
**Figure 17 - Autoradiography**



## 3.5 Measuring BP- Telemetry

Male rats were weaned at 21 days of age, maintained on a low salt diet (0.2% NaCl) and then fed a high salt diet (2% NaCl) from 35 days of age until the end of the experiment. Telemetry probes were implanted when the rats were 56 days old. The implantation surgery involved the following: the rats were anesthetized by the inhalation of isoflurane at a dose of 4% for 3 minutes. During the surgery, the state of anesthesia was maintained by isoflurane at a dose of 1.5-2%. The pressure catheter, which contained the biocompatible gel at the tip and the noncompressible fluid connecting the tip to the pressure sensor, was implanted through the femoral artery until it reached all the way up to the abdominal aorta. It was secured by sutures to the blood vessel. The telemetry device body, which included the pressure sensor, the reusable electronics module, and a battery, was fixed to the muscle wall and was left in the abdominal cavity. After the surgery, the rats were allowed to recuperate for ten to seventeen days. In the first three days of recuperation, the rats were fed Jell-O containing the analgesic buprenorphine at 0.5% mg/kg. Afterward, they were given a diet of Ensure milkshake with chocolate in addition to the 2% NaCl standard meal. This supplement usually lasted up to five days to facilitate their appetite and thus weight gain.At the implantation stage, the rats had been on 3 weeks of high salt diet and had body weights of 250 to 320g. The telemetry probe implantation procedure is the same for all rats studied in our laboratory (44). The Figure 18 describes the timeline for rats chosen to have their BPs measured. MAP, DAP, and SAP were measured individually. BP was measured at two different times or more, using different litters born at different times of the year, to exclude seasonal and environmental influences. The BP data were pooled from separately reproducible measurements for each strain. Once the time- period for BP measurement was concluded, the rats were sacrificed. The final body weights of the animals were measured, and certain organs such as the heart, kidney and brain were harvested for future studies. Only male rats were used in this study because in the female BP can vary due to the hormones, such as estrogen, involved in estrus.

**Figure 18 - Timeline for data collection**




Only male rats were used.

See text for details.

## *3.6 Statistical Approach*

BP is considered when comparing congenic strains to the S strain. The statistical test, ANOVA detects a difference between any of the groups being compared. For BP measures, the repeated measures' analysis of variance ANOVA is used, to compare the significance level for a difference or a lack of it, between a congenic strain and the control S Strain. This form of ANOVA takes into account, that for any given animal, BP is measured at several points in time. When the ANOVA detects no significant difference, it indicates that there is no significant difference between the groups being compared and no further analysis is required. If the ANOVA detects a significant difference, it indicates that there is a significant difference between the groups being compared.

To distinguish which pairs of groups are different, we also perform Dunnett *post* hoc comparison. The Dunnett test takes into account multiple pairwise comparisons to

determine which congenic strains are different from the control strain S. A probability value of $p < 0.05$ was considered statistically significant. The Dunnett test adjusts the $p$ values for differences in sample sizes among the compared groups and multiple comparisons. In the analysis, a BP component is compared each day for the period of measurement among the strains. We report data as mean ± standard deviation. These statistical computations were performed with the SPSS statistical program (1).

# Chapter 4

# Results

## 4.1 Congenic Strains Produced in My Study

By following the speed congenic method, two congenic strains were developed from hypertensive Dahl salt sensitive S rats and normotensive Lewis (LEW) rats. Using the speed congenic method, the congenic strains were developed in five generations instead of the eight generations that would have otherwise been required. The chromosome regions containing various LEW substitutions in the congenic strains are shown in figure 19. In the final congenic strains, the homologous regions of the LEW strain replaced each region of interest from the S strain.

In figure 19, the linkage map, indicated as a grey bar, is essentially the same as published previously in a linkage study(142) based on an $F_2$ (S x LEW) population. Numbers to the right of the map are units in cM, and to the left of the map are units in centiRays (cR) between markers. To locate a marker of interest onto an existing RH framework map, the results of genotyping of that marker were entered into the RHMAPPER program, available interactively on the website http://rgd.mcw.edu/RHMAPSERVER. The solid blue bars symbolize the S chromosome fragments that have been replaced by those of the LEW rat. The entire region indicated by solid bars and junctions between the solid and shaded bars are homozygous LEW alleles on the map for all the markers listed in the corresponding positions. Shaded bars on the ends of solid bars indicate ambiguities of crossover breakpoints between markers. The physical map consists of the alignment of rat supercontigs in an ascending order from top to bottom on chromosome 8 obtained by blasting a marker to the NCBI database (http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html). The name of a supercontig starts with NW_047 followed by three digits. Parenthesized numbers below a supercontig indicate its approximate size in bps. The congenic strains are S.LEW- (D8Chm14-D8Rat16) (abbreviated as C8S.L1) and S.LEW- (D8Rat56-D8Rat51) (C8S.L2), respectively. The white bar symbolizes the parental strain, S.

**Figure 19 - Map of Congenic Strains Constructed**

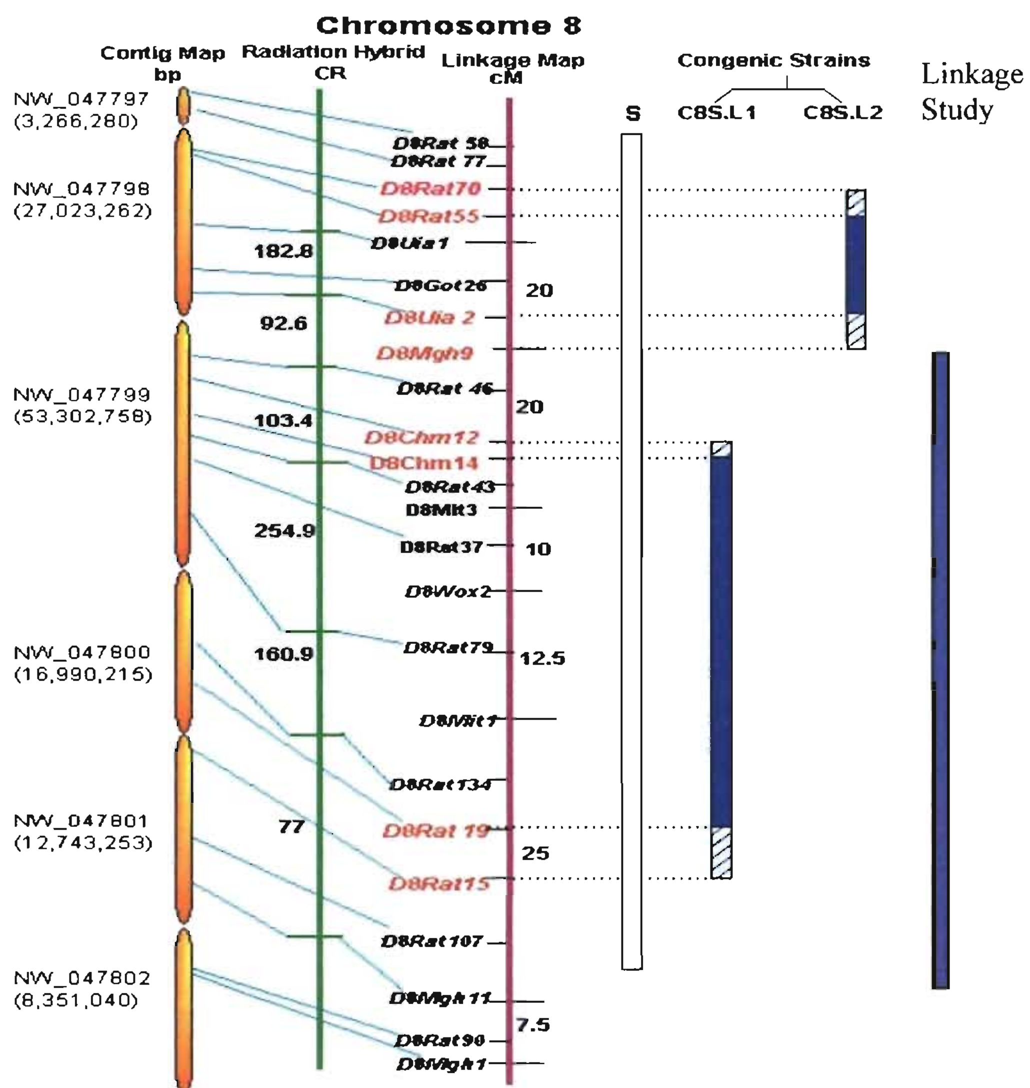


Table 7 shows, the new markers constructed in this study to aid in mapping the congenic strains. D8 indicates chromosome 8, and Chm, is an abbreviation for the 'Centre Hospitalier de l'Université de Montréal' research center where the study was conducted. The location of the markers within the supercontigs is indicated, as well as the primers and size of the PCR product. The temperature at which the PCR product is formed is indicated. Only the markers that had products and the resulting polymorphisms, if there are any when comparing S and LEW, are shown on Table 6.

| Rat Marker | Supercontig | Primers Sequences (5' → 3') | Product Size (bp) | Temp. (°C) | Polymorphism among rat strains |
|---|---|---|---|---|---|
| D8Chm2 | NW_047799 | agccatctcctcagcctgta caacagaaaagcaagcaagc | 512 | 60 | S>LEW |
| D8Chm3 | NW_047799 | aggggctgtaccacctttct gcatgcaatgcctctctctc | 191 | 60 | S=LEW |
| D8Chm6 | NW_047800 | gagcagtggagggtagcttg gctgagggcattgagaagtc | 369 | 55 | S=LEW |
| D8Chm11 | NW_047799 | catcctgggctcacaaactt aggtggggcagccatagtta | 457 | 60 | S>LEW |
| D8Chm12 | NW_047799 | aggctacatgggacactgtt tccaggctccacagtttttc | 591 | 60 | S<LEW |
| D8Chm14 | NW_047799 | catctgcatctgcatatctgg aggaaactgtgggttggaaa | 249 | 60 | S<LEW |
| D8Chm15 | NW_047799 | acctctgcccgaacttcttt ggggaaaggtgtcctgcta | 246 | 60 | S=LEW |
| D8Chm16 | NW_047799 | ctgtgctcacaggaggatga gctgtcctctgaccaacaca | 490 | 60 | S>LEW |
| D8Chm17 | NW_047799 | acgtgtcaccgtgtgtctgt gccttctcagtaccataacctca | 375 | 50, 55, 60 | S=LEW |
| D8Chm18 | NW_047799 | ggcagtgcctgagaagaaag cggatgaacttgttgatttctc | 458 | 50, 55, 60 | S=LEW |
| D8Chm19 | NW_047799 | accaaatgttttcccccagt tcctgagccttcttccagtt | 283 | 50, 55, 60 | S>LEW |
| D8Chm20 | NW_047799 | aggggcgtctgtatgttcac tcacacactggccttcatgt | 219 | 50, 55, 60 | S=LEW |
| D8Chm21 | NW_047799 | atcacaggggaaaacagtgg cagaaggctgggaatgtagc | 500 | 50, 55, 60 | S=LEW |
| D8Chm22 | NW_047799 | acatgagggaggctgaactg ctactccctccaccctgaca | 848 | 50, 55, 60 | S=LEW |
| D8Chm23 | NW_047798 | gaggccttctctgggagagt ctccttcatcaagaatcactgc | 241 | 50, 55, 60 | S=LEW |
| D8Chm24 | NW_047798 | tcctcattgaatgctgttgg accgggaaagggaataacac | 211 | 50, 55, 60 | S=LEW |

**Table 7 -New microsatellite markers constructed for rat chromosome 8**

### *4.2 Telemetry Data*

For all four strains, two parental and two congenic strains, the BP components were measured, including MAP, DAP, and SAP. One BP reading was taken every 2 minutes throughout the period of measurement. Each point on the graphs in Figures 20 and 21 represents a 6-hour average of these readings in order to show diurnal variations. For all the strains, BP was measured at two different times of the year using separate litters raised at different times, in order to exclude seasonal variations in the measurements. BP for all four strains was measured simultaneously each time. Figure 20 compares the BP readings of the two parental strains. The readings of the LEW and the S rats are indicated in blue and red respectively. The BP of the LEW rats had averaged around 96 ± 4 mmHg while the average readings of the S rats were higher, around 172 ± 4 mmHg. Results of DAP and SAP are not shown because they are similar to those of MAP.

**Figure 20 - BP readings comparing the two parental strains, S and LEW**

MAP

S vs. LEW

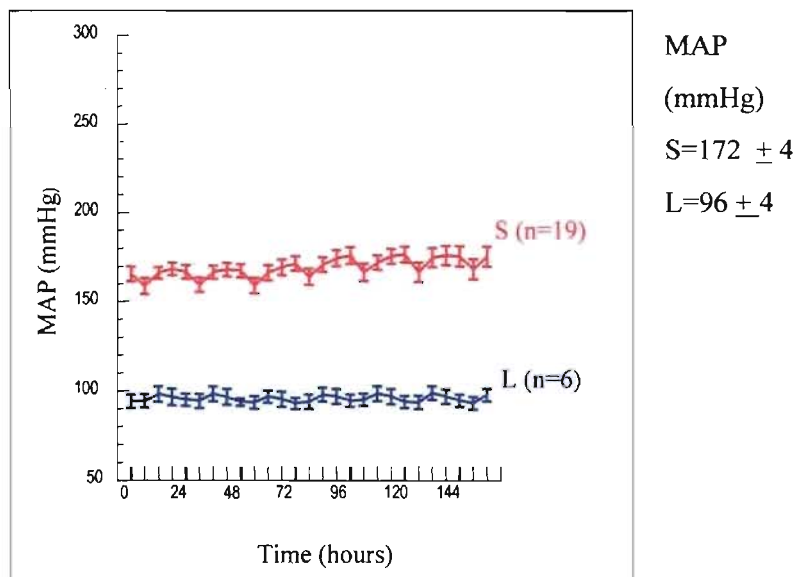


Figure 21 compares in MAP, DAP, and SAP between the C8S.L1 and C8S.L2 congenic strains and the S strain. Each time-point on the graph represents an average of 6-h readings. Each point on the 6-h graph is an average of readings taken at every 2-minute interval. Error bars represent the standard error of measurement. The number of rats is given as *n.* The congenic strains are S.LEW- (D8Chm14-D8Rat16) (abbreviated as C8S.L1) and S.LEW- (D8Rat56-D8Rat51) (C8S.L2), respectively. The pattern of BP in each strain was the same when comparing DAP, SAP and MAP. The MAP for C8S.L1 was 138 ± 5 mmHg while the MAP for C8S.L2 was 197 ± 5 mmHg, greater than that of the S rats. We will discuss this observation later.

Since BPs of strains C8S.L1, C8S.L2, LEW, and S were not different at the separate periods of measurement, the BP data was pooled from reproducible measurements for each strain. All the strains C8S.L1, C8S.L2, and S had higher BPs than LEW. The region that contains the QTL can be localized to the segment that showed significant changes in BPs. MAPs, DAPs and SAPs of C8S.L1 were lower ($p<0.001$) than those of the S strain. In contrast MAP, DAP and SAP of C8S.L2 were higher ($p<0.001$) than those of the S strain. Figure 22 summarizes the results obtained.

**Figure 21 - Comparisons of MAP, DAP and SAP of S rats with those of the congenics.**

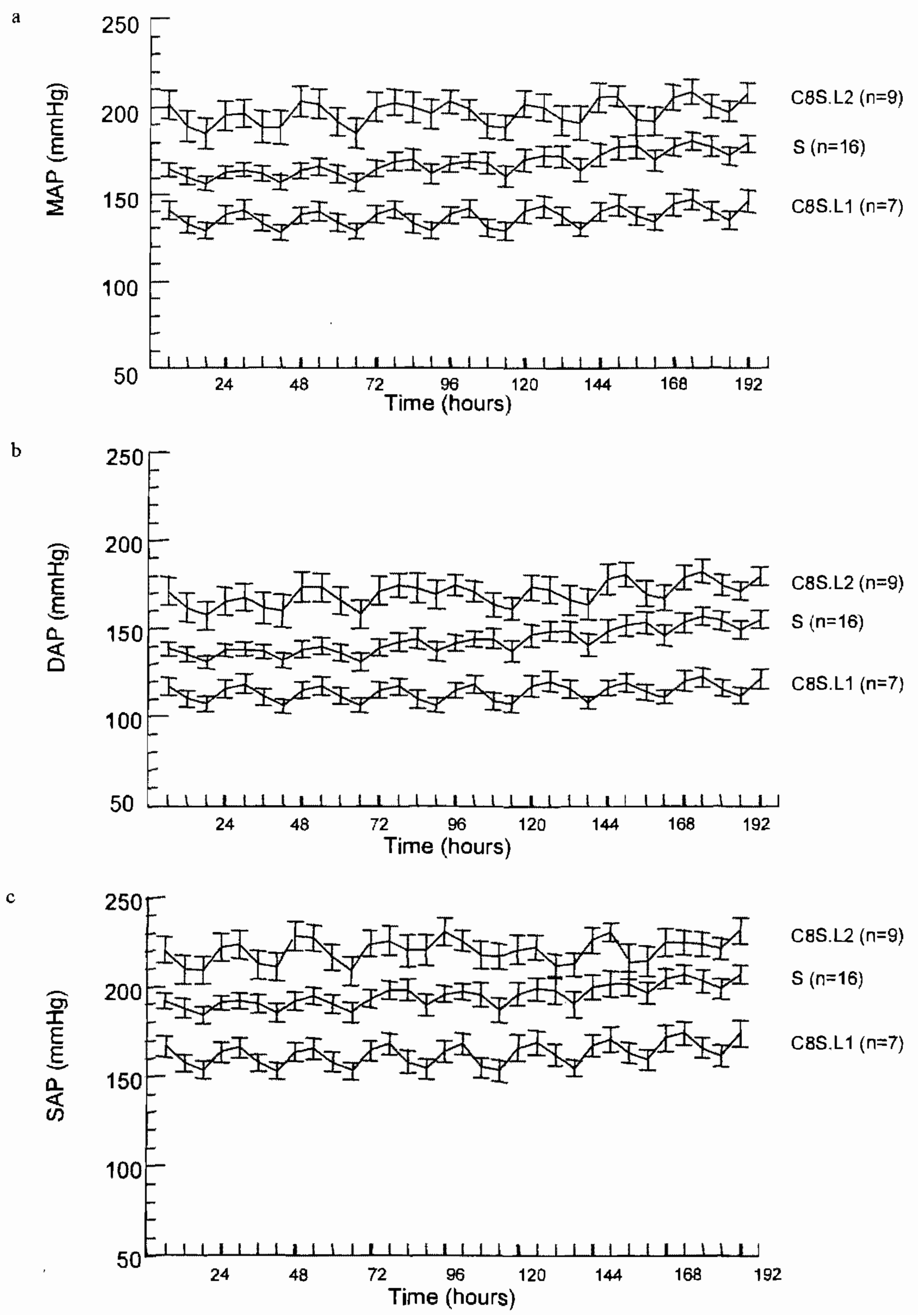
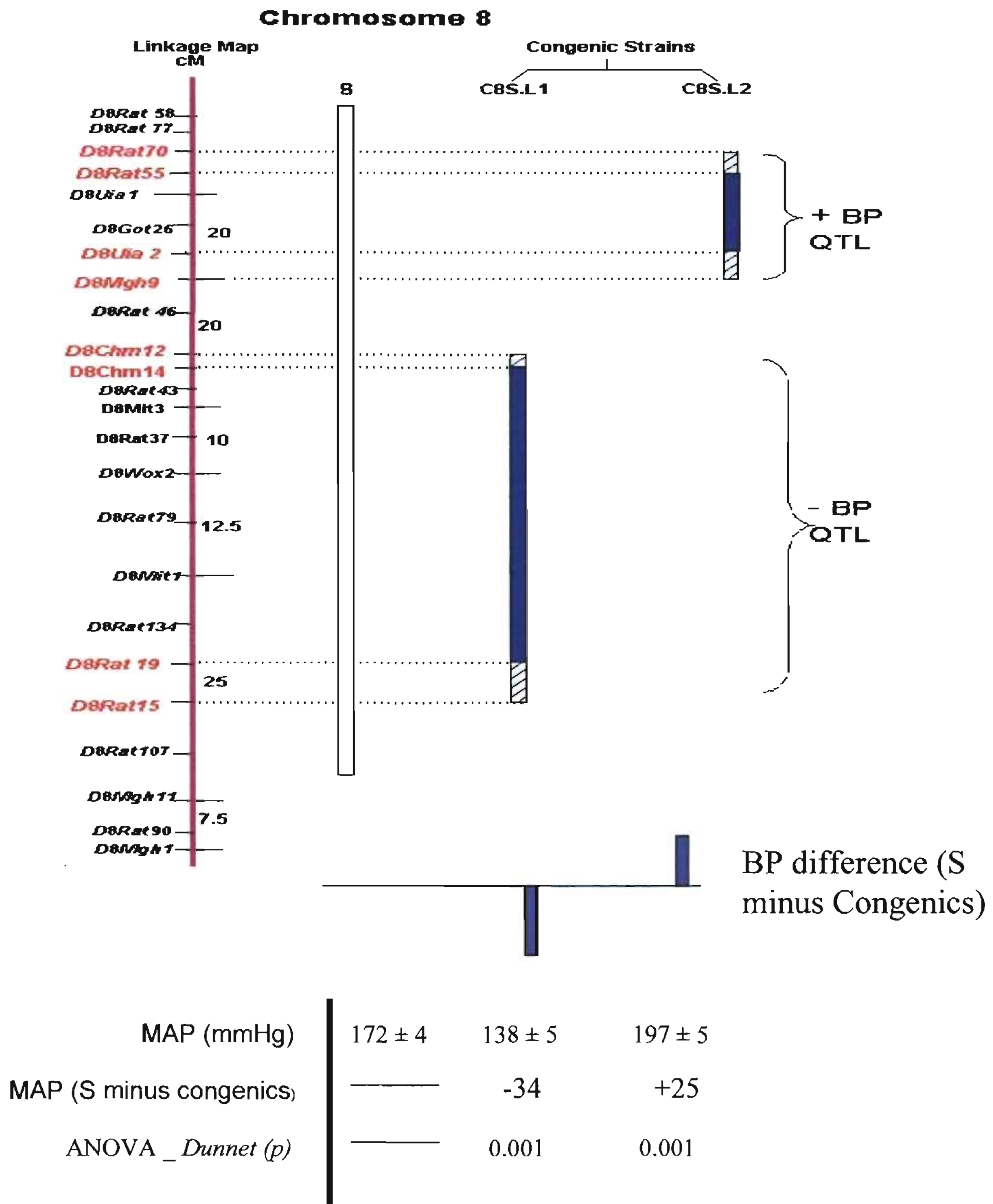


**Figure 22 - Summary including Statistical Analysis**



# Chapter 5

# Discussion

## 5.1 The Presence of Two QTLs with Opposing Effects

As described in Chapter 4, we bred two congenic strains of rats from normotensive LEW rats and hypertensive Dahl salt-sensitive S rats. In both of these, alleles from the LEW rats were substituted into the S background (since the LEW background is non-permissive (for further discussion refer to section 5.4), substitution into the LEW background would be pointless(17)). We called the congenic strains C8S.L1 and C8S.L2. A BP QTL was mapped to chromosome 8 for each strain. For the C8S.L1 strain the QTL is located between genetic markers D8Chm12 and D8Chm15, including ambiguous regions at both ends of the segment, while for the C8S.L2 strain it is located between D8Rat55 and D8Rat51, again including ambiguous regions at the ends (see Figures 20 and 22). The two QTLs are separated by 196 cR (centiRays).

The discovery that there are at least two distinct BP QTLs located near each other on chromosome 8 of the S rat is the first main result of this study.

The second result is that the two QTLs have opposite effects on BP. The C8S.L1 rat had lower BP than the S rat, while the C8S.L2 rat, surprisingly, had higher BP than the S rat (see Figure 22). Hence we have designated the two QTLs as the negative BP C8QTL and the positive BP C8QTL, respectively. In the latter region, the fact that the LEW alleles increased BP implies that the homologous S alleles would normally lower BP. In previous studies on chromosome 8, only a BP lowering effect has been reported. It was only when the two regions were separated by congenics that the opposing effects were observed.

In a previous linkage study (144),no QTL was detected in the region where we have found the positive BP C8QTL, but only a weak linkage for the negative BP C8QTL. It is only when congenic strains were developed to cover different sub-sections that two QTLs with opposite effects were discovered.

It is interesting that the C8S.L2 strain, created through selection, exhibited hypertension more severe than that of the S strain. This hyper-hypertensive strain had BP

raising alleles originating from the normotensive LEW strain. This result is surprising, because though many congenic strains have been reported by physical mapping of BP QTLs in S rats, most of the BP raising alleles have been associated with the hypertensive S rats, whereas the contrasting normotensive strains generally carried BP lowering alleles.

### 5.1.1 Epistasis

Epistasis is the effect of one gene or locus masking or modifying the effect of another. It is the relationship between two genes whose products act in the same biochemical pathway and in which the functional effect of one gene is masked by another. The masked gene is called a hypostatic gene and operates later on the pathway than the masking gene, which is called an epistatic gene. A hypostatic gene is unable to produce its usual effect when coupled with another gene that is epistatic toward it. So, for example, recessive alleles at one locus may override expression of alleles at another locus. In this case, the alleles at the first locus are said to be epistatic to the masked, hypostatic alleles at the second locus. When an allele or alleles at one locus require the presence of specific alleles at another locus, these pairs are said to complement each other; this is another example of epistasis. An additive relationship between two QTLs occurs when the sum of the BP effects of each QTL taken individually is equal to the BP effect of a congenic strain containing both QTLs. In contrast, an epistatic relationship occurs when the individual QTL BP effects are not equal to that of a congenic strain containing both QTLs resulting in synergism.

The BP of the C8S.L2 strain was higher than that of the S strain, due to the presence of alleles from the normotensive LEW strain. The result implies that alleles at other locations in the LEW strain interact with the positive BP C8QTL to prevent such a rise in BP, and that these alleles are not present in the S strain. It is possible that, in creating the C8S.L.2 strain, the genetic background of S rats was incapable of buffering the BP change that resulted.

Epistasis has been shown to exist in previous BP congenic studies, involving chromosome 3 (134) and chromosome 2(18). Since the positive BP C8QTL was not detected in the earlier linkage study of the F2 population, it is possible that the effect of the positive BP C8QTL might have been masked by other BP QTLs and only emerged after the latter were removed. Such a masking effect would indicate epistasis. Again, when moving the BP-raising LEW alleles into the S genetic background, the BP-raising alleles may have

found a complement to act in synergism with, in the S genetic background, resulting in the hyper-hypertensive phenotype.

In contrast to the positive BP C8QTL, LEW rats carry BP-lowering alleles at the negative BP C8QTL. In a previous linkage study (59), on which the present study is based, only a weak BP lowering effect was observed. In that study, the BP lowering effect reached a suggestive statistical level of significance. This could indicate that in the previous study the alleles located in the regions delimited by the two BP QTLs interacted with each other, resulting in an overall mild phenotype that lowered the BP in the F2 generation.

Since the earlier linkage study was only considering the F2 generation, which was heterozygous for the region in question, it is also possible that the effects on BP caused by the L alleles could have been masked. This masking could take the form of a biochemical product that is produced by the S alleles, but not by the LEW alleles. When the genotype is heterozygous SL, the presumed biochemical product that raises BP is produced by S, would compensate for the lack of the biochemical product not being produced by L alleles to a certain degree. Whereas, when the region is homozygous LL, there is no compensation, hence BP is reduced. Perhaps the effects of the negative BP C8QTL dominate those of the positive C8 BPQTL. The present work illustrates the value of dissecting suspected QTL fragments using congenic strains.

### 5.1.2 Overall Phenotype versus Individual QTLs

It seems surprising, as we have noted, that the normotensive LEW rats appear to carry BP-elevating alleles. When the QTL region containing LEW alleles was compared with the S alleles in the same region, it was found that the LEW rat raised BP higher than the hypertensive S rat.

This is not an extraordinary finding since we have seen that BP raising QTLs and BP lowering QTLs can be found within the same strain. Thus it appears that both the net normotensive LEW phenotype and the net hypertensive S phenotype may result from interactions between BP raising and BP lowering QTLs. Both LEW and S strains are inbred, and during their selective breeding various alleles could have become fixed randomly, a BP raising allele for that QTL could have become fixed in the LEW rat, while at the homologous region in the S rat, a BP lowering allele for that QTL could have become

fixed. It is only when the regions are isolated, as in a congenic strain that the effect of the individual QTL is observed.

Thus the hypertensive nature of the S strain could be explained by the fact that it has many more BP raising alleles compared to BP lowering alleles, while conversely the normotensive LEW strain could have a numerical advantage in terms of BP-lowering alleles. In both parental inbred strains only the overall phenotype is seen.

Similarly, it is possible that more than one BP QTL is located within each of the QTL segments defined by C8S.L1 and C8S.L2 congenic strains. Further subdivision of the concerned regions might bring to light the existence of smaller QTLs within the larger regions, as well as possible epistatic effects.

## *5.2 Physiological Analysis -Measuring BP*

Genetic studies can present evidence of genes implicated in hypertension, but they cannot show the mechanism by which hypertension is physically caused. Therefore, genetic studies cannot replace physiological studies; instead, each must complement the other. The measurement of arterial pressure, whether it be systolic, diastolic, or mean pressure, permits the direct association between a physiological, quantitative aspect of hypertension and a genetically delimited region on a chromosome. The three principal methods of measuring arterial pressure in rats are by tail cuff, by telemetry and by indwelling catheter(31). Each process has its own advantages and disadvantages (summarized in Table 8(31)).

### 5.2.1 Tail Cuff

In the tail cuff method of BP measurement, the rats are preheated in a chamber at 35°C for 10 minutes, then placed in plastic restrainers. A cuff with a pneumatic pulse sensor is attached to the tail and BP is then measured. The tail cuff method is best method of initial general targeting of BP differences in rats. It is a broadly applicable method to determine whether susceptible chromosomes contain a QTL for BP. However, the measurement is indirect, and the requirement for animal heating and restraint may cause stress-induced changes in BP. It is also unreliable for measuring DAP and MAP (53,82,101,202).

### 5.2.2 Telemetry

In telemetry, a radiotelemetric monitoring system continuously measures cardiovascular parameters in freely moving rats. When direct observation is impossible, telemetry allows animals to be monitored with minimum restraint and allows for reproduction of the transmitted data. It allows for monitoring physiological variables such as BP by providing a wireless link between the subject and the data collection equipment. The transmitting unit (probe) is surgically implanted in the abdomen, allowing for the subjects to be freely moving and conscious. If animals are tethered, they are prone to exit-site infections. Once the telemetry probe is implanted, data can be monitored 24 hours per day without human intervention or contact. Telemetry, being a more direct method than the tail cuff, is best for monitoring small changes in BP, such as diurnal and seasonal variations, as well as additive effects. It allows for a wide spectrum of environmental, physiological and behavioural data to be monitored.

### 5.2.3 Indwelling Catheter

In order to implant a rat with an indwelling catheter, it is first anaesthetized with drugs that cause hypotension and reduce HR. It then undergoes cannulation of the right carotid artery. The catheter is connected to a pressure transducer. BP measurements using the indwelling catheter allow for direct and accurate for measurement of SAP, DAP and MAP. Like telemetry, the catheter permits continuous, long-term monitoring. It is more economical than telemetry. Similarly to telemetry, it is invasive and may result in stress due to the necessary surgery to insert the catheter. There is also a high risk of infection resulting from the inserted catheter. Although BP can easily be measured in anesthetized rats in this way, repeated measurements taken over long periods of time in awake rats are much more difficult to make using this method.

**Table 8 -Advantages and disadvantages in the three principle methods for measuring BP in the rats**

| Method | Advantages | Disadvantages |
|---|---|---|
| Tail Cuff | - Non invasive<br>- Inexpensive<br>- Rapid for measuring SAP | -Low accuracy<br>-Stress imposed by immobilization<br>-Indirect measurement of BP<br>-Unreliable measure of DAP and MAP<br>- Vasoconstriction<br>- Cannot monitor for long-term<br>- Induce warmth in the experimental animal<br>- Changes in BP of less than 10 mmHg hard to detect |
| Telemetry | - Accurate for measuring SAP, DAP and MAP<br>- Direct measurement of BP<br>- Allows for long-term chronological monitoring | -Expensive in terms of equipment, monitoring and maintenance of equipment<br>-Invasive<br>-stress due to surgery |
| Indwelling Catheter | - Accurate for measuring SAP, DAP and MAP<br>- Direct measurement of BP<br>- Permits continuous, long-term monitoring<br>- Economical compared to telemetry | -Invasive<br>- Infections could arise |

Table 8 is based on Deng 1998 (31).

## *5.3 Gene-Environment Interaction*

Previous linkage studies (59)seem to have overlooked the strong positive BP raising effect we have observed in our congenic study. One possible source of this difference is the phenotyping protocol used. In the linkage study, phenotyping was done by tail cuff, while in the present work, it was done by telemetry since it is a less intrusive method of monitoring BP.

Another consideration is that in the linkage studies (59), the rats were fed an 8% NaCl diet, whereas in the present investigation, the rats were fed a 2% NaCl diet. The reason for using these differing diet protocols is as follows. It has been shown that LEW rats are extremely salt resistant, even on an 8% NaCl diet, while this diet is toxic to S rats (59). The rats in the F2 population of the linkage study contained more than 25% of the LEW genome, so that the F2 cohorts could survive on an 8% diet until the end of BP measurements (59). The S rat, which was the genetic background for the congenic strains, contained none of the LEW genome. A detailed survival study of S rats by Cicila et al. (22,24), showed that on a 2% NaCl diet for 42 days, S males were still alive, whereas on a 4% NaCl diet the mean survival time was only about 39.8 days. It was reasoned that S rats

would not survive on an 8% NaCl diet for the duration of our experiment, that is, 50-58 days starting from the onset of the salt-containing diet. For this reason, the S rats and congenic rats were fed a 2% salt diet uniformly to make sure that they would survive long enough to have their BPs measured (23,31,32,36,40,44,127,134) .The choice of the 2% NaCl diet in the present study is a compromise between survival of the animals and a sufficient life span to measure BP adequately.

The presence of a positive BP C8QTL in our LEW normotensive strain indicates that LEW does carry BP-raising alleles, despite its generally low BP. When placed into an appropriate genetic background, these BP raising alleles seem to be sufficient to increase the BP of the S strain.

Interestingly, in contrast to S rats, which normally live for 20–30 days under our experimental conditions, most of the C8S.L2 rats died by day 8 of measurements, probably due to fatally high BP. This result implies that in the genetic background of S rats there is an upper limit for BP in response to a high salt diet and C8S.L2 is close to reaching it. We now discuss genetic background effects.

## 5.4 The Genetic Background and its Effects

The genetic background effect is the influence of the genome as a whole on the materialization of the phenotype. The manner in which the genes located in the background interact with the genes in the targeted chromosomal region, together with the net effect of those genes, determines what the observable phenotype will be.

A strain is said to have a permissive genetic background when the net effect of the genes outside the targeted region does not overshadow the manifestation of the phenotype. If the effect of the genetic background overshadows the effect of the alleles located in the targeted region, the genetic background is non-permissive. A strain with a permissive genetic background will have variation in BP when compared to the parental strains. If the background is non-permissive, then no observable change in BP should be seen when compared to the parental strain.

One way to verify the effect of the genetic background of a strain is to conduct a test of reciprocity, which permits the isolation of the effect of contrasting alleles of a QTL on phenotype. In a test of reciprocity for strains A and B, say, one congenic strain is produced in which a region of A is transferred to the B background, while a second congenic strain is produced transferring the homologous region of B to the A background.

If a difference in phenotype is detected in the second congenic strain and not in the first, it is said that A is a permissive genetic background while B is non-permissive.

In this study, normotensive LEW alleles were introgressed into the S genetic background, which is seen to be permissive because the congenic strains show differences in BP compared to the LEW and S strains. Ongoing studies have shown, using congenics targeting other chromosomes together with a reciprocity test, that the LEW genetic background is non-permissive(17). Furthermore, in those studies, it is reasoned that because the BP of the S strain can be increased or decreased in congenic strains, the physiological capacity to buffer or modulate BP changes in the S rat has been weakened (17). For this reason, we did not develop any congenic strains using the LEW background.

Dahl salt resistant R rats, which are also normotensive, are the usual candidates for linkage and congenic BP analysis in conjunction with Dahl salt sensitive S rats. Both LEW rats and R rats have non-permissive backgrounds. But this is only true for the effects of particular chromosome sections from particular strains on a specific phenotype(148). Moreover, LEW rats are slightly more resistant to salt induced hypertension than R rats (59),even though R rats have been selectively bred for such resistance while LEW rats have not. However, LEW rats show approximately 45% of microsatellite markers polymorphic with S, but between R and S only 18% are polymorphic (83). The higher polymorphism rate between S and LEW is an advantage in a genome scan. This advantage is the reason we have used LEW rats rather than R rats as the normotensive control strain.

It is important to consider other potential confounding influences due to genetic background effects. These include epistasis, other influences of alleles outside the target regions, possible polygenetic effects, and effects due to difficulties in accurate mapping of the target region.

Ideally, if the genetic background has no influence on BP, the targeted QTL would be the cause of all variations in the congenic strain. However, there is a possibility that epistasis deriving from the genetic background might introduce masking or synergistic confounds into the results deriving from the congenic strains.

Furthermore, though our study tentatively interprets the variation of BP as a monogenic effect, it is possible that BP variation correlates to a group of several LEW alleles rather than to one specific allele.

Finally, congenic strains may produce misleading results due to the size of the chromosomal segment that is introgressed together with the gene of interest. (In our case,

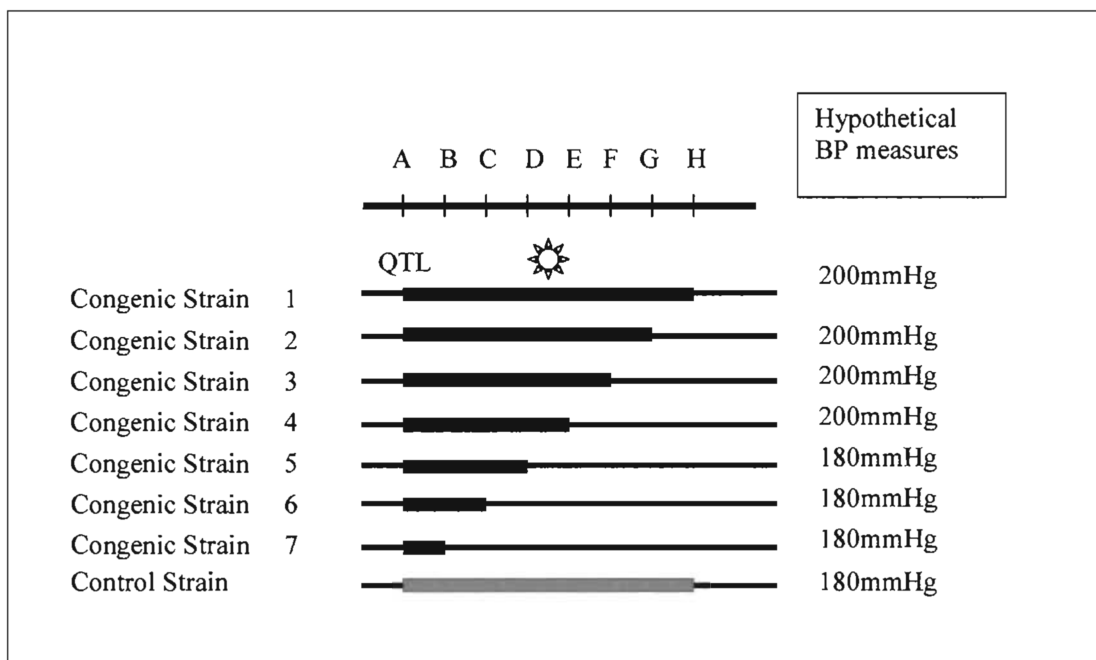
for example, after 10 backcrosses the fragment from the donor strain is still approximately 20 cM in length and contains approximately 1.3% of the genome.) If the trait analyzed is controlled by a large number of genes, as is the case, for example, for neural or behavioral processes, it can be difficult to distinguish between the effect of the gene of interest and the effects of genes located in the flanking chromosomal segment. One way to minimize this effect is to derive a series of partially overlapping congenic strains (Figure 23). If these strains are derived independently, the residual segments from the donor strain are different and the genes that are not tightly linked to the mutation or allele are not present in all strains. By comparing the phenotypes of the different congenic strains, it is possible to distinguish between the effects of the mutation or allele itself and the effects of the flanking genes.

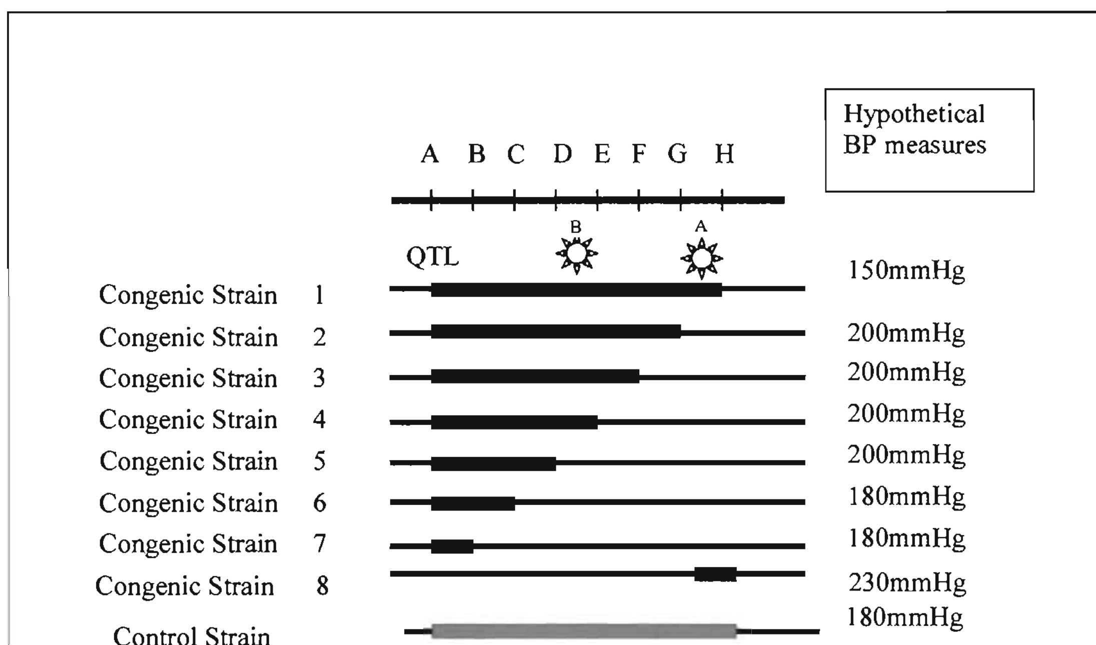
Despite these limitations, congenic strains are invaluable genetic tools for several reasons. First, a given phenotype can be studied in different genetic backgrounds. Second, congenic strains allow confirmation of results identified by linkage analysis. They are also helpful for study of the pathophysiologic features associated with a given genomic region. Because the congenic strain differs from the recipient strain by a small chromosomal segment selected during the backcross generations, any phenotypic differences observed between these two strains can be attributed to this segment, under limitations described above. Congenic strains are powerful tools to refine the location of a QTL, by generation of "subcongenic" strains carrying smaller, partially overlapping chromosomal segments (Figure 23). This strategy can narrow the candidate interval to <1 cM, a resolution that cannot be approached by linkage studies. An additional advantage of congenic strains is their usefulness for the detection of loci with minor phenotypic effects. After a major modifier locus has been mapped and introgressed in a congenic strain, a two-generation cross between this congenic strain and the donor strain can be generated. Because the major modifier locus is not polymorphic in the cross, its major effect no longer hides the more modest effects of other loci. Finally, congenic strains are ideal for testing epistatic interactions between modifier loci, because homogenous populations can be produced for each genotypic combination and their phenotypes can be compared. Although a single congenic strain might not always be sufficient to yield clear conclusions, multiple congenic strains are ideal tools for the analysis of interactions between the genetic background and genes responsible for Mendelian traits.

The fact that background genes can influence the phenotype induced by a single

gene mutation or allele should not be surprising. Probably few genes work in such a way that other genes cannot modulate the effects of altering them. Metabolic pathways, developmental processes, and the variation in levels of gene expression all result from protein-protein and protein-DNA interactions. The modification of any component of these networks may influence the other components.

In other words, a phenotype is context dependent. A mutation induces a phenotype in a particular genetic context, dependent on factors such as environment, experimental conditions, and genetic background. Since genetic mapping is a tool for associating physiological traits to the genome, it is context dependent as well, since genetic background effects have a significant impact on the traits that can be mapped. Before making general interpretations of physiological measurements, it is important to remember that the genetic background can significantly affect not only baseline measurements but also the response to, say, gender-related or environmental stimuli (103).




Deducing from the hypothetical situations above: In the first situation if there is one QTL, the phenotypic effect and location of the QTL can be deduced from the subcongenic lines. In the second situation: If the control strain had a BP of 180, QTL A increases BP when isolated, while QTL B not only decreases BP, but masks the effect of QTL A. Somehow QTL A and B are interacting to decrease BP more than when QTL B is acting by itself.

**Figure 23 - Deducing the Effect QTLs Using Congenics**

## 5.5 Other Limitations and Potential Sources of Error

Our study is subject to certain limitations and potential sources of error, due to decisions we had to make in order to focus the results. These include experimental measurement conditions, cross-specificity, age and gender-specific selection.

First of all, the conditions under which BP is measured during breeding are important. Many environmental factors can affect BP, e.g., the stress of being restrained, or dietary factors such as potassium intake. Therefore it is crucial to control the experimental environment and to treat the animals uniformly.

Secondly, linkage analysis and analysis using congenic strains are both limited in that they use a segregated population resulting from the backcrossing of two unique inbred strains. Therefore the results could be cross specific, due to the effect of the genetic background of the recipient strains on the expression alleles of QTLs of the donor strain. One must therefore mate individuals using a uniform selection process, in order to avoid fixing different alleles and confounding the genetic background. This is another argument for uniform treatment of the animals.

In fact, the detection of a QTL depends strongly on the identity of the two strains bred together to obtain the segregated population. The present study is limited to the comparison of the Dahl S strain and the Lewis strain. To bypass this limitation, one alternative is to study other contrasting strains, e.g., the SHR strain and the Milan normotensive strain.

Another limiting factor is the age of the animals. Most studies are done on adult animals, since BP increases with age and during development (145) . It has been pointed out that certain genetic effects on BP may be due to components that can only be observed during birth and development (163,187). At various stages in the lifespan of the rat, different genes come into play, all of which could have various effects on BP. Moreover, monitoring BP throughout a rat's lifespan is very costly. For all these reasons, the aspect of BP variation in development was ignored. Our analysis is limited to animals aged from 8 to 12 weeks.

Finally, we have studied only male rats. In terms of hypertension, specifically BP, there are differences between the sexes. Male rats develop hypertension faster than females(4). In a female rat in estrus, BP varies cyclically according to hormonal influences

(145). The variation in BP due to hormonal changes might result in background interference that would have complicated conclusions drawn from the results. If this study had been conducted on both sexes, it would have complicated the experimental design. It should be understood that conclusions drawn from this study should be restricted to males.

## *5.6 The QTL Regions*

### 5.6.1 Reproducibility of the Negative BP QTL

Comparing the results of the present study to those of studies conducted in other laboratories (59,94,98,169,190), we see that other investigators have found BP C8QTLs in regions near those where ours are located (Figure 24). It is possible that the QTLs we have found could be responsible, in part, for the hypertension seen by other researchers.

The data published by Garrett et al. (59) was the base for the development of the current congenic rats in this study. In Garrett's study, the region found is smaller and only a weakly suggestive linkage LOD was reached where BP was lowered mildly. This finding might indicate the presence of other QTLs involved in the phenotype of the negative C8 BP QTL.

Even though Kren et al.(98) and Khan et al. (94) both used congenic strains in their studies, only a negative BP QTL was found. In Kren's study this QTL exerted major effects on BP and cardiac mass in the SHR. Khan's study found reduced BP in a C8 region that contains a gene cluster for three nicotinic receptor subunits expressed in the nervous system. In the study, this cluster was implicated in nociceptive and BP hyperresponsiveness. Finally, our negative BP C8QTL falls within the same region as the BP lowering QTL found by Ueno et al. (190) in pharmacogenetic study in which the SNS was blocked.

In a linkage study conducted by Takami et al. (184) there was a hypotensive effect on C8 at the peroxisomal 3-keto acyl CoA locus in the F2 progeny obtained by crossing of Iwai salt-sensitive rats and Wistar-Kyoto rats. In a genome scan conducted by Schork et al. (169) using F2 progeny obtained from a cross between Brown-Norway and SHR rats, a BP increase was found when salt-loaded SBP was measured. Both of these regions coincide with regions we isolated in both the negative and the positive BP C8 QTL.

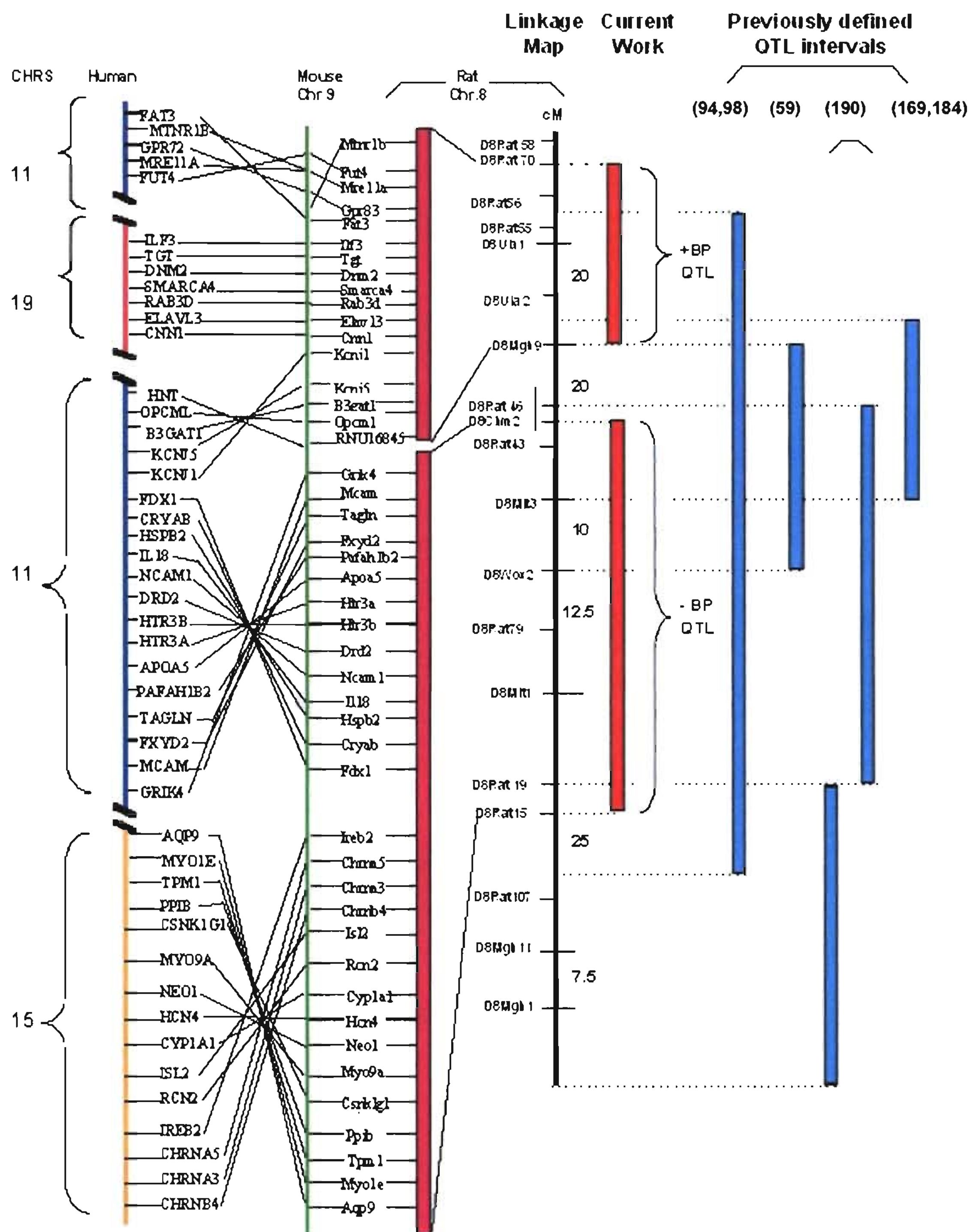



Figure 24 - Localizations of two quantitative trait loci (QTLs) and comparative mapping

Figure 24 is a comparative map of homologous regions found in the mouse and human. The rat linkage map shown is the same as in Figures 19 and 22. The positions of the QTLs are those defined by the congenic strains shown in Figure 19. Genes indicated on Figure 24 in the homologous regions on rat chromosome 8, mouse chromosome 9 and human chromosomes 11, 15 and 19 are based on information found in the NCBI database located at http://www.ncbi.nlm.nih.gov/mapview/. The figure lists most of known genes for the positive BP QTL region. Among over 80 genes found in the negative BP QTL region, only selected genes are listed based on their potential roles in BP regulation. QTL localizations from the present work and others are shown to the right of the linkage map for comparison. Genes are as follows: APOA5, apolipoprotein A-V; AQP9, aquaporin 9; B3GAT1, beta-1, 3-glucuronyltransferase 1 (glucuronosyltransferase P); CHRNA3, cholinergic receptor, nicotinic, alpha polypeptide 3; CHRNA5, cholinergic receptor, nicotinic, alpha polypeptide 5; CHRNB4, cholinergic receptor, nicotinic, beta polypeptide 4; CNN1, calponin 1, basic, smooth muscle; CRYAB, crystallin, alpha B; CSNK1G1, casein kinase 1, gamma 1; DNM2, dynamine 2; DRD2, dopamine receptor D2; ELAVL3, ELAV (embryonic lethal, abnormal vision, Drosophila)-like 3 (Hu antigen C); FAT3, fat3 protein; FDX1, ferredoxin 1; FUT4, fucosyltransferase 4 (alpha (1, 3) fucosyltransferase, myeloidspecific); FXYD2, FXYD domain containing ion transport regulator 2; GPR72, G protein-coupled receptor 72; Gpr83, G protein-coupled receptor 83 (glucocorticoid-induced receptor); GRIK4, glutamate receptor, ionotropic, kainate 4; HCN4, hyperpolarization activated cyclic nucleotide-gated potassium channel 4; HNT, neurotrimin; HSPB2, heat shock 27 kDa protein 2; HTR3A, 5-hydroxytryptamine (serotonin) receptor 3A; HTR3B, 5-hydroxytryptamine (serotonin) receptor 3B; IL18, interleukin 18 (interferon-gamma-inducing factor); ILF3, interleukin enhancer binding factor 3, 90 kDa; IREB2, iron-responsive element binding protein 2; Isl2, insulin related protein 2; ISL2, ISL2 transcription factor, LIM/homeodomain, (islet-2); KCNJ5, ATP-sensitive, inwardly rectifying potassium channel of the subfamily J; MCAM, l-gicerin, melanoma cell adhesion molecule; MRE11A, MRE11 meiotic recombination 11 homolog A (S. cerevisiae); MTNR1B, melatonin receptor 1B; MYO1E, myosin IE; MYO9A, myosin IXA; NCAM1, neural cell adhesion molecule 1; NEO1, neogenin; OPCML, opioid binding protein/ cell adhesion molecule-like; PAFAH1B2, platelet-activating factor acetylhydrolase, isoform Ib, beta subunit 30 kDa; PPIB, peptidylprolyl isomerase B (cyclophilin B); RAB3D, RAB3D, member RAS oncogene family, RCN2, reticulocalbin 2, EF-hand calcium binding domain; RNU16845, neurotrimin; SMARCA4, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4; TAGLN, Transgelin (smooth muscle 22 protein); TGT, tRNA-guanine transglycosylase; TPM1, tropomyosin 1, alpha.

## 5.6.2 Comparative Mapping and Potential Candidate Genes

The region containing the negative BP C8QTL in the rat shares regions of conserved synteny with a segment of mouse chromosome 9, and segments of human chromosomes 11 and 15. The region harboring the positive BP QTL is homologous to a fragment of mouse chromosome 9, and to sections of human chromosomes 11 and 19 (http://www.ncbi.nlm.nih.gov/mapview/). Since the general purpose of all animal hypertension studies is to gain more information in order to understand how human BP loci are implicated in BP control, it is important to do comparative mapping to find homologous genes in both mouse and human. Reproducible rat and mouse QTLs present good candidates for comparative mapping in human hypertension. Candidate genes in BP regulation include genes coding for or involved in the following: adrenergic receptors, ion channels or transporters, proteins regulation or involved in catecholamine biosynthesis, metabolism or transport, nicotinic cholinergic responses, the NO system including nitric oxide synthases, various peptides and receptors, prostacyclin; RAAS system, serotonergic responses, signal transduction, various oxygenases, proteases and nuclear hormone receptors.

We now describe selected genes that fall within the regions delimited by the BP QTLs we have located, which have been described in other studies as candidates for BP regulation.

### 5.6.2.1 Ion Channels

Ion channels are involved in determining the movement of ions into and out of cells, and so are crucial for the electrical activity of nerves, muscles and sensory organs and are central to homeostasis. They play an important role in regulating BP. It is believed that defects in ion channel proteins may underlie some forms of hereditary hypertension, thereby making ion channels attractive targets for study (8).

#### *5.6.2.1.1 Potassium Channel (Kcnj5)*

The gene Kcnj5 falls in the positive BP QTL region. Kcnj5 encodes for an ATP-sensitive, inwardly rectifying potassium channel of the subfamily J. Potassium channels are present in most mammalian cells, where they participate in a wide range of physiologic responses. The protein encoded by this gene is an integral membrane protein. The encoded

protein, which has a tendency to allow potassium to flow into rather than out a cell, is controlled by G-proteins. It may associate with two other G-protein-activated potassium channels to form a heteromultimeric pore-forming complex (96). Kcnj5 is expressed in heart tissue. Knockouts of this gene have suggested that Kcnj5 is involved in vagal regulation of heart rate as well as spatial memory.

#### *5.6.2.1.2 FXYD2 Protein-Sodium Potassium Pump Activity Modifier*

There is a gene coding for the FXYD2 protein in the negative BP QTL region. This protein modulates the activity of the sodium-potassium pump. The sodium-potassium ATPase or Na/K pump belongs to the protein family of P-type ATPases that are characterized by the formation of a phosphorylated intermediate during the catalytic cycle. By using the energy of hydrolysis of ATP, Na-K-ATPase located in the plasma membrane, transports three $Na^+$ ions out of the cell in exchange for moving two $K^+$ ions into the cell.

Na-K-ATPase is an oligomeric protein. Its catalytic subunit, having ten transmembrane segments, transports the cations, hydrolyzes ATP, and is the pharmacological receptor for cardiac glycosides. Na-K-ATPase is the transport system responsible for the maintenance of the $Na^+$ and $K^+$ gradients across the plasma membrane. These gradients are essential to maintain cell volume and membrane potential, and the $Na^+$ gradients provide the energy for the activity of secondary $Na^+$ dependent transport systems that supply the cell with nutrients and regulate intracellular pH and $Ca^{2+}$ concentrations.

Na-K-ATPase is present in all cells to ensure basic cellular homeostasis, but it also contributes to specialized tissue functions. In the kidney, Na-K-ATPase is exclusively located in the basolateral membrane of epithelial cells, and thus becomes the driving force for $Na^+$ reabsorption essential to maintain extracellular volume and BP. Numerous mechanisms are involved in the regulation of the Na-K-ATPase to adapt its activity or expression to accommodate changing physiological demands. Because intracellular $Na^+$ is the limiting factor for Na-KATPase activity, any change in the intracellular $Na^+$ concentration affects its transport rate. Moreover, peptide hormones or neurotransmitters may provoke phosphorylation of Na-KATPase by protein kinases, which modulate its cell surface expression. Finally, steroid hormones such as aldosterone affect gene transcription, leading to an increased number of Na-K-ATPase pump units.

In addition to regulatory effects mediated by hormones and neurotransmitters, there is a novel regulatory mechanism that involves interaction of Na-K-ATPase with small-membrane proteins of the FXYD family. In contrast to hormonal regulation, interaction of FXYD proteins does not produce a change in Na-K-ATPase expression but rather modifies the transport properties of the Na-K-ATPase in a tissue- and isoform-specific way. The gene family of FXYD proteins is based on the invariant amino acids in a signature sequence containing the FXYD motif together with two conserved glycine and a serine residues. In mammals, this family contains seven members. FXYD proteins contain 61 to 95 amino acids.

There is a FXYD2 gene in the region delimited by the negative BP QTL. FXYD2 is the γ-subunit of Na-K-ATPase. It is a type I membrane protein with a single membrane span and the COOH terminus exposed to the cytosol, having no signal peptide. The transmembrane domains of FXYD2 adopt a α-helical conformation. FXYD2 is associated with renal Na-K-ATPase and it modulates Na-K-ATPase activity, to produce a functional effect on its transport properties. FXYD2 is predominantly expressed in the kidney, with segment specific distribution of FXYD2a and FXYD2b . FXYD2a and FXYD2b colocalize with Na-KATPase in the basolateral membrane of renal epithelial cells. FXYD2 has been shown to increase the apparent $K^+$ affinity of Na-KATPase at high negative membrane potentials in both the presence and absence of extracellular $Na^+$. FXYD2 decreases the apparent $K^+$ affinity at less negative membrane potentials but only in the presence of extracellular $Na^+$. FXYD2 also increases the affinity for ATP. FXYD2 increases the $K^+$ antagonism of intracellular $Na^+$ binding, suggesting an additional effect of FXYD2 on intrinsic binding of $K^+$ at cytoplasmic sites. Finally, FXYD2 decreases the $Na^+$ activation of Na/K pump currents and produces a parallel decrease in the $Na^+$ and $K^+$ activation of Na-KATPase activity. It is speculated that in the kidney, FXYD2, which decreases the $Na^+$ affinity of Na-K-ATPase, favors the existence of low-$Na^+$ affinity Na-K-ATPase in the kidney, which may in turn be favorable for an efficient reabsorption of $Na^+$ in renal segments with high cellular $Na^+$ load (68).

### 5.6.2.2 Cytochrome P450 Enzyme CYP1A1

CYP1A1 is a member of a large class of cytochrome P450 enzymes. Human CYP1A1 is expressed extrahepatically, and contributes to the detoxification of a variety of

xenobiotics, such as the conversion of polycyclic aromatic hydrocarbons or aromatic amines that are constituents of tobacco smoke. This enzyme transforms arachidonic acid and eicosapentaenoic acid into vasodilator compounds.

Induction of a CYP1A1 by various environmental products, including cigarette, smoke is regulated by the aromatic hydrocarbon receptor (AHR). A polymorphism of the gene CYP1A1 (T3801C polymorphism), which is associated with higher CYP1A1 inducibility and enhanced catalytic activity, has been linked to stroke and triple vessel disease, and may, thus, be associated with BP. In addition, genetic deletion of AHR in mice can lead to cardiac hypertrophy and elevated arterial BP by five months. Based on the known relationship between CYP1A1 T3801C and t AHR G1661A polymorphisms and the cigarette-smoking-related risk of cardiovascular disease, it has been speculated that a dysregulation of CYP1A1 expression, due to AHR G1661A polymorphism or CYP1A1 T3801C polymorphism or both, would lead to significant changes in vascular homeostasis and thus BP(57,58,165).

Men carrying the less frequent CYP1A1 allele (TC and CC genotype) tend to have higher SBP and DBP than homozygous carriers of the more frequent allele (TT genotype). It has been demonstrated that AHR-null mice develop an elevation in arterial BP as early as two months of age, indicating that AHR contributes to BP regulation. AHR-null mice also develop cardiac hypertrophy associated with increased expression of vascular endothelial growth factor. AHR is transcriptionally activated by cigarette smoke condensates. Human carriers of both rare CYP1A1 and AHR alleles exhibited the highest SBP when they quit smoking or never smoked. This indicated to the researchers that AHR contributes to the regulation of BP via an interaction between the G1661A polymorphism and the CYP1A1 T3801C polymorphism(57,58,165).

It had been found that smooth muscle cells of AHR knockout mice respond in a very divergent way to classic inducers, when compared to cells of wild-type mice. As many as 107 genes have been seen to respond differently to inducers between wildtype and AHR cells, including transforming growth factor-beta and insulin-like growth factor-I. TGF-b and IGF-I signalling have been reported to contribute to the modulation of BP. Three other different CYPs, namely CYP2C9, CYP3A5 and CYP4A11, have been reported as candidate genes in hypertension, according to gender and ethnicity. Healthy control Chinese women have higher CYP2C9-3 allele frequency than hypertensive women. The CYP3A5-1 allele is more common in hypertensive African–American individuals, but this

allele appears not to contribute to BP in Caucasians. A loss of function variant of CYP4A11 (8590C allele) is associated with hypertension in Caucasian individuals(58).

### 5.6.2.3 Alpha B Crystallin

Alpha B-Crystallin is a small heat-shock protein which is constitutively expressed in various tissues, including the lens of the eye (9). It has been suggested that alpha B-crystallin provides lens transparency, but its function in nonlenticular tissues is unknown. It has been reported that alpha B-crystallin is involved in the stabilization and the regulation of parts of the cytoskeleton, such as intermediate filaments and actin. There is a possibility that alpha B-crystallin interacts with microtubules, which are a component of the cytoskeleton. One of the functions of alpha B-crystallin is to bind microtubules via microtubule associated proteins and to give the microtubules resistance to disassembly (56). It is also implicated in cardiac stress(80).

During ischemia, alpha B-crystallin has been shown by immunoelectron microscopy to translocate to the N(2)-line area of myofibrillar I-bands of rat cardiomyocytes where it resisted extraction with 1 m NaSCN and 2 m urea, as did titin, which is also known as connectin (71). Titin is a protein involved in the contraction of striated muscle tissues. Titin connects the Z line to the M line in the sarcomere. It limits the range of motion of the sarcomere in tension, thus contributing to the passive stiffness of muscle. Variations in the sequence of titin between different types of muscle (e.g. cardiac or skeletal) have been correlated with differences in the mechanical properties of the muscles (76). Binding of alpha B-crystallin to titin during cardiac ischemia could serve to stabilize titin against denaturation and might provide an endogenous mechanism to delay ischemic damage of this important elastic component of myofibrils (71).

### 5.6.2.4 Neuropeptide Y Family Member- GPR72

The neuropeptide Y gene family mediates important physiological effects on appetite, regulation of BP, reproduction and anxiety in humans through interaction with specific G-protein-coupled receptors including GPR72. According to the GenBank Database, GPR72 is related to a glucocorticoid-induced receptor found in the brain which may be involved in behavioral sensitization. GPR72 has been identified as the human homologue of the mouse orphan receptor GIR, showing 85% amino acid identity. GPR72 is

expressed in regions of the hypothalamus, hippocampus and amygdala in both rodents and humans (70). GPR72 was previously suggested to be a new NPY receptor, but experiments showed that it does not bind NPY-receptor ligands in standard binding assays.

### 5.6.2.5 Nicotinic Cholinergic Receptors

The genes of the cholinergic nicotinic receptor cluster, which are implicated in Khan et al. (94) as candidate genes in BP control and nociception, fall within the negative BP QTL region. In the congenic strains studied by Khan et al. and in the C8S.L1 strain of the current study, BP was lower than that of the S strain despite the differences in the parental strains. The neuronal nicotinic acetylcholine receptor subtypes α3, α5, and β4 are members of a ligand-gated ion channel family that affect sodium and potassium transients.

Cholinergic receptors can be divided into two types, muscarinic and nicotinic, based on the pharmacological action of various agonists and antagonists. Muscarinic receptors were originally distinguished from nicotinic receptors by the selectivity of the agonists muscarine and nicotine respectively. Nicotinic receptors produce pharmacologically and physiologically distinct responses from muscarinic receptors, although acetylcholine stimulates both types of response. Nicotinic responses are of fast onset, short duration and excitatory in nature. The pharmacology of nicotinic receptors has been studied in detail and our understanding of how ion channel-coupled neurotransmitter receptors work is based largely on the study of this class of proteins.

Nicotinic receptors are found in a variety of tissues, including the autonomic nervous system, the neuromuscular junction, and the brain in vertebrates. They are also common in the electric organs of various electric eels and rays. The large quantities of receptors in these tissues and the use of neurotoxins from snake venom that bind specifically to the nicotinic receptor have been used in the purification of the receptor protein. Agonists such as acetylcholine, carbamylcholine and nicotine produce the physiological responses associated with nicotinic cholinergic activation. Acetylcholine produces an influx of sodium through a ligand-gated ion channel.

The nicotinic receptor consists of five polypeptide subunits. The amino acid sequence for the α subunits consists of a glycolipid region with four hydrophobic regions that span the membrane. In the neuromuscular junction, d and g subunits also have been identified. The g subunit is replaced by an e subunit in the adult muscle. α-Bungarotoxin

binds to the a and b subunits and probably blocks both the receptor channel and the ACh binding site. Local anesthetics and other compounds such as phencyclidine bind to the receptor at the site of the sodium channel and modulate the binding of acetylcholine to the active site. Local anesthetics also prevent ion conductance through direct action on the channel. The nicotinic cholinergic receptor shares some properties with the sodium channel.

Acetylcholine binds to the a-subunits of the receptor, making the membrane more permeable to cations and causing a local depolarization. The local depolarization spreads to an action potential or leads to muscle contraction when summed with the action of other receptors. Nicotinic receptors possess a relatively low affinity for acetylcholine at rest. At high concentrations of acetylcholine, the affinity of the receptor for acetylcholine becomes higher and it subsequently becomes desensitized. The ion channel is open during the active state and local anesthetics may bind to it.

As discussed in section 2.3, there is a connection between nociception, BP and baroreceptors. Khan et al. (94) suggest that the cholinergic nicotinic receptors are candidates for nociception and hyper-responsiveness of BP. If our negative BP QTL is in fact the same as the one in Khan's study, then there is a possibility that BP variation due to salt sensitivity (high salt diet) is somehow connected to nociception, which in turn is associated to cholinergic nicotinic receptors and baroreceptors. Knockout mouse strains lacking the genes encoding the subtypes $\alpha 5$ and $\beta 4$ of the nicotinic receptors showed cardiac autonomic dysfunction, affecting the heart rate response to vagal electrical stimulation. In humans, although intragenic polymorphisms on the nicotinic receptor subunits have been identified (42,110,157), these gene mutations have not yet been associated with cardiovascular phenotypes. Perhaps there is a relationship between heart rate and BP, which may eventually be clarified by finding the specific alleles on rat chromosome 8 of C8S.L1 that cause hypotension.

### 5.6.2.6 Dopamine Receptor 2

The brain dopamine system is organized into four anatomically distinct pathways. Each of these pathways is associated with particular neural functions and disease states. Further separation and refinement of dopaminergic function is achieved, in part, via actions at different receptor subtypes. Dopamine mediates its neural effects via actions at both

presynaptic and postsynaptic dopamine receptors. Five separate dopamine receptor subtypes have been identified, all belonging to the transmembrane channel G-protein coupled receptor family.

The DRD1-like subfamily, comprised of DRD1 and DRD5 subtypes, is differentiated from the DRD2-like subfamily, which contains subtypes DRD2, DRD3 and DRD4. These subfamilies are distinguished from each other by their pharmacological and signaling properties. DRD1-like receptors stimulate signal transduction by coupling to Gs proteins and subsequent activation of adenylyl cyclase and cAMP production. DRD2-like receptors couple to Gi/o-like proteins and suppress signal transduction via inhibition of adenylyl cyclase and cAMP production as well as modulation of ion channels.

Intra-renally produced dopamine plays an important role in the regulation of renal sodium excretion. The dopamine-induced natriuretic mechanism is activated during high salt intake, and attenuated dopamine-induced natriuresis is thought to be involved in the mechanism of salt sensitivity in BP. Both DRD1-like and DRD2-like receptors are present in the kidney. Although it is well established that DRD1-like receptors mainly mediate dopamine-induced natriuresis, it is not clear what independent role DRD2-like receptors may perform in renal tubular and vascular functions.

Results of in vitro experiments have suggested that DRD2-like receptors are necessary to potentiate DRD1-like receptor-induced natriuresis in renal tubular cells. The dopamine receptors in renal tubules play a central role in the regulation of tubular sodium reabsorption.

DRD2 is an adrenergic receptor. DRD2 receptors are expressed in the renal tubules. It has not been determined whether DRD2 is involved in the mechanism of sodium handling and BP control. Ueda et al.(189) have investigated the function of DRD2 in mice with a disrupted DRD2 gene (DRD2 knockout mice). Six-week-old male DRD2 knockout mice and wild-type mice were fed high-salt or low-salt diets for 8 weeks. The high-salt diet caused a significant elevation in systolic BP in the DRD2 knockout mice but not in wild-type mice. There was a high level of sodium retention in DRD2 knockout mice placed on the high-salt diet. Administration of nemonapride, a specific DRD2-like receptor antagonist, to wild-type mice given 0.9% NaCl in drinking water caused suppression of urinary sodium excretion but had no effect in mice without salt loading. This effect suggested to the researchers that DRD2 receptors promote sodium excretion during a period of high salt intake and that a defect in this mechanism may result in sodium-dependent BP

elevation, since DRD2 knockout mice also show increased BP and susceptibility to sodium-induced hypertension.

In transgenic DRD2 knockout mice, it was found that DRD2 plays a significant role in sodium excretion in response to sodium loading. A defect in this system may be a cause of sodium-sensitive hypertension. Therefore, it is thought that the sodium retention in DRD2 knockout mice was caused by the loss of function of DRD2 in the renal tubules. It was found that mice without DRD2 displayed an impaired ability to excrete an excessive sodium intake, resulting in a significantly high level of sodium retention and sodium-related BP elevation. The DRD2 mediated modulation of sodium excretion had a significant impact on total body sodium balance specifically during high sodium but not low sodium intake. This indicated that DRD2 is involved in the mechanism for natriuretic response to sodium loading, and that DRD2 is one of the key factors that determine the sodium sensitivity of BP. The precise mechanism of this effect remains to be determined.

## *5.7 Proposals for Future Research*

### 5.7.1 QTL-QTL Interactions

It would be interesting to investigate how the BP QTLs found on chromosome 8 in the S rat relate to BP QTLs found on other chromosomes and how they interact. Methods involving linkage analysis, coupled with epistatic QTL mapping, which partition individuals of a F2 generation according to the genotypes at multiple loci and measures a phenotype, simultaneously, are available for a global genome search for epistatic interactions(15). They indicate possible regions that need further investigation. Since these types of studies are essentially linkage analyses, as they are based on probability, not on certainty, they must be verified by double congenic strains.

Double congenic strains are created when two different regions containing BP QTLs are transferred simultaneously to a permissive genetic background. Such strains could be used to assess how various BP QTLs interact with each other. Epistatic interactions between BP QTLs have already been found on chromosomes of the S rat using double congenics (31). This method allows one to describe the relationships between the different BP QTLs without knowing the specific physiological role that each gene plays.

#### 5.7.1.1 Double Congenics within the Same Chromosome

It will be interesting to examine if rat strains combining the two QTLs isolated in the current study would retain or lack the salt-sensitive nature of the S strain. Another question is whether such a combination would have higher or lower BP than S rats. By constructing a double congenic strain within the same chromosome, one could investigate these effects as well as possible epistatic effects due to the combination.

#### 5.7.1. 2 Double Congenics Using Different Chromosomal Segments

It would also be interesting to know whether and how the isolated BP QTLs in chromosome 8 interact with BP QTLs that have been isolated on other chromosomes. Will the effects of the BP QTLs on the different chromosomes cancel each other? Is one of the QTLs dominant over the other? Are there epistatic effects? These questions can be answered by constructing double congenic strains with chromosomal fragments from different chromosomes.

#### 5.7.1.3 Construction of Congenics Using Other Contrasting Strains.

This study could have missed some BP QTLs because the alleles for BP QTLs could be the same in both LEW and S rats. Since there would be no apparent difference in the measured BP, such QTLs would be overlooked. A more comprehensive way to study BP QTLs located on rat chromosome 8 is to create congenic strains using other contrasting strains such Wistar-Kyoto, Milan, or SHR rats. In this way one would reduce the chances of missing a QTL (44,45,98,144).

Moreover, it would be of interest to see if the high mortality rate of C8S.L2 is attributable to the transducer surgery, is a result of the specific strains crossed, or is a result of their elevated BP, as suggested earlier. Regions homologous to the positive BP QTL could be transferred from other normotensive strains into the genetic background of S, to see whether the hyper-hypertensive nature of C8S.L2 is specifically due to the alleles transferred from the LEW strain, and whether other normotensive strains have the same alleles.

#### 5.7.1.4 Narrowing the QTL Regions Using Congenics.

The QTL regions isolated in this study are quite large. This fact was made more evident from the sequence of the rat genome, which indicates that the negative BP QTL spans approximately 60,000,000 bp, while the positive BP QTL spans 25,000,000 bp. Using congenic strains, the QTL regions can be refined, as mentioned earlier, until the region of interest spans from 1 to 2 cM. In this way, gene sequencing could be performed to look for specific mutations causing elevated BP.

### 5.7.2 Functional and Positional Candidate Genes

Once high-resolution mapping methods and the delineation of the QTL regions by congenics results in a narrower region, spanning 1 to 2cM, the question remains of how to determine which genes correspond to the QTL.

Co-segregation of intragenic recombinant genotypes in a candidate gene with the QTL phenotype would be strong evidence that the QTL corresponds to the candidate gene. Functional complementation, in which the trait phenotype is rescued in transgenic organisms, is another method for gene identification. These methods are facilitated by QTLs with pronounced effects, and which have a dominant gene action.

If the effect of the QTL was not large, it would be necessary to construct multiple independent transgenic lines to account for changes in expression of the transgene due to different insertion sites.

The number of genes that are present within the narrower region becomes smaller and thus allows for the sequencing of those genes within the parental lines. By sequencing, one might be able to find mutations, such as frame shifts and translocations.

#### 5.7.2.1 Analysis of Differential Gene Expression

As the QTL region is narrowed through mapping, the number of candidate genes becomes smaller. Eventually one should examine compare the parental strains and the congenic strains to locate differential gene expression of possible candidate genes (such as those discussed in section 5.6) located in the QTL regions. Possible qualitative and quantitative approaches for differential gene expression analysis include Northern blot analysis, real-time PCR and micro-array analysis.

The Northern blot analysis remains the standard for detection and quantification of

mRNA levels. In this technique, sample RNA is separated by electrophoresis on a denatured agarose gel transferred to a solid support and immobilized. An RNA or DNA probe, radiolabeled or not, is then used to detect the message of interest. Typically, ethidium bromide-stained or radiolabeled RNA markers are run on the same gel as sample RNA to provide an accurate sizing ladder in gels or on autoradiographs. Technically, this analysis presents several important advantages. First, the method is "low-tech" in that it uses standard electrophoresis equipment and requires minimal finesse in the physical processing of samples. The RNA undergoes very little manipulation; no enzymatic reactions or amplification need be carried out before analysis.

Northern blot analysis is the easiest method for determining both transcript size and the presence of alternatively spliced or multiple transcripts generated from a single locus. It allows a direct relative comparison of message abundance between samples on a single blot. Northern blotting is versatile in the type of probe that can be used for hybridization. High specific activity random-primed or PCR-generated DNA probes, in vitro transcribed RNA probes, and oligonucleotide probes can all be used successfully. Additionally, probes with only partial homology may be used.

There are limitations associated with Northern blot analysis. First, if RNA samples are even slightly degraded, the quality of the data and the ability to quantify expression are severely compromised. Northern blot analysis is also less sensitive than reverse transcription coupled with PCR (RT-PCR).

RT-PCR has revolutionized the study of gene expression. It is possible to detect the RNA transcript of any gene regardless of the amount of starting material or the relative abundance of the specific mRNA. In the RT-PCR method, an RNA template is copied into a complementary DNA (cDNA) transcript using a retroviral reverse transcriptase. The cDNA sequence of interest is then amplified exponentially using PCR. Detection of the PCR product is typically performed by agarose gel electrophoresis and ethidium bromide staining or by the use of radiolabeled nucleotides or primers in the PCR(179).

RT-PCR is one of the most sensitive techniques currently available for mRNA detection and quantification. Since most RT-PCR methods amplify only a few hundred bases rather than the complete mRNA sequence, the sample RNA can be slightly degraded. RT-PCR can be used for relative or absolute quantification. Relative quantification compares transcript abundance across multiple samples, using a co-amplified internal control, which ideally has invariant expression within those samples, for sample

normalization. Absolute quantification using competitive RT-PCR measures the absolute amount of a specific mRNA sequence in a sample. Dilutions of a synthetic RNA (identical in sequence, but slightly shorter than the endogenous target) are added to sample RNA replicates and are co-amplified with the endogenous target. The PCR product from the endogenous transcript is then compared to the concentration curve created by the synthetic "competitor RNA." It is also possible to do real-time RT-PCR quantification by measuring an internal control in replicate samples. Because of its sensitivity, the technique of RT-PCR requires that samples be free of genomic DNA or other DNA contaminants. RT-PCR can be a very technically challenging RNA quantification method.It often requires substantial pre-experimental planning to design suitable primers and controls(141,179).

Microarray analysis has become a popular technique for identifying differentially expressed genes. It is a very costly method. Once specific mRNAs are identified, their expression levels must be confirmed using another previously described technique.

Each of the techniques described can be used to determine precisely the level of a specific RNA within a population. In practice, each technique has inherent technical advantages and limitations.

## 5.8 Conclusion

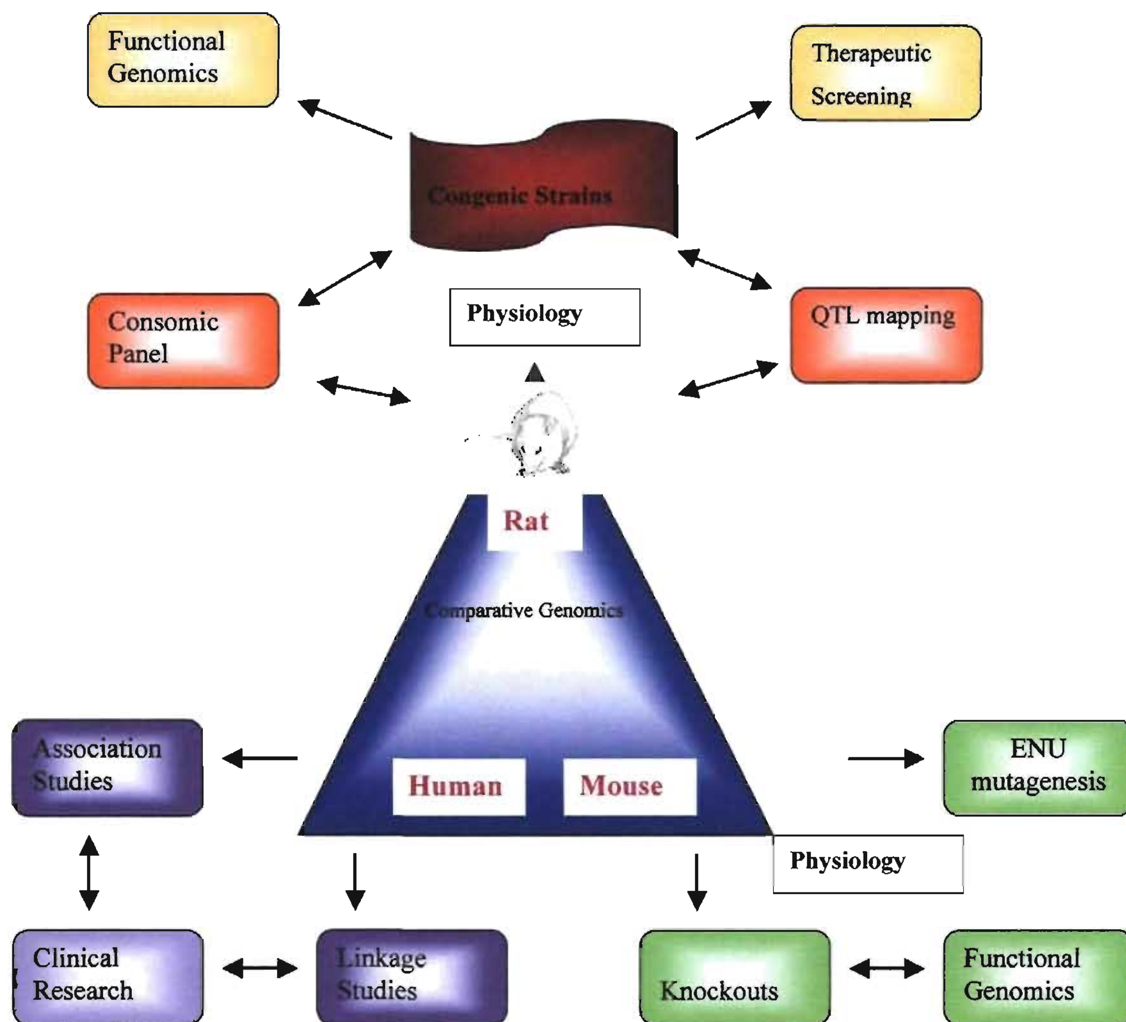
In this study, two QTLs in close proximity on rat chromosome 8 were separated by the use of congenic strains. One QTL has a BP-lowering effect, whereas the other has a BP-raising effect, compared to the Dahl salt sensitive S rat. The BP-raising QTL was not detected in linkage analyses. As a result of applying the congenic strategy, a strain with higher BP even than that of the hypertensive strain S was produced. This strain differs from the S strain by about 20 cM in the genome.

This study is the first step in refining the study of BP regulating genes on rat chromosome 8 and understanding the precise way in which they are implicated in the control of BP. A fine mapping of the QTLs in question would aid in identifying the exact portion of the human chromosome with which the rat chromosome has a conserved synteny, and eventually help unravel some of the genetic determinants in human hypertension. This further refinement will enhance our understanding of the various mechanisms already known (see Chapter 1.5), and might even contribute to the discovery of new mechanisms of BP regulation.

Through comparative genomics (Figure 25), the information gathered from humans,

rat and mice is integrated to obtain a clearer picture of the genes implicated in essential hypertension (84). As the genome projects for the human, the mouse and the rat progress, the genomic information will converge and might also contribute to the identification of the BP QTLs in humans (see Figure 25).

Figure 25 - Comparative Genomics




Comparative genomics integrates the information gathered from humans, rats and mice to obtain a clearer picture of the genes implicated as causative in essential hypertension. In rats, QTL mapping through the use of congenics and consomic panels is used to narrow down candidate genes. Congenic strains can be used in functional genomics and therapeutic screening. Mice are of great use in identifying the structural or functional action of discovered genes and for validating the importance of candidate gene knockouts. One of the advantages of the mouse as a model organism is the availability of gene knockout technology. In humans, through linkage and association studies, the information gathered from comparative mouse and rat genomics can culminate in clinical research, once drugs that can modify or affect disease genes are discovered. Diagram based on figure 1 in Jacob and Kwitek (84).

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