Identification and Dissection of Cardiovascular and Cerebrovascular Quantitative Trait Loci in the Stroke-Prone Spontaneously Hypertensive Rat

by

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DECLARATION

I declare that this thesis has been composed entirely by myself and it has not been accepted in any previous application for a degree. The work, of which it is a record, has been done by myself, except as indicated in the acknowledgements, and all sources of information have been specifically referenced.

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ABBREVIATIONS

AII	Angiotensin II
ACA	Anterior cerebral artery
ACE	Angiotensin converting enzyme
AGT	Angiotensinogen
AME	Apparent mineralocorticoid excess
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
BAC	Bacterial artificial chromosome
BC	Back cross
BN	Brown Norway rat
BNP	Brain natriuretic peptide
BP	Blood pressure
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
cDNA	Complementary DNA
cM	centiMorgan
Dahl RR	Dahl salt resistant rat
Dahl SS	Dahl salt sensitive rat
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
F1	First filial generation
F2	Second filial generation
FHC	Familial hypertrophic cardiomyopathy

GH	Genetically hypertensive rat
GRA	Glucocorticoid remediable aldosteronism
LEW	Lewis rat
LV	Left Ventricle
LVH	Left ventricular hypertrophy
MAP	Mean arterial pressure
MCA	Middle cerebral artery
MHS	Milan hypertensive rat
MNS	Milan normotensive rat
mRNA	Messenger RNA
PAC	Phage P1 clones
PCA	Posterior cerebral artery
PCR	Polymerase chain reaction
PHA-1	Pseudo-hypoaldosteronism type 1
PP	Pulse pressure
QTL(s)	Quantitative trait locus (loci)
RFLP	Restriction fragment length polymorphism
RH	Radiation hybrid
RNA	Ribonucleic acid
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
SHRSP	Stroke-prone spontaneously hypertensive rat.
WKY	Wistar-Kyoto rat
YAC	Yeast artificial chromosome

SUMMARY

Human essential hypertension is a classic example of a complex, multifactorial, and polygenic trait. That a substantial fraction of the variation in blood pressure between individuals is genetically determined has been well established by classic epidemiologic, twin, and adoption studies, as well as the existence of rare Mendelian forms of hypertension. Similar evidence exists for a genetic basis to human stroke and left ventricular hypertrophy, unrelated to the effects of blood pressure. Given the heterogeneity of the human condition, however, little progress has been made towards the identification of the genes involved in these common disease states. Instead, a major strategy has been successfully developed using inbred animal models which results in the identification of a quantitative trait locus (QTL), a large chromosomal region which contains a gene or number of genes responsible for the quantitative trait under investigation. Known as a genome wide scan, this strategy involves the high fidelity phenotyping of a large segregating F2 population derived by crossing hypertensive and normotensive inbred rat strains, and the genotyping of a large panel of dimorphic microsatellite markers with a thorough coverage of the entire rat genome.

The research described in this thesis incorporated the use of two genome wide scans to identify QTLs containing genetic determinants of hypertensive cardiovascular and cerebrovascular disease in the Glasgow SHRSP x WKY F2 cross. The first genome wide scan was performed in 140 male and female F2 hybrids (M:F = 65:75). Blood pressure at baseline and after 1% sodium chloride administration was measured by radio-telemetry; other phenotypes included heart rate, motor activity, and left ventricular hypertrophy.

This was the first genome scan where physiological phenotypes were measured with a radio-telemetry system in an entire F2 cross. The second genome scan was performed in 59 male and female F2 hybrids (M:F 33:26) phenotyped by the volume of cerebral infarction following the experimental occlusion of their middle cerebral artery.

QTLs affecting a given phenotype were mapped relative to microsatellite markers with the aid of the MAPMAKER/QTL 1.1 computer package. This resulted in the identification of three blood pressure QTLs; two on rat chromosome 2 and one on rat chromosome 3. The QTL close to the microsatellite markers *D2Mgh12* (suggestive linkage with a maximal LOD score of 3.1) and *D3Mgh16* (significant linkage with a maximal LOD score of 5.6) showed sex specificity being only present in the male F2 cohort. This was further confirmed by the likelihood ratio test for the difference in the locus effect between the sexes. This study was the first to show such sex specificity of autosomal QTLs for genetic hypertension. This genome scan also identified a new QTL for left ventricular hypertrophy on rat chromosome 14 (suggestive linkage, with a maximal LOD score of 3.1).

The second genome scan identified a single highly significant QTL on rat chromosome 5 for the increased sensitivity to experimentally induced focal cerebral ischaemia, with a LOD score of 16.6. This QTL accounted for 67% of the phenotypic variance and was blood pressure independent. Sex also had a significant effect with male F2 hybrids having larger infarcts than females ($p = 0.012$), and explained 10% of the phenotypic variance. The microsatellite marker in the centre of this QTL was *Anp*, a marker within the gene encoding the atrial natriuretic peptide. DNA sequencing analysis of the coding regions of

the *Anp* gene, and the gene for brain natriuretic peptide (*Bnp*) which is known to co-localise with the *Anp* gene, revealed no significant nucleotide differences between the SHRSP and the WKY strains that are potentially important to their function. Consequently the *Anp* and *Bnp* genes were not supported as candidates for the QTL for severity of cerebral ischaemia.

The identification of the large QTLs is only the first step towards the ultimate goal of gene identification. The next step is the production of congenic strains and substrains containing progressively smaller chromosomal regions with the final task being positional cloning of the causal gene. In this thesis, the process of dissection was begun for the two blood pressure QTLs identified on rat chromosome 2 using a speed congenic strategy only previously verified in mice. Five congenic strains (SP.WKYgla2a-e) were produced by introgressing regions of chromosome 2 from WKY rats into the recipient SHRSP strain, and four congenic strains (WKY.SPgla2a-d) were produced by introgressing regions of chromosome 2 from SHRSP rats into the recipient WKY strain. The number of generations of backcrossing required for each strain to achieve complete homozygosity of the background genetic markers in a best male varied between 3 and 5. In total 542 progeny were necessary for screening in order to produce the congenic strains, an average of 60 animals per strain, 15 animals per backcross. The average reduction in background marker heterozygosity mirrored that theoretically expected over 8 generations utilising the traditional congenic approach. It follows that the presentation of nine congenic strains derived for rat chromosome 2 clearly demonstrated the applicability of a speed congenic strategy in this species.

The success of the speed congenic strategy will pave the way for the construction of congenic strains of the remaining QTLs identified in this study. Phenotyping these strains should further the evidence for, and the genetic dissection of, the QTLs involved. This in turn should lead to the construction of sub-strains containing smaller relevant sections of chromosome until, via substitution mapping, positional cloning and comparative mapping to human chromosomes, the causal genes and their pathophysiologic pathways are identified. This will hopefully improve prospects for the prevention and improved treatment of these complex traits which are the major causes of morbidity and mortality in the UK.

1. INTRODUCTION

1.1 Complex Cardiovascular Traits

Complex traits, which encompass most cardiovascular as well as other epidemiological important disorders, do not exhibit classic Mendelian inheritance attributable to a single genetic locus (Lander & Schork, 1994). Rather their inheritance can be described as polygenic and multifactorial, displaying the concurrent morbid effects of many genetic and non-genetic factors. It follows that unravelling the precise primary genetic determinants of a complex trait is a daunting task (*Figure 1.1*). The effects of individual genes may be independent and thus simply additive to the trait, or more complex, characterised by gene-gene (epistatic) and gene-environment (ecogenetic) interactions. Gene expression can also be gender and/or age dependent. The genetic background may itself be heterogeneous, the particular set of genes operative in one affected individual differing from that in another, despite similar phenotypic features (Schork, 1997). Success in this effort, however, will afford insights into pathophysiology, permit clinical identification of subjects with specific inherited susceptibility, and provide opportunities to tailor therapy to specific underlying abnormalities (Lifton, 1995).

1.1.1 Hypertension

Blood pressure is the hydrostatic force that blood exerts against the vascular walls. In simple mechanical terms it is determined by the amount of blood ejected by the heart and the resistance to blood flow offered by the bottlenecks of the circulatory system, the arterioles. The provision of a blood pressure level at which adequate tissue perfusion is maintained, however, reflects the interaction of a myriad of neural, hormonal and circulatory

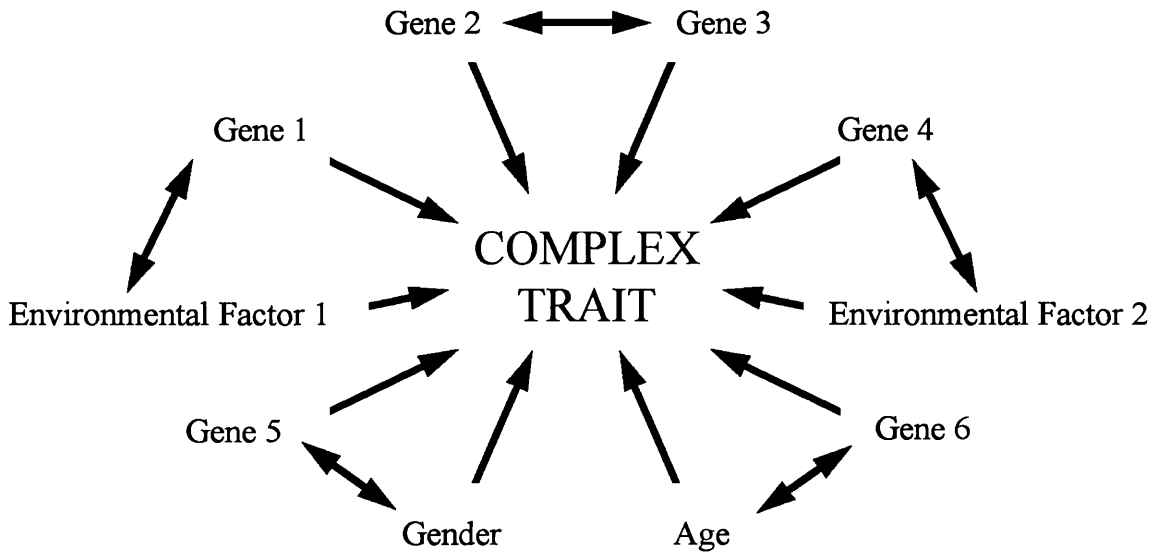

Figure 1.1 Multifactorial determination of a complex trait (modified from Lifton, 1995). Each arrow represents the potential interaction of genetic, environmental and demographic factors.

systems and sub-systems, the complexity of which creates enormous potential for a variety of mutant genes to induce deleterious effects (Schork, 1997).

Hypertension refers to the maintenance of an excessively high blood pressure (Swales, 1994). Although without conspicuous symptoms, hypertension can have severe detrimental effects on a number of important tissues in the body, including the heart and brain. This so-called "end organ damage" contributes to the genesis of various coronary, cerebral, and peripheral vascular diseases which account for the leading causes of adult death, morbidity, social disruption, and loss of income in westernised societies (Whelton *et al*, 1994). Unfortunately, whilst 5% of those with elevated blood pressure display a potentially remediable aetiology (secondary hypertension), such as hormone excess or coarction of the aorta, the primary determinants of the more frequent "essential" hypertension remain unknown, hindering the efficacy of any preventative and therapeutic measures (Dominiczak & Lindpaintner, 1994).

Numerous population studies (Smirk, 1949; Pickering, 1959) have proven blood pressure to be a quantitative trait with essential hypertension representing the upper end of its distribution. This implies that variation in blood pressure, including essential hypertension, is under the influence of several independently segregating genes for each of which the effects on blood pressure are generally smaller than the sum of the whole and the effects of environment (Kearsey & Pooni, 1996).

All lines of evidence are concordant in supporting the existence of a genetic component in the aetiology of human essential hypertension. Epidemiologic studies have revealed highly

significant familial aggregation of blood pressure. Individuals with essential hypertension are twice as likely as normotensive individuals to have a parent who is hypertensive, and hypertension is 2- to 8-fold more common in the offspring of hypertensive parents than in the offspring of normotensive individuals (Perera, *et al*, 1971; Ibsen, 1984). *Table 1.1* demonstrates that when blood pressure measurements are compared in household members, correlations increase with the degree of genetic relationship between the members. Identical twins who share all their genes demonstrate higher blood pressure correlations than non-identical twins who share 50% of their genes (Feinleib *et al*, 1977). Similarly, correlations are higher for first-degree relatives such as sib-sib and child-parent pairs than for more distant relatives. Studies of families with adopted and biological children have shown a significant correlation in the blood pressure of parents and their biological children that is not seen when compared to their adopted children's (Biron *et al*, 1976; Mongeau *et al*, 1986).

Whilst these findings have led to the estimate that 20-40% of the variation in blood pressure can be attributed to genes (Annest *et al*, 1979; Mongeau, 1989), they are also indicative of the multifactorial nature of essential hypertension. Not only does blood pressure not typically segregate in families in a fashion consistent with Mendelian transmission but spouses have also shown significant correlations despite sharing no genetic material (*Table 1.1*, Mongeau *et al*, 1986). Indeed, a variety of other factors have been shown to chronically influence blood pressure, including high salt, low calcium and low potassium intake, low levels of physical exercise, exposure to noise and stress, age, gender, and body mass (Horan & Lenfant, 1990). It is likely that these only produce elevated blood pressure when appropriate, genetically determined susceptibility is present, indicating a specific

Relationship	Correlation	Reference
Twins, monozygotic	0.54-0.55	Havlik *et al*, 1979; Feinleib *et al*, 1977
Twins, dizygotic	0.25-0.40	Havlik *et al*, 1979; Feinleib *et al*, 1977
Sib-sib	0.34-0.38	Zinner *et al*, 1971; Annest *et al*, 1979
Parent-child	0.16-0.27	Zinner *et al*, 1971; Annest *et al*, 1979
Aunt/uncle-niece/nephew	0.05	Weinberger *et al*, 1981
Spouse-spouse	0.15	Mongeau *et al*, 1986
Parent-adopted child	0.09	Biron *et al*, 1976

Table 1.1 Estimates of systolic blood pressure correlation coefficients between household members (adapted from Mongeau, 1989).

interaction between environmental/demographic factors and certain allelic variants. A classic example of such genetic heterogeneity concerns the different responses of blood pressure to dietary salt. Both normotensive and hypertensive human subjects have been demonstrated to be sensitive or resistant to the blood pressure increasing effects of salt intake (Weinberger, 1996).

1.1.2 Stroke

Stroke is the third most common cause of mortality and life-long disability following heart disease and combined malignancies in Western society (Sharma, 1996). It follows that any advancement in both preventive and therapeutic measures would be most welcome. Unfortunately, stroke is also a highly variable clinical state. Its cause, late-onset, location, and pre-existing disease all contribute to variability in stroke outcome and thus confound clear interpretation of its primary determinants (Macrae, 1992).

Stroke describes the sudden onset of a focal loss of neurologic function which lasts longer than 24 hours (Dept. of Health, 1994). Occlusion of a cerebral artery causes some 84% of strokes, comprising 53% from *in situ* thrombosis, and 31% from thrombi originally formed in the heart or atherosclerotic neck arteries impacting on the cerebral circulation. Haemorrhage accounts for 16%, 10% of which are intracerebral and 6% subarachnoid (Kannel & Wolf, 1983). Whatever the cause, a marked focal reduction in cerebral blood flow is the common result. The brain derives all its energetic needs from the oxidative catabolism of glucose supplied from arterial blood. If the supply of oxygen and glucose is interrupted for more than a few minutes, neurones in the most vulnerable areas of the brain

will be irreversibly damaged. The area of the brain where all cell types are dead is termed an infarction (Fisher & Garcia, 1996). Most infarcts (~50%) are seen in the cortex, basal ganglia and internal capsule supplied by the middle cerebral artery, or MCA (McAuley, 1995). The posterior cerebral artery (PCA) is the next most frequent, followed by the anterior cerebral artery, or ACA (Bogousslavsky *et al*, 1988). In the acute period following ischaemia, the infarction is surrounded by a "penumbra", a region where cerebral blood flow is low but sufficient to maintain neuronal survival. Collateral vessels play a vital role in preserving blood flow to the penumbra and thereby limiting the extent of the ischaemic damage (Heistad *et al*, 1993).

Historically most strokes have been attributed to hypertension, either by accelerating atherosclerosis and thus thrombosis and occlusion of a large cerebral artery, or by weakening the vessel wall of smaller penetrating arteries of the brain by a process known as fibrinoid necrosis, leading to haemorrhage (Gunel & Lifton, 1996). In a series of 420,000 adults aged 25-70 years in 9 prospective studies the difference between a diastolic blood pressure of 75mmHg and 105mmHg was enough to increase 10-fold the risk of stroke (Whelton *et al*, 1994). However, the aetiology of stroke remains unknown in approximately 40% of patients despite extensive investigation (Sharma, 1996) suggesting elevated blood pressure is not the only prerequisite for a stroke event. Far from all hypertensive individuals develop fibrinoid necrosis or experience a stroke, and fibrinoid necrosis and strokes are reported in normotensive individuals (Gunel & Lifton, 1996). Indeed, it has been shown that similar atherosclerotic occlusion of a major cerebral artery in humans may cause a catastrophic stroke or be entirely asymptomatic, with an apparently continuous distribution of neurological damage in between (Allen, 1983; Jacewicz, 1992). This raises the possibility

that multiple genetic factors may be important in the pathogenesis of stroke, whether contributing to the elevated blood pressure or acting independently.

An increasing body of evidence from human epidemiological and twin studies does suggest a genetic contribution to stroke onset (Alberts, 1990). Most convincingly, analysis from the Framingham study (Kiely *et al*, 1993) examining patterns of familial aggregation of stroke showed that offspring whose mothers experienced a stroke or a transient ischaemic attack were 2.3-fold more likely to experience a similar event than those with negative maternal histories. In another study, Graffagnino *et al* (1994) examined the family histories of 90 patients with cerebral infarction and found a significant positive family history of stroke in 47% of patients and 24% of controls. Data from a classic twin study demonstrated concordance rates for stroke of 17.7% for monozygotic twins and 3.6% for dizygotic twins with a relative risk of 4.3 (Brass *et al*, 1992), providing strong evidence for a genetic component.

Like essential hypertension, these epidemiological studies which have identified a family history of stroke have also served to highlight its multifactorial nature. *Table 1.2* lists many of the lifestyle and risk factors identified so far (Khaw, 1996). Whilst direct associations have been reported between some of these factors and stroke, others appear to influence stroke by their effect on blood pressure. Indeed many of these factors, including blood pressure, fibrinogen, and obesity, are known to have their own genetic and environmental determinants either potentially in common with, or distinct from, stroke. This scenario, indicative of a highly complex trait, offers a vast array of putative stroke risk genes which demand further investigation.

Major	Implicated
Age	Alcohol
Gender	Potassium
Body Mass	Fruit & Vegetables
Blood Pressure	Total Calories
Cigarette Smoking	ω-3 Fatty Acids
Diabetes	Fibre
Physical Activity	White Cell Count
Obesity	Fibrinogen Level
Dietary Sodium	Albumin Concentration
Saturated Fat	Blood Homocysteine Level

Table 1.2 Biological and lifestyle factors for stroke (adapted from Khaw, 1996).

1.1.3 Left Ventricular Hypertrophy

The heart adapts to the abnormal sustained haemodynamic burden that hypertension imposes with a compensatory growth of the myocardium (Straurer *et al*, 1994). This primarily involves enlargement of the myocyte cross-sectional area (Anversa *et al*, 1986), the actual number of myocytes increasing only when a critical heart weight of approximately 500g is reached (Linzbach, 1960). This response maintains systemic perfusion until a time when ventricular dysfunction evolves despite continued hypertrophy, the result of which is heart failure, myocardial infarction or arrhythmia (Pfeffer *et al*, 1982). As such hypertensive left ventricular hypertrophy (LVH) has proved a major independent risk factor for cardiovascular mortality and morbidity (Dominiczak *et al*, 1997).

Although elevated blood pressure is the most important determinant of LVH (Kannel, 1983), the blood pressure level does not always parallel the degree of hypertrophy in humans (Dominiczak *et al*, 1997) indicating the existence of a more complex relationship than a simple dose-response effect. Indeed, the distribution of cardiac mass within populations of unselected normotensive or hypertensive individuals appear to be continuous for the most part (Nunez *et al*, 1996), with 50% of hypertensives without any sign of cardiac hypertrophy (Gross & Jeger, 1949; Devereux *et al*, 1987). It follows that as this unexplained variance in LVH can not be totally explained by haemodynamics, it is also likely to include the additional effects of as yet unidentified genetic and non-genetic determinants (Nunez *et al*, 1996).

The quantitative distribution of cardiac mass within populations supports the view that any heritable effects are likely to be polygenic. Unfortunately, there is a lack of robust data on the extent and importance of inherited variability in the response of the heart to hypertrophic stimuli. This is not surprising since large families with hypertension have been studied with respect to blood pressure rather than cardiac phenotype. The most convincing data on the heritability of cardiac mass comes from various twin studies (Nunez *et al*, 1996). In the largest of these, Verhaaren *et al* (1991) analysed 254 11-year old white twins, finding that genetic variance accounted for 63% of the total variance of cardiac mass in boys and 71% in girls. They found that genes that control LV mass include those which regulate LV mass alone and others which affect both LV mass and body weight. In boys the genetic effects were for the most part due to the latter, whereas in the girls the former predominated. Again this serves to highlight the existence of both several independently modifying cardiac mass genes and the multifactorial influence of environmental and demographic factors as befits a complex trait.

1.2 Genetic Dissection of Human Hypertensive Cardiovascular Disease

From the evidence cited above it is clear that complex human cardiovascular diseases such as essential hypertension all have important genetic components, although the genes responsible, and their specific interaction with environmental and demographic factors, remain mostly unknown. Human studies have pursued two main routes to gene identification; the in-depth deconstruction of rare Mendelian forms of cardiovascular disease and the similarly intense interrogation of numerous candidate genes.

1.2.1 Mendelian Disorders

Part evidence to support an appreciation of essential hypertension, stroke and LVH as a genetically determined group of illnesses has been based on the observation of a number of uncommon syndromes which display a Mendelian inheritance pattern indicative of a monogenetic disorder (*Table 1.3*). Although these syndromes occur rarely, considerable effort has been devoted to the identification of the genes responsible. The reasons for this are several (Lifton, 1996). Firstly, the effects of segregation of single alleles in families can be easily discerned, thereby simplifying the molecular genetic analysis necessary. Secondly, these disorders generally result in a severe disease state, making phenotypic selection easier. Finally, it is hoped that the genes and physiologic pathways involved in these severe forms may also be involved in the cardiovascular disease states more commonly seen in the general population. It follows that understanding these disorders may not only be of importance to the families that are affected but could also provide more broadly relevant diagnostic and therapeutic tools (Karet & Lifton, 1997).

Mendelian Forms of Disordered Blood Pressure

At present, three Mendelian forms of human hypertension have yielded mutations which impart large elevating effects on blood pressure levels:

1) - Glucocorticoid remediable aldosteronism (GRA) was first described (Sutherland *et al*, 1966) as an autosomal dominant form of potentially severe early onset hypertension with the cardinal symptom being a therapeutic response to glucocorticoid administration.

Syndrome	Chromosome	Gene(s)
Hypertension		
Glucocorticoid-remediable hyperaldosteronism	8q22	CYP11B1/CYP11B2
Apparent mineralocorticoid excess	16q22	HSD11B2
Liddle's syndrome	16p12-p13	SCNN1B/SCNN1G
Gordon's syndrome	1q31-q42	Unknown
	17p11-q21	Unknown
Isolated pheochromocytoma	1p	Unknown
Multiple endocrine neoplasia, type II A	10q11.2	RET proto-oncogene
Multiple endocrine neoplasia, type II B	10q11.2	RET proto-oncogene
Male pseudo-hermaphroditism	10q24.3	CYP17
Female pseudo-hermaphroditism	8q22	CYP11B1
von Hippel-Lindau syndrome	3p26-25	VHL tumour suppressor
Neurofibromatosis, type I	17q11.2	NF1 gene
Hypertension plus brachydactyly	12p	Unknown
Ischaemic Stroke		
CADASIL	19q12	Notch 3
MELAS	Mitochondria	tRNA-3243
Inherited homocystinuria	21q22.3	Cystathionine β-synthase
Haemorrhagic Stroke		
Hereditary haemorrhagic telangiectasia-1	9	Endoglin
Hereditary haemorrhagic telangiectasia-2	12q13	Activin receptor-like kinase 1
Cerebral cavernous malformation	7q22	Unknown
Hereditary cerebral haemorrhage with amyloidosis-Dutch type	21	Amyloid precursor protein
Hereditary cerebral haemorrhage with amyloidosis-Icelandic type	20	Cystatin C
Polycystic kidney disease	16p13.3	Polycystin
Marfan syndrome	15q3	Fibrillin
Cardiac Hypertrophy		
Familial hypertrophic cardiomyopathy	seven loci	Myofibril proteins
Friedreich's Ataxia	9q	X25 (Frataxin)
Fabry's disease	X	α-Galactosidase

Table 1.3 Mendelian forms of human cardiovascular diseases (adapted from Hamet *et al*, 1998; Gunel & Lifton, 1996, & Nunez *et al*, 1996).

Affected subjects have variable elevated plasma aldosterone levels in conjunction with suppressed plasma renin activity, and secrete high levels of 18-oxocortisol that unaffected individuals produce in negligible amounts (Sutherland *et al*, 1966; Ulick *et al*, 1990). Using 18-oxocortisol secretion as a phenotype, a large GRA kindred demonstrated linkage to a segment of chromosome 8 harbouring CYP11B2, or the aldosterone synthase gene (Rich *et al*, 1992; Lifton *et al*, 1992a). This gene is 95% identical in DNA sequence to another involved in steroid biosynthesis, the 11β-hydroxylase gene (CYP11B1), which also lies on chromosome 8 (Lifton *et al*, 1992b). Unequal crossing over between the aldosterone synthase and 11β- hydroxylase genes results in a chimeric gene duplication fusing proximal sequences of 11β-hydroxylase onto more distal sequences of aldosterone synthase (Pascoe *et al*, 1992; Lifton *et al*, 1992a). The presence of this novel mutation cosegregates with GRA at a recombination fraction of zero and is thus clearly responsible for the clinical features observed in affected individuals.

2) - Apparent mineralocorticoid excess (AME) is an autosomal recessive form of Mendelian hypertension also associated with defects in steroid metabolism. Inactivating mutations in the renal isozyme of the 11β-hydroxysteroid dehydrogenase (HSD11B2) gene, which normally converts cortisol to cortisone, create hypertension by failing to provide affected subjects with protection against the potent mineralocorticoid effect of cortisol (Mune *et al*, 1995; Stewart *et al*, 1996).

3) - Liddle's syndrome (pseudoaldosteronism), an autosomal dominant form of hypertension, is related to abnormalities in renal ion transport and is clinically characterised by the suppressed secretion of aldosterone, suppressed plasma renin activity and

hypokalaemia (Shimkets *et al*, 1994). Initially linked to a short segment of human chromosome 16, subsequent research identified that constitutive over-activation of the aldosterone-regulated epithelial sodium channel (expressed in the renal nephron) arises from a mutation in the β subunit (SCNN1B) of this channel (Shimkets *et al*, 1994) and/or a mutation truncating the carboxy terminus of the γ subunit (SCNN1G) of the channel (Hansson *et al*, 1995). Both lead to hypertension via increased renal salt and water absorption independent of mineralocorticoid action.

The molecular causes of three rare inherited forms of hypotension have also been reported. Autosomal recessive pseudohypo-aldosteronism type 1 (PHA-1) is a mirror image of Liddle's syndrome with causative mutations in the α- and β-subunits of the epithelial sodium channel (Chang *et al*, 1996). Gitelman's syndrome, characterised by hypokalaemic alkalosis with elevated renin and aldosterone, hypomagnesemia, and hypocalciuria maps to a region on chromosome 16 inseparable from the gene encoding the renal thiazide-sensitive sodium-chloride co-transporter (NCCT) present on the surface of the distal convoluted tubule cells (Simon *et al*, 1996a). Bartter's syndrome features salt wasting, hypokalaemic alkalosis and hypercalciuria and is due to mutations in the renal sodium-potassium-chloride co-transporter gene (NKCC2) that is expressed apically in the thick ascending limb of Henle's loop (Simon *et al*, 1996b). Given the common genetic heterogeneity observed, it seems likely that these alleles which lower blood pressure may be of equal importance in determining blood pressure levels (Karet & Lifton, 1997).

It is noteworthy that all the above mutations, whether elevating or lowering blood pressure, do so through a common pathway (*Figure 1.2*), affecting salt and water reabsorption in the

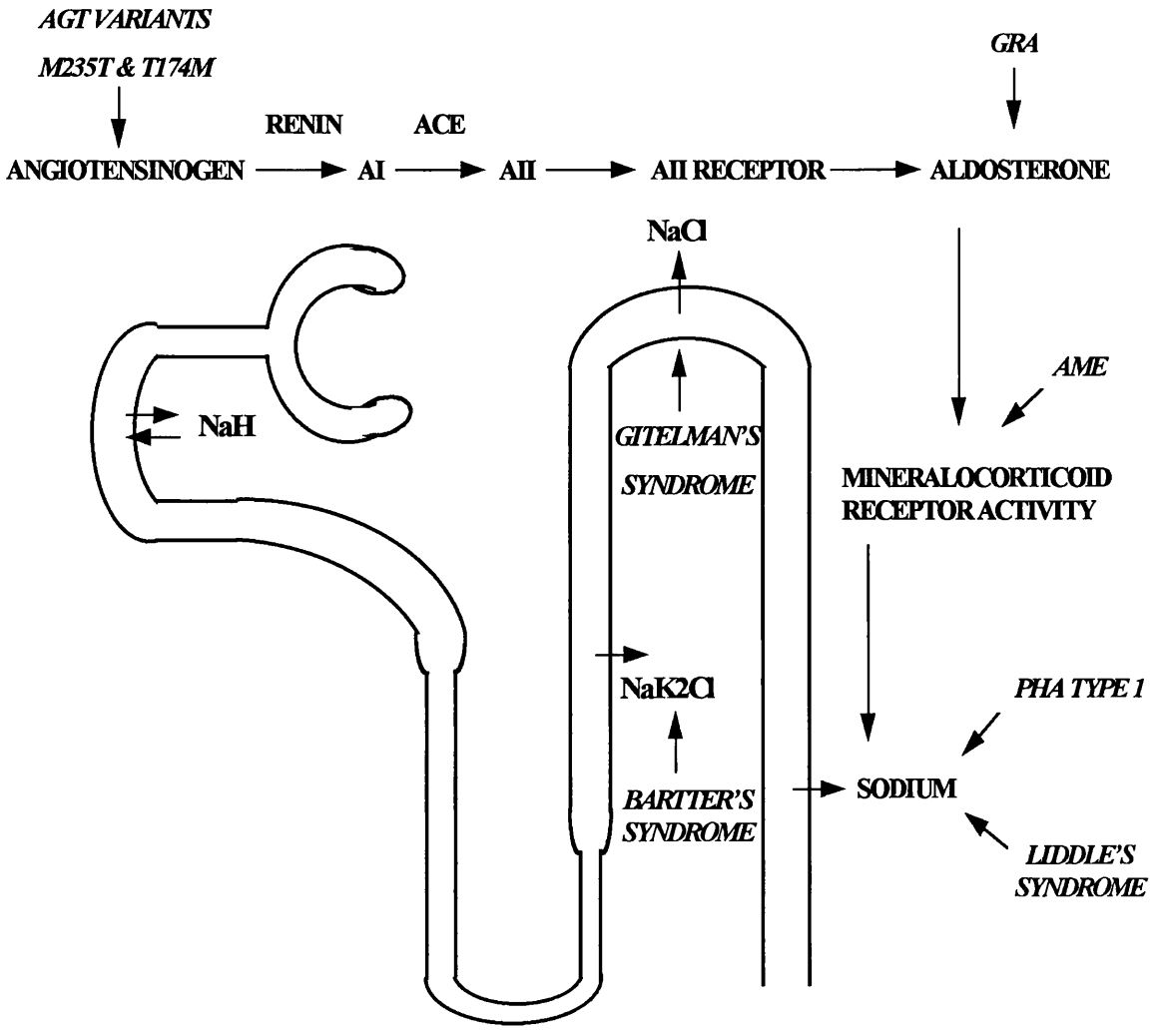

Figure 1.2 Common pathway of all mutations contributing to blood pressure variation identified so far - the regulation of salt reabsorption by the kidney (adapted from Karet & Lifton, 1997). Genetic conditions affecting this pathway are given in italics. All abbreviations are described in text.

kidney (Lifton, 1996). Mutations causing GRA, AME and Liddle's syndrome all result in a constitutive increase in renal sodium absorption, whereas mutations causing PHA-1, Bartter's and Gitelman's syndromes reduce blood pressure by causing renal salt wasting. No causal gene has yet been identified for an autosomal dominant form of severe hypertension plus brachydactyly which has been described in a single, large Turkish kindred (Biliginturan *et al*, 1973). Linkage analysis concerning families with this disease have localised the genetic defect to a region of chromosome 12p in between markers D12S364 and D12S87 (Schuster *et al*, 1996). The eventual identification of the genetic cause may elucidate new mechanisms of blood pressure elevation as this is uniquely a non salt-sensitive syndrome; the renin-angiotensin system and sympathetic nervous system have been shown to respond normally in this kindred (Biliginturan *et al*, 1973).

Mendelian Forms of Stroke

As *Table 1.3* shows, convincing evidence of an underlying genetic predisposition to human stroke independent of blood pressure is also provided by the existence of several well-documented Mendelian forms which do not require hypertension as a permissive factor (Gunel & Lifton, 1996). Of these, most is known of the genetic basis of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).

Originally mapped to chromosome 19q12 in two families (Tournier-Lasserve *et al*, 1993), analysis of 13 additional pedigrees placed the CADASIL locus within a 2cM interval bracketed by the markers *D19S199* and *D19S253* (Ducros *et al*, 1996). The defective gene was subsequently identified as *Notch3* (Joutel *et al*, 1996). Members of the *Notch* gene

family encode evolutionary conserved transmembrane receptors, and are involved in cell fate specification during embryonic development (Artavanis-Tsakonas *et al*, 1995). The *Notch3* gene includes 33 exons encoding a protein of 2321 amino acids and its extracellular domain contains 34 epidermal-growth-factor-like (EGF-like) repeats. Clustering of mutations within the two exons encoding the first five EGF-like repeats has been observed and all lead to a loss or gain of a cysteine residue and therefore to an unpaired number of cysteine residues within a given EGF domain. These findings suggest that aberrant dimerisation of *Notch3*, due to abnormal disulphide bridging with another protein, may be involved in the pathogenesis of this disorder (Joutel *et al*, 1997). Further molecular analysis of the human Notch3 receptor carrying CADASIL mutations will determine the molecular pathogenesis underlying the CADASIL phenotype (Goate & Morris, 1997), whilst a study of the CADASIL gene in the general stroke population will determine any involvement it may have in more common forms of stroke.

Mendelian Forms of Cardiac Hypertrophy

A number of primary inherited disorders are also associated with left ventricular hypertrophy independent of each other and of secondary stimuli such as hypertension (*Table 1.3*). Most is known of familial hypertrophic cardiomyopathy (FHC), a genetically heterogeneous autosomal dominant disorder which has been linked to seven disease loci so far; cardiac troponin T, cardiac myosin binding protein-C, cardiac β-myosin heavy chain, α-tropomyosin, ventricular myosin essential light chain, ventricular myosin regulatory light chain and cardiac troponin I (Kimura *et al*, 1997). These seven genes all encode components of the myofibril and as such it has been proposed that sacromere

disorganisation may play a role in the pathogenesis of the disease, the abnormal protein impairing the correct packaging of proteins into functional macromolecular structures (Nunez *et al*, 1996). Whilst classical FHC causes death at an early age several molecular genetic studies have shown that some FHC causing mutations are compatible with a long lifespan (Watkins *et al*, 1992; Anan *et al*, 1994). It follows that carriers of mutations of the FHC-type may be more common in hypertensives and the general population then currently appreciated.

Taken alone, all the rare mutations identified above prove beyond doubt that genetic defects can produce human cardiovascular disease. The question now stands as to whether they play a role in the more common forms of each disease. It follows that their impact on clinical outcome must now be assessed by studies in the heterogeneous general population just as for any other candidate gene.

1.2.2 Candidate Gene Analysis

The general premise of a candidate gene approach is that the pathophysiological process of interest is due to the altered expression or structure of a gene product which in turn is reflected by changes in the gene. The selection of a candidate gene requires prior knowledge of the physiologic pathways known to influence the disease state (Dominiczak & Lindpaintner, 1994) and is therefore based either on the known, or suspected, function of the gene, sequence homology with other known genes, interaction of the protein product with another protein, or tissue specificity of expression. Once selected the direct search for molecular variants of the candidate gene is followed by linkage studies using affected sibling

or relative pairs and/or association studies contrasting the allele frequencies between large cohorts of unrelated cases and controls (Lifton, 1995). Most studies of human polygenic multifactorial cardiovascular disease have employed a candidate gene approach.

Essential Hypertension

In light of the common pathway shared by all the functional variants identified in Mendelian forms of disordered blood pressure (*Figure 1.2*), it is not surprising that the vast majority of candidate genes tested for a contribution to human essential hypertension have been chosen from those encoding enzymes and peptides of the renin-angiotensin system, or related to water and sodium handling. These include the renin gene (Jeunemaitre *et al*, 1992a); the angiotensin-converting enzyme, or ACE gene (Jeunemaitre *et al*, 1992b), and the angiotensin II receptor AT_1 gene (Bonnardeaux *et al*, 1994). However, only the angiotensinogen (AGT) gene has provided mutually reinforcing lines of evidence supportive of a causative role.

AGT is secreted by the liver, cleaved sequentially by renin and ACE, eventually producing the active hormone angiotensin II (AII), which promotes a rise in blood pressure (*Figure 1.2)*. That this gene plays a role in the development of hypertension was initially supported by the observation that plasma angiotensinogen levels cosegregate with blood pressure in families (Watt *et al*, 1992). Additionally, transgenic mice overexpressing the rat angiotensinogen gene develop hypertension (Kimura *et al*, 1992), knockout mice with a disrupted *agt* gene lack angiotensinogen and develop low blood pressure (Kim *et al*, 1995), and mice carrying from zero to four copies of the normal mouse *agt* gene display plasma

angiotensinogen levels which increase progressively with the number of *agt* copies and correlate with increases in blood pressure (Kim *et al*, 1995; Smithies, 1997).

The human *AGT* gene has been localised to chromosome 1q42-43 and specific mutations in the gene, most commonly the M235T polymorphism which encodes a change of amino acid from methionine to threonine, have shown positive linkage and association with essential hypertension in several human populations (Jeunemaitre *et al*, 1992c; Caulfield *et al*, 1994; Caulfield *et al*, 1995; Schmidt *et al*, 1995). Indeed, a recent meta-analysis summarising a total of 11 association studies of the *AGT* M235T variant with hypertension in 14 Caucasian populations (Kuntz *et al*, 1997) found a significant association between the *AGT* M235T allele and hypertension in five of the studies. Whilst the remaining six studies failed to confirm these findings, when the *AGT* M235T-allele frequency was pooled using the common odds ratio estimator of Mantel-Haenszel it became significantly associated with hypertension. The strength of this association increased in those with more severe hypertension, positive family history or recruitment of cases from referral centres. This variant has been shown to be in tight linkage disequilibrium with a guanine-to-adenosine transition -6bp upstream of the initiation site of transcription, which may result in an increased transcription rate (Inoue *et al*, 1997).

Stroke and Left Ventricular Hypertrophy

Excluding the reported associations in small numbers of cases with rare Mendelian diseases where stroke or left ventricular hypertrophy is part of the phenotype (Natowicz & Kelley, 1987; Nunez *et al*, 1996) very few studies have investigated the molecular genetics of either

in the wider human population. Of those which have, the majority of the candidate genes have been genetic components of lipid metabolism, the clotting cascade and the renin-angiotensin-aldosterone system which are directly implicated in secondary risk factors such as atherosclerosis, myocardial infarction and hypertension. Whilst worthy of investigation, these remain compounding factors only, with their own potentially unrelated genetic determinants. For example, whilst evidence clearly links the *AGT* M235T polymorphism with essential hypertension (Jeunemaitre *et al*, 1992c; Caulfield *et al*, 1994) and cardiac hypertrophy (Ishanov *et al*, 1997), an association has not been shown with cerebrovascular disease (Barley *et al*, 1995). However, attention is beginning to turn to candidate genes putatively involved in free radical determination of eventual infarct size (the nitric oxide synthases and superoxide dismutases), blood-brain barrier breakdown (the matrix metalloproteinases), maintenance of cardiac infra-structure (myofibrillar genes), and those others identified by the dissection of related Mendelian traits (Sharma, 1996).

1.2.3 Limitations

Until proven otherwise by candidate gene analysis, it seems likely that single gene disorders of cardiovascular diseases will cumulatively explain only a small fraction of the total genetic variance in the general population, thereby necessitating continued analysis of their more common forms with less clear-cut modes of inheritance (Karet & Lifton, 1997). Unfortunately the strategy of applying a candidate gene approach in the human population has not enjoyed a great deal of success in identifying important disease susceptibility genes (Mitchell & Dyke, 1997). The reproducibility is poor between different populations and, even within the same or ethnically similar populations, different results have been obtained

depending on whether an association study or a linkage study was employed (Kuntz *et al*, 1997; Brand *et al*, 1998). Such observations serve to highlight the numerous methodological limitations imposed by the complex, polygenic, and multifactorial nature of human cardiovascular diseases.

The first major limitation lies in the fact that candidate genes are, by necessity, derived from the relatively small pool of genes recognised so far compared with the estimated total number of genes (> 50,000) in the human genome (O'Connor *et al*, 1996). It follows that if the disease-relevant genes are, as is likely, among the unknown majority, they will never be found by this approach (Dominiczak & Lindpaintner, 1994).

Secondly, the use of blood pressure as the phenotype in human studies has been a source of criticism since it is known that this phenotype is not stable, fluctuates greatly throughout the day and is particularly effected by stress (Lander & Schork, 1994). Furthermore, diagnostic thresholds for hypertension can vary from country to country and are arbitrary, having been based on cut-off points at which therapeutic interventions have been perceived to be of benefit in reducing health risks rather than on true qualitative demarcations. It follows that while the optimal method of determining "the" blood pressure of an individual in population studies remains to be determined, technical differences between clinics may produce artefactual data.

Association studies do not concern familial inheritance patterns but are case-control studies based on a comparison of unrelated affected and unaffected individuals from a population. An allele at a gene of interest is said to be associated with the trait if it occurs at a

significantly higher frequency among affected compared with control individuals. Positive associations can arise if the allele is a cause of the disease, in which case the association should be found in all populations, or if the allele is in linkage disequilibrium with a disease causing allele (Mitchell & Dyke, 1997). Unfortunately the presence of an association can also arise as an artefact of population admixture as any trait present at a higher frequency in an ethnic group will show positive association with any allele that also happens to be more common in that group. It follows that association studies are only meaningful if performed within relatively homogeneous populations, which are rare (Lander & Schork, 1994).

Classical parametric linkage analysis involves proposing a model to explain the inheritance pattern of phenotypes and genotypes observed in a pedigree. It is the method of choice for simple Mendelian traits because the allowable models are few and easily tested. However, fitting such a precise model that adequately explains the inheritance pattern in common complex traits is impossible (Lander & Schork, 1994). Some members of a family may present the putative susceptibility gene and not the trait (incomplete penetrance), or present the trait and not the gene (phenocopy), either because of the polygenic and heterogeneous diversity of the disease and human population at large, epistatic and ecogenetic interactions, environmental factors alone, or phenotypic misdiagnosis. Model-free non-parametric methods relying only on allele sharing by affected first-degree relatives have been developed, the idea being that affected relatives should show excess allele sharing even in the presence of incomplete penetrance, phenocopy, and genetic heterogeneity (O'Connor *et al*, 1996). However, allele-sharing methods are often less powerful than a correctly specified linkage model as the relatively late-onset of most complex diseases leads to a shortage of sib-sib and parent-offspring pairs.

It follows that all the above methodological difficulties imposed by trying to study directly the genetic determinants of complex human cardiovascular traits have given major impetus to the use of similar, but inherently simpler, paradigms in experimental animal models of genetic hypertensive cardiovascular disease, where the importance of heredity factors has long been clearly and unquestionably demonstrated (Tanase *et al*, 1970; Lindpaintner, 1993).

1.3 Genetic Models of Hypertensive Cardiovascular Disease

Animal models, and in particular the rat, offer several advantages for genetic research. Their low cost, ease of handling and breeding, access to sophisticated measurements, short generation time, and large litters all serve to remove some of the complexity inherent in studying human subjects and families (Lovenberg, 1987). This has been extended further by the production of inbred, genetically homogeneous rat models of hypertensive cardiovascular disease exhibiting particularly relevant phenotypes (Dominiczak & Lindpaintner, 1994).

The approach most commonly used to develop inbred models of hypertensive cardiovascular disease has been to measure blood pressure in a large number of non-inbred animals and then selectively breed those having the highest blood pressures (Kurtz *et al*, 1994). In each successive generation the offspring with the highest blood pressures are brother x sister mated to produce an inbred strain. After 20 generations of brother x sister mating the offspring should be homozygous at >99% of loci and therefore all animals within the strain nearly isogenic (Rapp, 1983). This solves the problem of aetiological

heterogeneity such as is present in the human disease state, while also allowing the production of a large number of progeny. Moreover, the ability under conditions of standardised animal husbandry to control confounding environmental factors provides the opportunity to investigate far more complex environmental and genetic interactions than is ever possible in even the most closely supervised clinical trials (Dominiczak & Lindpaintner, 1994).

A useful parallel to the diversity found in human hypertensive cardiovascular disease is maintained by the existence of a number of commonly used in-bred strains (*Table 1.4*). Each of these rat strains has some unique pathophysiological features linked to the development of the disease and thus resemble disorders found in subgroups of hypertensive subjects worthy of independent genetic investigation, including left ventricular hypertrophy and stroke (Ganten, 1987). In particular, development of rat models derived from the spontaneously hypertensive rat are now widely used for hypertension-related diseases and others (Yamori & Swales, 1994).

1.3.1 The Stroke-Prone Spontaneously Hypertensive Rat

In 1963, Okamoto and Aoki reported the production of a colony of spontaneously hypertensive rats (SHR) by selective in-breeding of individual normotensive Wistar-Kyoto (WKY) rats with elevated blood pressure from the Animal Center Laboratory, Kyoto University Faculty of Medicine, Japan (Yamori & Lovenberg, 1987). The typical lesions often associated with human hypertensive cardiovascular disease, including cerebral (infarction, haemorrhage), myocardial (infarction, fibrosis) and nephrosclerotic (benign,

Name and Origin of Strain	Systolic Blood Pressure (mmHg)
Spontaneously Hypertensive Rat (SHR) Kyoto, Japan	200
Stroke-Prone Spontaneously Hypertensive Rat (SHRSP) Kyoto, Japan	230*
Milan Hypertensive Rat (MHS) Milan, Italy	175
Dahl Salt-Sensitive Rat (SS/Jr) Brookhaven, USA	170
Lyon Hypertensive Rat (LH) Lyon, France	180
Sabra Hypertensive Rat (SBH) Jerusalem, Israel	185
Genetically Hypertensive Rat (GH) Dunedin, New Zealand	180

Table 1.4 Genetically inbred hypertensive rat strains in common use. *Refers to mean systolic blood pressure recorded in Glasgow colony of SHRSP (Davidson *et al*, 1995).

malignant), were all observed in SHR after three months of age, indicative of their potential as a good model for the human condition (Okamoto & Aoki, 1963).

The SHR were separated into 3 main substrains in 1971 and at that time it was confirmed that the incidence of spontaneous cerebrovascular disease was different among these - high in substrain A (80%) and low in B and C (50%) - despite the similar blood pressures. Suspecting the involvement of independent genetic factors in the pathogenesis of stroke, Okamoto and co-workers undertook successive selective breeding of substrain A by maintaining the offspring only from the SHR which died with stroke. This resulted in the production of a stroke-prone spontaneously hypertensive rat, or SHRSP (Okamoto *et al*, 1974).

The SHRSP displayed a rapid onset of hypertension after birth and by three months of age had a systolic blood pressure of 230mmHg, compared to the 125mmHg of the normotensive WKY control. The SHRSP had a high tendency to naturally develop cerebrovascular lesions, particularly in the anteromedial and occipital cortex and the basal ganglia, with over 90% of all males suffering stroke while on a Japanese diet of high salt, low potassium and protein (Nagaoka *et al*, 1976). In contrast to regular SHR strains which were known to be resistant to salt, the SHRSP were found to be salt sensitive (Yamori *et al*, 1981).

Given the immediate interest in the SHRSP and its relatives for hypertension and stroke research, they were rapidly distributed around the world. In particular, WKY may have been issued from Japan as early as the F10 generation (Kurtz *et al*, 1989). This world-wide distribution, prior to complete in-breeding (F20 required at least), has resulted in significant

genetic heterogeneity between different colonies (Matsumoto *et al*, 1991; Samani *et al*, 1989; Kurtz *et al*, 1989; Nabika *et al*, 1991). Whilst this does not effect their utility, it does necessitate an awareness of the source of the SHRSP, SHR, or WKY, when making any comparison of experimental results obtained by different research groups.

There are numerous pathophysiological similarities between hypertensive cardiovascular disease in SHRSPs and human subjects which promote their application to genetic studies, although classical atheroma does not develop (Frohlich, 1986). For example, in common with human essential hypertension, male SHRSPs have higher blood pressures than females. Left ventricular hypertrophy is also a common feature of the SHRSP (Yamori *et al*, 1979). Furthermore, their intracerebral perforating arteries show fibrinoid necrosis similar to that observed in severe clinical hypertension (Yamori *et al*, 1976a). Whilst the most preponderant site for stroke in SHRSP is the cortical region (69.8%), the next highest is the basal ganglia (24.5%), in common with humans. Indeed, a branch of the middle cerebral artery (the lenticulostriate artery) is responsible for basal ganglia lesions in both the SHRSP and humans.

It follows that the SHRSP represents the most suitable in-bred model of human hypertensive cardiovascular disease in which to begin the dissection of the specific genetic determinants of hypertension, stroke and left ventricular hypertrophy, their interactions, and the influence of environment, in particular salt. However, as indicated by the studies of the Mendelian forms of human hypertension, any success in elucidating the causal phenomena is as dependent on strong and defined phenotyping as on the appropriateness of the genetic approach. For this reason, great efforts are being made to provide an error free, high fidelity

and wholly encompassing method of continuous blood pressure measurement. In addition, clearly quantifiable models of organ damage, such as experimental focal cerebral ischaemia, are also being considered as alternative phenotypes.

1.3.2 Measurement of Blood Pressure in the Rat

In genetic studies the greatest consideration must be given to the circumstances under which blood pressure is measured (Kurtz *et al*, 1994). If measurements of blood pressure are obtained in stressed animals not only will they have an added element of environmental artefact but one may be selecting largely for stress-induced hypertensive genes rather than those contributing to spontaneous hypertension in a less stressful environment. There are three commonly used methods of measuring blood pressure in the rat; tail-cuff plethysmography, in-dwelling arterial catheter and radio-telemetric monitoring.

Originally described by Byrom & Wilson (1938), the most commonly used indirect method of blood pressure measurement involves the use of a cuff for the occlusion and slow release of blood flow in the tail of the rat as well as a variety of techniques to ascertain the occluding pressure. Whilst this permits quick and inexpensive repeated measurements on a large number of animals, this method possesses several prerequisites known to induce stress (Bunag, 1983). Firstly, the recording procedure requires considerable restraint. Secondly, a system for warming the rats before using a tail-cuff is absolutely essential. Recording at room temperature the blood flow through the tail arteries is too low to allow reliable reading (Sponer *et al*, 1988). However, as rats regulate their body temperature primarily by changing blood flow in their tails (Rand *et al*, 1965), heating to approximately 34°C ensures

that arterial pulsations during deflation of the cuff are easily detected. Both factors can introduce pronounced pressor effects and thus errors which falsely exaggerate the incidence of experimental hypertension.

That this method is of limited value for detailed genetic analysis is made clearer by the fact that it is unable to provide long-term continuous monitoring and measurements of any parameters of blood pressure other than systolic. Should diurnal variation in blood pressure or any other component be under different genetic control to systolic pressure, it will be missed using this method. It follows that whilst it may still be useful for an initial screening of a genetic strain, the stress induction involved and its narrow parameters are likely to introduce damaging artefacts when attempting the identification of subtle additive effects in ecogenetic and epistatic interactions.

The most commonly used method to directly examine blood pressure in conscious rats, the chronic catheterisation of a major systemic artery, possesses many of the same artefact-inducing errors as tail-cuff plethysmography (Bunag, 1983). During surgical implantation, the fluid filled arterial catheter's outer end is externalised subcutaneously to emerge at the nape. This is connected to pressure transducers which have been calibrated with a recording device. Whilst this method gives accurate and direct measures of systolic, diastolic and mean arterial pressure it is invasive, the trauma of the operation and anaesthesia inducing stress. Furthermore, maintenance of infection free post-operative animals and unclogged cannulae represent significant problems. Stagnation of blood flow resulting after arterial cannulation encourages the formation of blood clots and fibrous outgrowths which can clog

up the catheter. Emboli from carotid catheters may cause renal infarction while those from iliac or femoral catheters may result in hind-leg paralysis (Bunag, 1983).

Radio-telemetry systems for chronic implantation have progressed over the last 30 years from simple devices allowing the measurement of one physiological parameter (Mackay, 1970) to more complex systems capable of simultaneously monitoring several cardiovascular variables (Rubenson *et al*, 1984), including systolic, diastolic, pulse and mean arterial blood pressure, heart rate and motor activity. As a consequence of continued advances in miniaturisation techniques, telemetry units small enough to be implanted inside the abdominal aorta and remain inside the rat body cavity are now available. The Dataquest IV system (Data Sciences International) consists of the implantable transmitter which measures the pressure, the receiver panel, which detects the signal from the implantable transmitter and converts it to a form readable by computer, the pressure reference module which measures atmospheric pressure to be converted to a gauge pressure, and the data acquisition software which accepts data from the reference module and receivers, converts the telemetered pressure to mmHg, subtracts atmospheric pressure from the telemetered pressure and stores the data for retrieval, plotting and analysis.

Whilst implantation of the transmitter does require invasive surgery, the capacity for long-term monitoring (measurement is continuous night and day and sampling as frequent as every five minutes over a six month period or more) allows time for blood pressure and heart rate to stabilise post-operatively. For example, Davidson and co-workers (1995) observed a necessary 12 day period before collection of haemodynamic measurements for data analysis. There is no requirement for heating, and the animals are unrestrained and free

to move within their cages placed over the receiver panel. It follows that several studies have begun to indicate that radio-telemetry is an accurate and reliable, if expensive, means of determining cardiovascular parameters in long-term studies, yielding chronic measurements that are repeatable and free of stress-induced artefacts (Brockway *et al*, 1991; Guiol *et al*, 1992; Bazil *et al*, 1993).

The considerable utility of radiotelemetry in genetic research has already been demonstrated. Davidson *et al* (1995) performed two large reciprocal crosses, with the SHRSP as a male progenitor of one and with the normotensive WKY a male progenitor of the other. The resulting F2 hybrids were phenotyped using radiotelemetry. Their major finding (*Figure 1.3*) was a highly significant elevation of systolic, diastolic and mean arterial pressure at both baseline and after salt-loading in male F2 hybrids with the Y chromosome originating from the SHRSP grandfather (Ys) compared to those hybrids with the Y chromosome originating from the WKY grandfather (Yw) . These results suggested that the SHRSP Y chromosome contains a locus or loci which contribute to hypertension in this model, an association which has been confirmed by the production of congenic strains utilising the SHR (Turner *et al*, 1991; Ely *et al*, 1993). Previous studies had shown conflicting evidence on the contribution of the Y chromosome locus or loci to the hypertension in genetically hypertensive models (Ely & Turner, 1990; Hilbert *et al*, 1991; Vincent *et al*, 1994). It is conceivable that the artefacts associated with the methods used to record blood pressure in these studies (tail-cuff and direct femoral artery catheter) were responsible for the discrepancies.

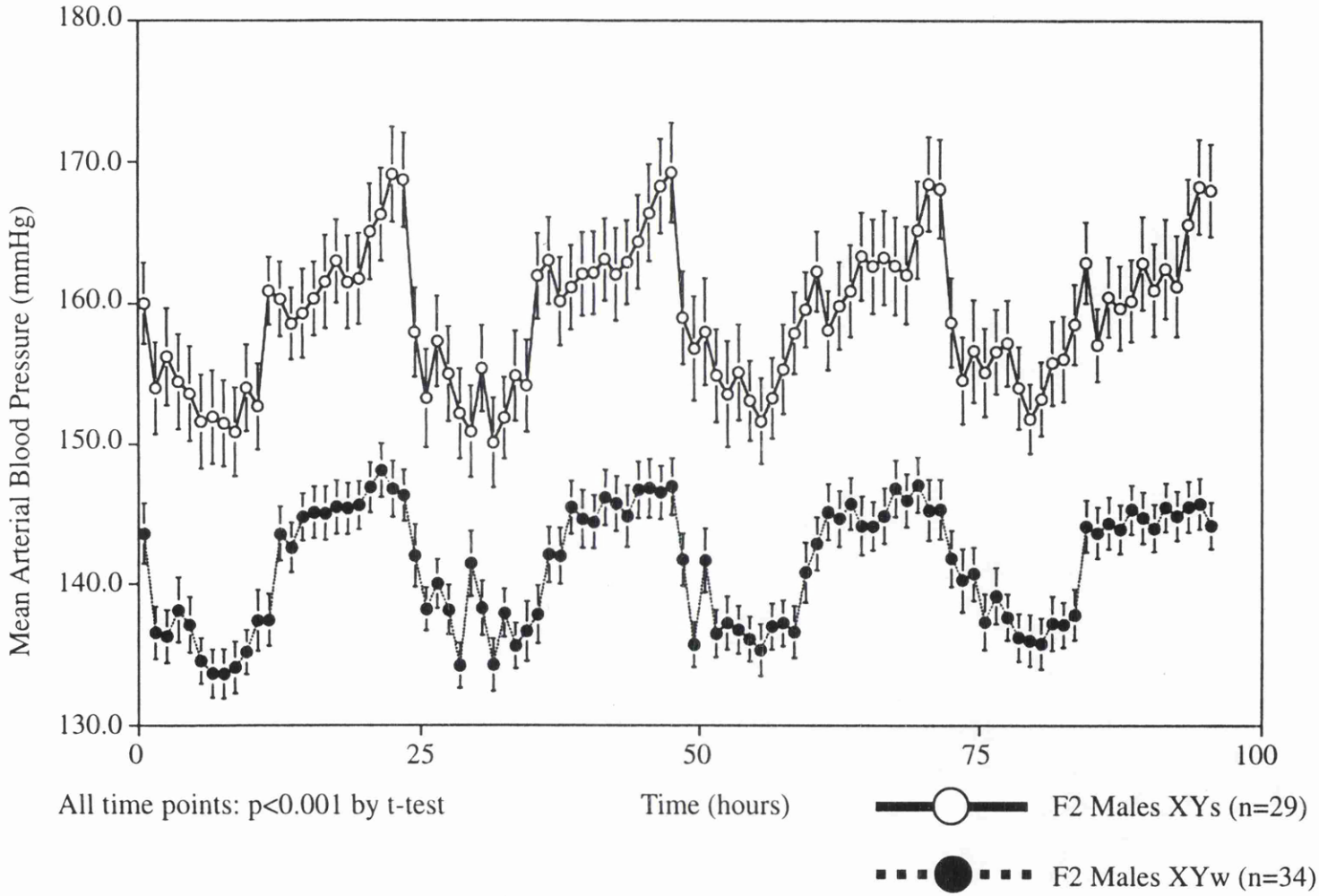

Figure 1.3 The effect of the Y chromosome on mean arterial blood pressure (MAP) in SHRSP x WKY F2 hybrids. XYs denotes F2 males who have inherited their Y chromosome from a SHRSP grandfather, XYw from WKY grandfather. (adapted from Davidson *et al*, 1995).

1.3.3 Experimental Focal Cerebral Ischaemia in the Rat

Animal models of stroke have provided an invaluable contribution to the current understanding of the pathogenesis of cerebral ischaemia by removing variables which confound the interpretation of human stroke data (Macrae, 1992). The severity, duration, location and cause of the ischaemia can be controlled, co-existent disease states and variations in cerebrovascular anatomy avoided, and physiological parameters such as blood pressure, blood gases, temperature and plasma glucose, all of which influence the magnitude of the ischaemic lesion, closely monitored and controlled.

A wide diversity of animal species have been used to study cerebral ischaemia but the majority of experiments are now carried out in rats, not least because of the relatively low cost, the small brain size suited to fixation methodology, and the close similarity between the anatomy of the arterial supply to the cerebral hemispheres in the rat and man (Yamori *et al*, 1976a). Each has anterior, middle and posterior cerebral arteries which give rise to cortical and basal perforating branches. In the rat, all three arteries are derived mainly from the internal carotid arteries but connect via an azygos interior cerebral artery and via posterior communicating arteries to form a modified circle of Willis. The middle cerebral artery (MCA) supplies the lateral part of the cerebral hemisphere through its cortical branches, whilst the penetrating branches supply the putamen, the caudate nucleus, the outer globus pallidus, the posterior limb of the internal capsule and the common radiata (Adams & Victor, 1989). Both the size of the MCA and the territory are larger than those of the anterior cerebral artery (ACA) and posterior cerebral artery (PCA).

The permanent occlusion of the middle cerebral artery (MCA) in the rat is the definitive model of focal cerebral ischaemia and one of the most relevant animal stroke models to man as this is reported to be the vessel most commonly affected in human stroke syndromes (Berkow & Fletcher, 1987; Mohr *et al*, 1986; Karpiak *et al*, 1989). The standard subtemporal approach (Tamura *et al*, 1981) involves microbipolar coagulation of the MCA proximal to the lenticulostriate arteries, reducing cerebral blood flow to below 25ml/100g/min within the core territory of the MCA. This produces, without mortality, a significant, reproducible infarction in both the frontal cortex and lateral part of the caudate nucleus equivalent to a massive hemispheric ischaemic stroke in man. A range of autoradiographic and histopathological methods have been developed that quantify in a precise anatomical manner the severity of the ischaemic insult (Osbourne *et al*, 1987). Area and distribution of the ischaemic infarction are normally assessed at eight coronal levels throughout the MCA territory and these data are computed to provide a total volume of ischaemic damage.

A subsequent variation in the model included the use of alternative strains of rat in order to improve the reproducibility of the infarct size or to incorporate a recognised risk factor such as hypertension (Duverger & MacKenzie, 1988). The SHR and SHRSP have been shown to have much larger and less variable infarcts after MCA occlusion than most, if not all, other strains (*Figure 1.4*), including the WKY (Coyle, 1984; Coyle, 1986; Brint *et al*, 1988; Duverger & MacKenzie, 1988; Gratton *et al*, 1998). They display a greater infarcted surface area at any given coronal section and the caudal limit of the infarction is situated more posteriorly.

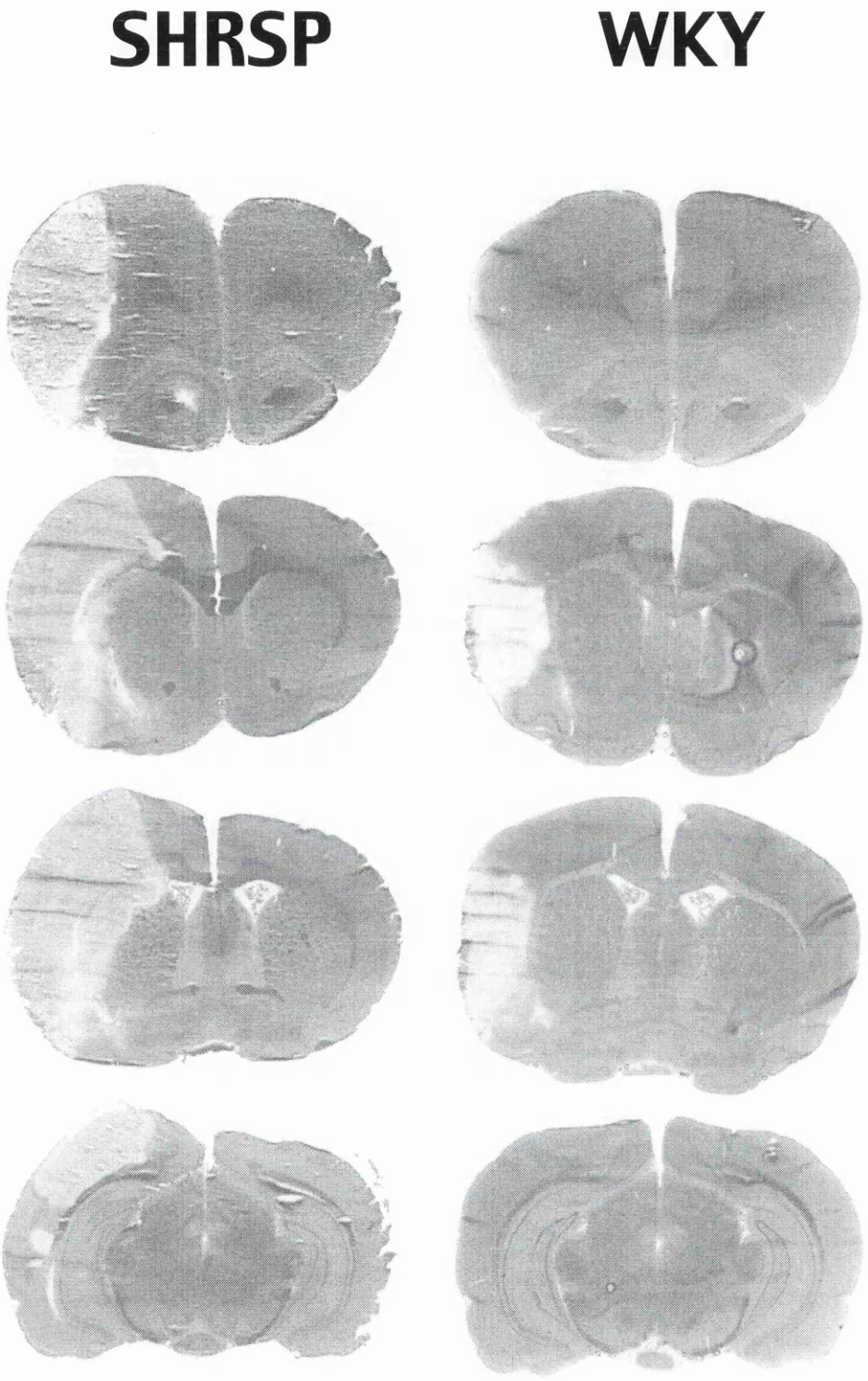

Figure 1.4 Cerebral infarction in the SHRSP and WKY following MCA occlusion showing the bimodality of this phenotype. MCA territory stained with haematoxylin and eosin to show the location and extent of ischaemia.

It was initially thought that the inherent hypertension was solely responsible for this increased susceptibility to focal cerebral ischaemia in the SHRSP. Vascular hypertrophy, a physiological adaptation of the vasculature to maintain circumferential wall stress relatively constant under high blood pressures, was believed to infringe on the vascular lumen (internal diameter), decreasing functional compliance and thus aggravating the infarct process (Folkow *et al*; 1973; Baumbach & Heistad, 1989). However, the reduction in the external diameter observed in cerebral arterioles (Baumbach & Heistad, 1989) cannot be wholly attributed to vascular hypertrophy. If hypertrophy increased the stiffness of arterioles, external diameter might be reduced, but hypertrophied cerebral arterioles actually become more distensible (Baumbach *et al*, 1988). In fact, it has been shown that hypertrophy of the vessels may actually protect against stroke as when vascular hypertrophy is reduced by chronic sympathetic denervation in SHRSP the incidence of spontaneous cerebral haemorrhage and infarction increases rather than decreases (Sadoshima *et al*, 1981 & 1983).

That hypertension is of secondary importance in determining susceptibility to cerebral infarction in SHRSP has been reinforced by other observations. Large infarcts after MCA occlusion have been observed to occur in a minority of normotensive rats suggesting that whatever governs infarct susceptibility it can be dissociated from hypertension and presumably occur with variable frequency in normotensive populations (Duverger & MacKenzie, 1988). Similarly, Coyle (1986) observed that chronic hypertension does not uniformly predispose rats to infarction as ~10% of young and adult SHRSP and SHR suffer small infarcts or none at all after distal, single point MCA occlusion, and correlation between pre-occlusion blood pressure and infarction size is poor. Coyle (1984) noted that

rats made hypertensive by deoxycorticosterone acetate and salt administration fail to develop large infarcts after MCA occlusion, whilst adult SHR in which hypertension is treated early and continuously with the angiotensin-converting enzyme inhibitor cilazipril develop infarcts that are only at most 20% smaller than the very large infarcts in the untreated hypertensive cohorts (Slivka, 1991; Fujii *et al*, 1992). Furthermore, SHRSP suffer larger infarcts in early youth before either hypertension or pathological changes in the vasculature become established (Coyle & Jokelainen, 1983; Gratton *et al*, 1998).

Several lines of evidence have subsequently led Baumbach & Heistad (1989 & 1991) to propose that infarct susceptibility in SHRSP arises from a detailed remodelling of cerebral arterioles and vascular collateral blood flow that is associated with, but not directly linked to, the vascular lesions of chronic hypertension. Collateral blood supply in all rats is by numerous small diameter pial surface collaterals of the MCA anastomosing with rami of the ACA or PCA to form an extensive network which plays a vital role in preserving blood flow following the occlusion of a major cerebral artery and thus limiting the volume of ischaemic damage (Coyle & Jokelainen, 1983). Coyle & Heistad (1986) identified that only five minutes after MCA occlusion blood flow through collateral vessels was significantly less in SHRSP than in WKY, clearly implicating collateral impairment as a major contributor in the creation of the large cerebral infarcts observed in SHRSP. This is supported indirectly by neuroprotection studies where drugs with flow-enhancing properties (e.g. L-type calcium channel blockers) are found to be more effective in SHR and SHRSP than NMDA glutamate antagonists with proven efficacy in normotensive strains (McCulloch, 1996; Sauter & Rudin, 1995). Indeed, whilst no difference has been observed in the number of ACA-MCA anastomoses, or their distribution, the internal diameter measured in SHRSP is

only 60% of that in WKY and a strong correlation exists between lumen diameter and of cortex spared ischaemic damage at the zone of anastomoses (Coyle, 1986). As to the cause of remodelling, and its interrelationship with hypertension, much research remains to be completed, although the demonstration of geometric disorganisation of medial layers of cerebral vessels in the SHRSP independently of blood pressure (Arribas *et al*, 1996) does suggest a genetic component.

That infarct susceptibility in the SHRSP may have a genetic cause led Coyle and co-workers (1984) to investigate its pattern of inheritance in a F2 generation and series of subsequent backcrosses derived by crossing a male outbred normotensive Wistar (NW) rat with several SHRSP females. They showed a bimodal distribution of this phenotype and by observing the frequency of infarcting and non-infarcting animals concluded that susceptibility to experimental ischaemia is inherited through a single gene locus best described as autosomal recessive (Coyle *et al*, 1984). Subsequently Gratton *et al* (1998) observed that the distribution of infarct volumes in the first filial (F1) generation produced by crossing in-bred SHRSP and WKY rats virtually matched that in parental SHRSP, strongly suggesting a dominant mode of inheritance for this phenotype. Possible reasons for the two different conclusions may include Coyle's use of outbred normal Wistar rats in place of inbred WKY rats, and a less severe, more distal occlusion in much younger animals (Gratton *et al*, 1998). That a role exists for genetic factors in spontaneous stroke of SHRSP had previously been highlighted by the stroke-resistance of the related SHR despite similar hypertension and diet (Okamoto *et al*, 1974). Indeed, by studying the latency to stroke, or age of stroke occurrence, on a high salt diet, Nagaoka and colleagues (1976) observed this phenotype was characterised by a polygenic inheritance. It seems likely therefore that the genes

governing latency to stroke could well be unrelated to those governing infarct size and that the pathophysiological mechanisms may be quite different.

1.4 Genetic Dissection of Hypertensive Cardiovascular Disease the Rat

The successful development of experimental inbred strains of hypertensive cardiovascular disease presents a useful adjuvant to the genetic study in humans. They preserve the polygenic character of the disorder whilst providing a reduction in complexity due to the lack of genetic heterogeneity, the standardisation of environmental influences, and the availability of high fidelity phenotyping as described above. Indeed, crude biometric estimates from breeding experiments in various hypertensive strains have shown the number of independent genes affecting blood pressure to be between 2 and 6 (Rapp, 1983). This reasonably low number of loci suggests the feasibility of identifying the effects of these genes, as well as those independently influencing stroke and left ventricular hypertrophy, in such models (Harrap, 1994).

Traditionally genetic research using inbred hypertensive rat strains was undertaken in a solely comparative fashion by contrasting them with a control (Lander & Schork; 1994). A variety of biochemical, anatomic and histological characteristics have been used as phenotype parameters for this approach, and over a 100 differences found (Dominiczak & Lindpaintner, 1994). Unfortunately most of these have subsequently proved unrelated to the pathogenesis of hypertension or its sequelae (Yamori, 1982). Rather, these differences have evolved as a consequence of genetic drift (random selection and fixation of alleles unrelated

to the regulation of blood pressure) during the process of selective breeding and subsequent inbreeding (Rapp, 1983).

In order to tackle this, Rapp (1983) formulated a set of criteria that must be fulfilled for differences between normotensive and hypertensive strains to be considered pathogenetically important. The most powerful of these is the requirement that the (intermediate) phenotype investigated must cosegregate with an increment in blood pressure in an F2 or backcross population of rats derived from a cross of the hypertensive and control strains, as it allows the distinguishing of random association of two phenotypes from fairly solid evidence for a causal relationship between the two (Rapp, 1983). Among phenotypic traits that have been shown to cosegregate with blood pressure in the SHRSP are noradrenaline induced oscillatory activity and increased lymphocyte potassium efflux (Dominiczak & Lindpaintner, 1994). Unfortunately, the confounding question remains whether the cosegregating trait represents a causative factor, or is simply a secondary phenomenon of the hypertension.

This problem has been solved in recent years by the development of a series of practical DNA-based markers which are genetically distinguishable (dimorphic) between the hypertensive and the normotensive strains in terms of differences in DNA structure, and thereby allow the cosegregation of both genotype and phenotype to be tested in a F2 generation (Lindpaintner, 1992). The earliest markers resulted from the recognition of restriction fragment length polymorphisms (RFLPs). Digestion of genomic DNA with restriction enzymes results in DNA cleavage at specific sequences in a candidate gene of interest. If a dimorphism exists between two strains, the restriction enzyme will cut at

different sites and the resulting DNA fragments will differ in size. This can be visualised after electrophoretic fractionation by hybridisation against complementary molecular probes (Jeffreys, 1979).

As a probe for the sequence of interest is required, it follows that RFLP analysis has been limited to the cosegregation analysis of several characterised candidate genes with hypertension. Given the relative paucity of characterised genes (see *Chapter 1.2.3*) this may mean that a chromosomal region containing a gene of interest is missed. Indeed, even when a positive cosegregation is determined it need not automatically implicate this gene as pathogenic; rather, it may arise as a result of a close association to the actual disease-causing gene which is as yet unknown. For example, whilst a significant association between the renin gene marker and hypertension has been found in the Dahl strain of sodium-sensitive hypertension (Rapp *et al*, 1989), similar analyses in the SHR have proved negative (Lindpaintner *et al*, 1990; Kurtz *et al*, 1990), and there is no evidence from biochemical studies for a pathogenic role of the renin-angiotensin system in the Dahl rat.

Alternative strategies without *a priori* hypotheses regarding candidate genes have been made possible by the identification of variable DNA sequences composed of randomly organised stretches of the same sequence of nucleotides repeated over and over. These sequences are widely dispersed throughout the entire genome, show a high degree of polymorphism, and in themselves are usually not associated with any particular gene and thus functionally meaningless (Weber & May, 1989). The simplest types of repeats, termed microsatellite markers, are characterised by short sequence motifs, such as a two base core unit of dCdA, repeated up to a dozen times (Weber & May, 1989). Locus specificity is

coupled to length polymorphism (Beckman & Weber, 1992) and large numbers of individuals are easily typed by polymerase chain reaction (PCR). It follows that a genome wide scan utilising both candidate genes and anonymous microsatellite markers to identify quantitative trait loci (QTLs) has successfully emerged as the first major step towards ultimate identification of genes influencing complex traits.

1.4.1 Genome Wide QTL Analysis

A QTL is a broad chromosomal region containing a gene, or set of genes, influencing a quantitative trait such as blood pressure, heart weight, or volume of cerebral infarction. The main tools necessary for QTL mapping include linkage analysis, a large segregating F2 population constructed from two inbred strains, high fidelity phenotyping of the F2 cohort such as described in *Chapters 1.3.2* and *1.3.3*, and a large panel of markers which are dimorphic between the two inbred strains and complete a thorough coverage of the rat genome.

Genetic linkage occurs when two genes or markers are physically close together on the same chromosome. During Prophase I of meiosis, homologous chromosomes pair. Each consists of two sister chromatids as DNA replication has taken place. Chiasmata form between homologous chromatids and crossing over and exchange of genetic material occurs. The closer together on a chromosome two genes are, the less likely a chiasma will form between them, the less likely they will recombine and the closer the linkage. If genes are far away from each other or on another chromosome, they will recombine frequently and segregate independently.

Genetic linkage is measured by the recombination fraction, θ, the number of recombinants observed divided by the total number of meiotic events (progeny). If loci are unlinked, whether on different chromosomes or far away from each other on the same chromosome, approximately half of the progeny will be recombinants, $\theta = 50\%$. If genes are linked, $\theta < 50\%$ and parental combinations will predominate. If genes are very closely linked and no recombination events take place between them then $\theta = 0\%$. As the frequency of recombination events occurring between two genetic loci is a function of their physical distance on a chromosome, the recombination fraction may be converted into distance. This distance is expressed in centiMorgans (cM) where one cM represents 1% likelihood of recombination, which corresponds to approximately 1-2 million base pairs of the mammalian genome (Lindpaintner, 1992).

The principle underlying identification of QTLs by linkage to marker loci is conceptually simple. A segregating second filial (F2) population is derived by crossing affected (for example SHRSP) and wild-type (WKY) inbred strains to produce a uniformly heterozygous first filial (F1) population, which is then brother-sister mated. This is a powerful population in which to detect QTLs statistically by linkage analysis as many informative meiotic events are available for study. Any marker which is dimorphic has three possible genotypes in the F2 progeny; homozygous ss (possessing both alleles from the SHRSP), homozygous ww (possessing both alleles from the WKY), and heterozygous ws (possessing one allele from both strains). Individuals are scored for their genotype at the marker loci spread throughout the genome, and their phenotype for the quantitative trait. If there is a significant difference in quantitative trait levels between the genotype groups at a particular marker, one can infer the presence of a QTL. Alleles at a locus not genetically linked to the

causative gene will segregate independently and there will be no statistical association of the marker genotypes with the phenotype.

Traditionally, marker loci across the genome have been considered singly using a one way analysis of variance (ANOVA) to calculate the contribution of alleles to the genetic variation of the phenotypic trait (Sax, 1923; Soller & Brody, 1976), a significance level of $p < 0.05$ indicative of linkage with 95% probability (Rapp & Deng, 1995). However, as a given marker may be some distance from a QTL, simultaneous analysis of markers is preferable to establish greater significant evidence for the QTL's existence. It follows that sophisticated computer packages such as MAPMAKER (Lander *et al*, 1987) have been developed which undertake multipoint maximum-likelihood linkage analysis using an interval mapping approach (Lander & Botstein, 1989).

The first task of MAPMAKER is to construct a genetic linkage map with the aid of a mapping function. These are used to describe the relationship between recombination fraction and genetic map distance, converting θ into cM distances (Lander *et al*, 1987). Haldane's mapping function is widely used and is given as $\theta = 0.5(1-e^{-2\omega})$ where ω is equal to the map distance in Morgans (Haldane, 1919). Alternatively, Kosambi's mapping function may be used as a more realistic formula, $\omega = 0.25 \ln (1+2\theta/1-2\theta)$. This takes chiasma interference into account which occurs when the presence of a chiasma inhibits the formation of chiasmata in nearby regions (Kosambi, 1943; Coneally & Rivas, 1980).

Once a map has been constructed, MAPMAKER examines each marker for linkage to the trait and compares them with adjacent markers. The significance of genetic linkage is

determined by the logarithm of the ratio of the odds (or likelihood) of obtaining a set of linkage data (a QTL) over the odds of obtaining the same data by chance (no linkage). Termed a LOD score, it is described as $Z = \log_{10} [L(\theta)/L(0.5)]$ and is calculated at various levels of θ to identify the value at which Z is greatest (Zmax). This is considered the best estimate.

Traditionally, the evidence for a putative QTL and the amount of phenotypic variance it explains is considered significant when Z was equal to or greater than 3.0, which corresponds to 1000:1 odds in favour of linkage. A LOD score of -2 or less indicates evidence of exclusion of linkage. More recently, Lander & Kruglyak (1995) have proposed stringent LOD thresholds (*Table 1.5*) to distinguish between suggestive linkage (statistical evidence that would be expected to occur one time at random in a genome scan) and significant linkage (statistical evidence that would be expected to occur 0.05 times at random in a genome scan). Whilst suggestive linkage results may be incorrect, they are worth reporting if accompanied by the appropriate label of significance. It also follows that as a LOD score of 3.0 may occur by chance 5% of the time, the greater the LOD value the greater the confidence in the linkage result.

The multipoint maximum likelihood linkage approach was first used for mapping QTLs controlling fruit characteristics in tomato plants (Paterson *et al*, 1988) and has since been successfully applied in mammals (Rise, 1991; Hilbert *et al*, 1991; Jacob *et al*, 1991). Its success depends on many factors, among them the genetic make-up of the cross. In strains such as the SHR with few effective genes, each with a relatively large contribution to the overall genetic variance of the phenotype, it will be easier to detect QTLs than in strains

Mapping Method in the Rat	Suggestive Linkage	Significant Linkage
Backcross (1 d.f.)	$p = 3.4 \times 10^{-3}$; LOD = 1.9	$p = 3.4 \times 10^{-4}$; LOD = 3.3
Intercross (1 d.f., co-dominant)	$p = 3.4 \times 10^{-3}$; LOD = 1.9	$p = 3.4 \times 10^{-4}$; LOD = 3.3
Intercross (1 d.f., recessive)	$p = 3.4 \times 10^{-3}$; LOD = 2.0	$p = 3.4 \times 10^{-4}$; LOD = 3.4
Intercross (1 d.f., dominant)	$p = 3.4 \times 10^{-3}$; LOD = 2.0	$p = 3.4 \times 10^{-4}$; LOD = 3.4
Intercross (2 d.f.)	$p = 3.4 \times 10^{-3}$; LOD = 2.8	$p = 3.4 \times 10^{-5}$; LOD = 4.3

Table 1.5 LOD thresholds for mapping loci underlying complex traits (adapted from Lander & Kruglyak, 1995). d.f. refers to degrees of freedom.

such as the genetically hypertensive (GH) rat with a larger number of genes each making a small contribution (Okamoto & Aoki, 1963; Smith & Hall, 1958). Similarly the inter-observation variance of phenotype measurements correlates inversely with the likelihood of success in a linkage study due to the introduction of excessive noise (Dominiczak & Lindpaintner, 1994), necessitating the development of high fidelity phenotyping. Obviously, the quality of genome coverage by the marker panel used, *i.e.* the even distribution of the markers and their spacing, will also critically effect the chances of success in a mapping project (Rapp, 1991).

1.4.2 Blood Pressure QTLs

A $SHRSP_{Heidelberg}$ x WKY F2 cross phenotyped for both baseline and salt-loaded systolic and diastolic blood pressure by femoral artery cannulation was the first mammalian model to undergo genome wide QTL analysis in search of the genetic determinants of high blood pressure (Hilbert *et al*, 1991; Jacob *et al*, 1991). Screening a panel of 240 mini- and microsatellite markers revealed three chromosomal loci which showed LOD scores of over 3.0. Indeed, both Jacob *et al* (1991) and Hilbert *et al* (1991), in utilising the same data from the F2 cross, mapped a locus (*BP1* or *BP/SP-1*) on rat chromosome 10 which contributed significantly to salt-loaded systolic and diastolic blood pressure with LOD scores of over 4.8 and p-values of less than 0.0001, respectively (*Figure 1.5*). This locus contains a particularly relevant candidate gene coding for the angiotensin converting enzyme (ACE). ACE mediates the synthesis of angiotensin II and the degradation of bradykinin, two vasoactive peptides involved in the regulation of blood pressure.

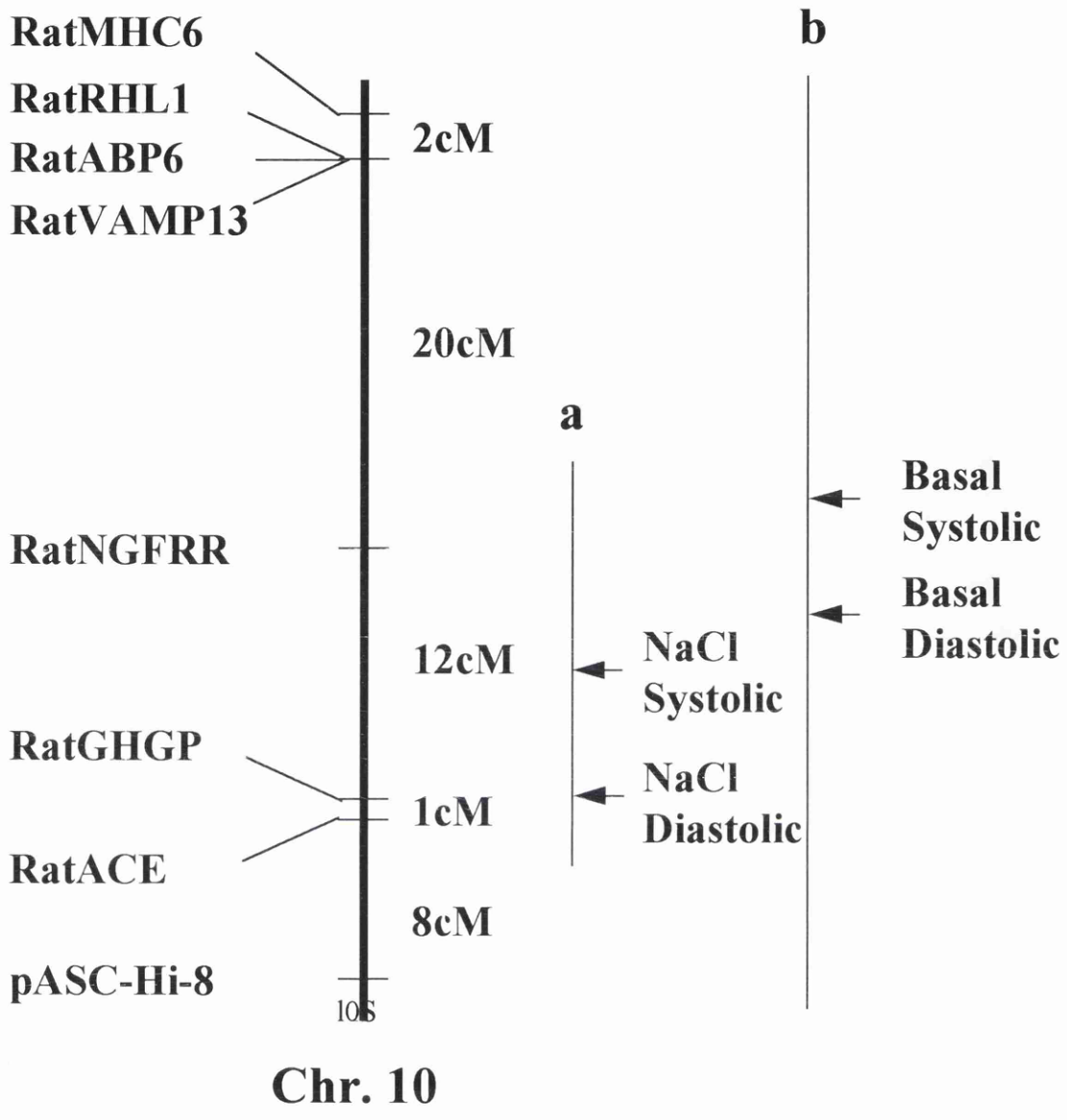

Figure 1.5 100:1 odds support intervals for the *BP-1* locus on rat chromosome 10 (Hilbert *et al*, 1991; Jacob *et al*, 1991). Lines **a** and **b** indicate the determined 100:1 odds support interval for salt-loaded (NaCl) and baseline blood pressure respectively. RatACE represents a marker within the angiotensin converting enzyme gene.

Jacob *et al* (1991) mapped a second locus (*BP2*) on chromosome 18 which was linked to baseline diastolic blood pressure with a LOD score of 3.23 whilst Hilbert *et al* (1991) mapped a X-linked locus contributing to hypertension. Subsequently, additional blood pressure QTLs have been identified in the $SHRSP_{Izumo}$ (Nara *et al*, 1993; Nara *et al*, 1996) on chromosomes 1 (basal blood pressure only) and 3 (basal and salt-loaded). As *Table 1.6* shows, many other groups have identified these same loci in other experimental crosses indicating that they are of general relevance in rats. Indeed many additional blood pressure QTLs have been located. As a QTL allele may have differing effects when bred onto a different genetic background (Rapp & Deng, 1995), these genetic mapping results need not be consistent among different crosses and thus clearly demonstrate that hypertension is a truly polygenic disease.

Having confirmed the polygenic nature of hypertension, several groups have gone on to investigate its complex pathophysiological heterogeneity with the identification of loci that may specifically influence the blood pressure response to salt (Hilbert *et al*, 1991, Jacob *et al*, 1991, Gu *et al*, 1996), the temporal change in blood pressure (Samani *et al*, 1996), and individual haemodynamic components of blood pressure (Dubay *et al*, 1993). However, few of these have proved wholly comprehensive due to several limitations in experimental design. For example all have concentrated on males only whilst it is likely that autosomal sex-specific QTLs exist (Ely & Turner, 1990). Secondly, the majority rely on artefact ridden methods of blood pressure measurement which may either mask true genetic determinants, or mis-report false ones, all of which results in making a complex task doubly so.

Chr	Rat Strains	Central Gene/Markers	Lod score	Reference
1	WKY x $SHRSP_{(Izm)}$	*Lsn, Myl2*	4.5	Nara *et al*, 1993
	Lewis x Dahl SS	*D1Mco1, Cytp450*	3.4	Gu *et al*, 1996
	Lewis x Dahl SS	*Sa*	2.5	Gu *et al*, 1996
	ACI x FHH	*Mt1pa*	4.2	Brown *et al*, 1996
	Sabra HR x Sabra HP	*D1Mit2, Sa*	4.9	Yagil *et al*, 1998
	Sabra HR x Sabra HP	*D1Mit1, Cytp450*	4.7	Yagil *et al*, 1998
	Donryu x SHR	*D1mit3*	4.3	Innes *et al*, 1998
2	Lyon N x Lyon H	*Cpb*	7.0	Dubay *et al*, 1993
	WKY x Dahl SS	*Nakα1*	3.4	Deng *et al*, 1994
	MNS x Dahl SS	*Camk*	2.6	Deng *et al*, 1994
	RI (BN x SHR)	*D2N35*	n/a	Pravenec *et al*, 1995
	BN x SHR	*Mt1pb*	3.0	Schork *et al*, 1995
	BN x SHR	*Gca*	6.3	Schork *et al*, 1995
	BN x SHR	*R598*	3.0	Schork *et al*, 1995
	BN x GH	*Gca*	n/a	Harris *et al*, 1995
	WKY x $SHRSP_{(Gla)}$	*D2Mit6*	3.4	Clark *et al*, 1996
	WKY x $SHRSP_{(Gla)}$	*D2Mit14*	3.1	Clark *et al*, 1996
	WKY x SHR	*D2Wox7*	5.6	Samani *et al*, 1996
	Lewis x Dahl SS	*D2Mco19*	2.9	Garrett *et al*, 1998
3	WKY x $SHRSP_{(Izm)}$	*D3Mgh16*	n/a	Matsumoto *et al*, 1995
	WKY x $SHRSP_{(Gla)}$	*D3Mgh16*	5.6	Clark *et al*, 1996
	WKY x $SHRSP_{(Izm)}$	*D3Mgh12*	6.2	Nara *et al*, 1996
	Lewis x Dahl SS	*D3Mgh6*	3.0	Garrett *et al*, 1998
4	RI (BN x SHR)	*Il-6*	n/a	Pravenec *et al*, 1995
	BN x SHR	*Npy2*	4.6	Schork *et al*, 1995
5	WKY x SHR	*Mitr1678, Anp, Bnp*	4.2	Zhang *et al*, 1996
	Lewis x Dahl SS	*Edn2*	4.5	Garrett *et al*, 1998
8	BN x SHR	*R850*	5.1	Schork *et al*, 1995
10	WKY x $SHRSP_{(Hd)}$	*Ace*	n/a	Hilbert *et al*, 1991
	WKY x $SHRSP_{(Hd)}$	*Ace*	5.1	Jacob *et al*, 1991
	WKY x Dahl SS	*Nos2*	2.3	Deng *et al*, 1995
	MNS x Dahl SS	*Nos2*	6.3	Deng *et al*, 1995
	MNS x Dahl SS	*Ace*	4.8	Deng *et al*, 1995
	BN x GH	*Ace*	n/a	Harris *et al*, 1995
	Lewis x Dahl SS	*D10Wox6*	5.5	Garrett *et al*, 1998
13	Lyon N x Lyon H	*Renin*	5.6	Dubay *et al*, 1993
	WKY x SHR	*D13Mit2*	5.7	Samani *et al*, 1996
16	BN x SHR	*R220*	4.3	Schork *et al*, 1995
17	Sabra HR x Sabra HP	*D17Mgh5*	3.4	Yagil *et al*, 1998
18	WKY x $SHRSP_{(Hd)}$	*Rr1094*	3.2	Jacob *et al*, 1991
19	RI (BN x SHR)	*D19Mit7*	n/a	Pravenec *et al*, 1995
X	WKY x $SHRSP_{(Hd)}$	*Per-Ha-2/Per-Ha-7*	n/a	Hilbert *et al*, 1991

Table 1.6 QTLs for blood pressure. Normotensive strain cited first on all occasions.

1.4.3 Non Blood Pressure Dependent QTLs

Whilst the identification of QTLs influencing left ventricular hypertrophy and stroke does not currently match the number located for blood pressure, it has proven a worthwhile exercise with the isolation of a several putative susceptibility and/or severity loci confirming a role for genetics in both these phenomena. For example, three blood pressure independent QTLs for susceptibility to spontaneous stroke have been mapped to chromosome 1 (*STR-1*), 4 (*STR-3*) and 5 (*STR-2*) in a SHR x $SHRSP_{Heidelberg}$ cross by Rubattu *et al* (1996) which account in total for 28% of the overall phenotypic variance. Interestingly, both *STR-2* and *STR-3* appear to protect from stroke in the SHRSP as the SP/SP homozygotes had significantly greater latency to spontaneous stroke on a Japanese diet (high salt, low potassium and low protein) as compared to the SH/SH homozygotes. *STR-2* localised in close proximity to the genes encoding atrial (*Anp*) and brain (*Bnp*) natriuretic peptides, both possessing important vasoactive properties.

Genome wide scans in two different crosses have revealed two different QTLs responsible for a proportion of left ventricular (LV) mass which are blood pressure independent. Pravenec *et al* (1995) studying recombinant inbred strains derived from the SHR and the normotensive Brown-Norway (BN) found a QTL around *Drd1a*, a microsatellite marker within the dopamine 1a receptor gene on rat chromosome 17, to be linked to LV weight. The animals homozygous for the SHR allele at this locus had the lowest LV weight to body weight ratios whereas those homozygous for the BN allele had the highest LV weight. This suggests that the identified QTL plays an inhibitory or protective role during the development of LV hypertrophy in the SHR. By contrast, Innes *et al* (1998) identified a

locus on rat chromosome 2 linked to relative LV mass with a LOD score of 4.3 in a cross of SHR and normotensive Donryu rats which accounted for 29.5% of the genetic variance. The average value for relative LV mass for rats homozygous for the SHR alleles at the central *D2Mgh15* marker was approximately 7% greater than rats homozygous for the Donryu alleles. A QTL for left ventricular hypertrophy has not previously been searched for in the SHRSP.

1.4.4 Fine Genetic Mapping

It is recognised that the identification of large QTLs (20-30cM) is only the first step towards understanding the fine genetic control of a trait. It is subsequently necessary to identify whether a QTL actually comprises a single genetic locus, and then locate the actual gene(s) involved. Darvasi *et al* (1993) have shown that confidence intervals for QTL mapping are approximately 10cM. Isolation of the DNA structure of an unknown disease-causing locus by positional cloning, however, requires the locus to be localised to within a 1- to 2-cM region. It follows that the practise of first distinctly establishing the chromosome regions containing the QTL by using congenic strains, and then narrowing down these regions to 1-2 cM in size using congenic substrains containing progressively smaller chromosomal regions, has evolved to enable the final task of gene identification (Rapp & Deng, 1995).

A congenic strain is a strain in which a small portion of a chromosome from one strain has been selectively replaced by the homologous portion of the same chromosome of another strain. A classic genetic procedure, the use of congenic strains was originally pioneered by

George Snell as a component of his Nobel Prize-winning strategy for the dissection of mouse histocompatibility and tumour resistance (Snell, 1948), and has been widely used in the study of genetic problems. Congenic strains are traditionally developed by repeatedly backcrossing a donor strain that harbours a QTL of interest with a recipient inbred strain (*Figure 1.6*). According to Mendel's laws it is expected that, on average, half of the unrelated genomic material will be transmitted to a subsequent backcross generation. Ten generations of backcross matings results in animals heterozygous for the loci of interest that are approximately 99.90% recipient genome and carry an introgressed region. The introgressed region is then made homozygous by brother-sister mating, resulting in the congenic strain (Rapp & Deng, 1995). Congenic strains can be constructed in two directions (*Figure 1.6*); that is a plus allele which increases the phenotype can be transferred from a hypertensive to a normotensive strain, or a minus allele which decreases the phenotype can be transferred from a normotensive to a hypertensive strain. Whichever, the successful production of a congenic strain allows precise and unequivocal localisation of genes contributing to complex traits and removes the inherent ambiguity involving the statistical nature of QTL localisation.

The utility of congenic strains can be clearly seen in *Table 1.7*. Whilst no further dissection of the QTLs for left ventricular hypertrophy or spontaneous stroke has yet been achieved, several congenic lines have been successfully produced, all more precisely localising regions of linkage to blood pressure. For example, new data on the $SHRSP_{Heidelberg}$ x WKY cross was generated via congenic strains which revealed that the *BP/SP-1* locus on chromosome 10 could be dissected into two loci, *BP/SP-1a* linked to baseline blood pressure and *BP/SP-1b* which cosegregated with blood pressure after salt-loading (Kreutz *et al*, 1995). These

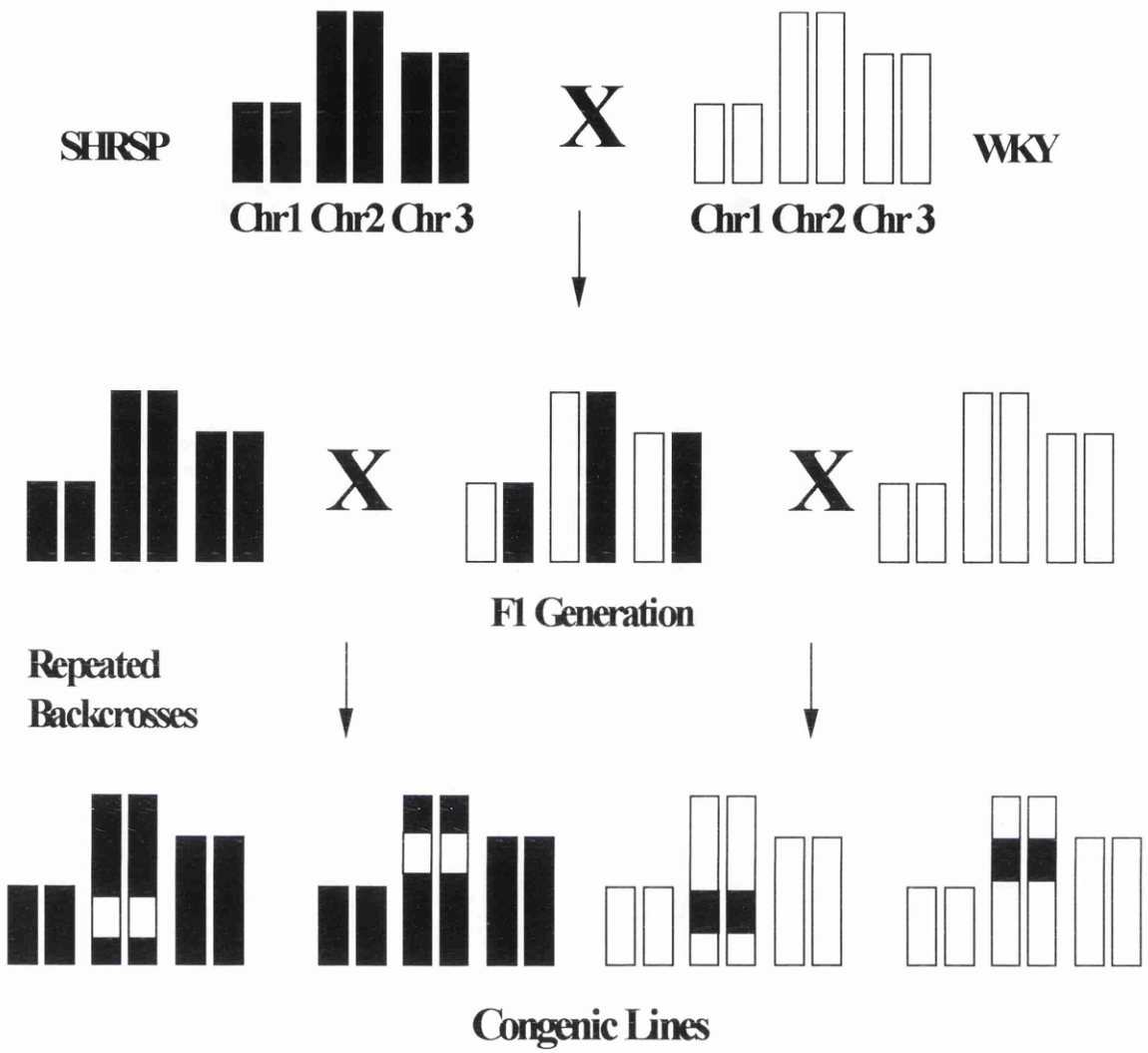

Figure 1.6 Congenic breeding theory. Each congenic line represents a new strain which, through a series of repeated backcrosses, has replaced a small portion of a chromosome (here chromosome 2) from the recipient strain with the homologous portion of the same chromosome of the donor strain. The donor and recipient strains are interchangable, that is the chromosomal region of interest may be transferred from the WKY to the SHRSP or *vice versa*.

Chr	Congenic Strain	Chromosomal Region Transferred (cM)	BP Change (mmHg)	Reference
1	SHR.BN	22	-10	St. Lezin *et al*, 1997
	Dahl SS.LEW	75	-26	Garrett *et al*, 1998
2	Dahl SS.WKY	41	-44	Deng *et al*, 1997
	Dahl SS.MNS	76	-29	Deng *et al*, 1997
5	Dahl SS.LEW	70	-15	Garrett *et al*, 1998
7	Dahl SS.Dahl RR	16	-40	Cicila *et al*, 1997
8	SHR.BN (*Lx*)	31	-20	Kren *et al*, 1997
9	Dahl SS.Dahl RR	21	-19	Rapp *et al*, 1998a
10	WKY.SHRSP$_{Hd}$	6	+5	Kreutz *et al*, 1995
	Dahl SS.MNS (10a)	31	-42	Dukhanina *et al*, 1997
	Dahl SS.LEW	60	-42	Garrett *et al*, 1998
13	DahlRR.DahlSS_{renin}	19	-8	St. Lezin *et al*, 1996
	DahlSS.DahlRR_{renin}	6	+27	Jiang *et al*, 1997
	DahlSS.DahlRR_{renin}	24	-35	Zhang *et al*, 1997
Y	SHR.WKY	Whole	-15	Turner *et al*, 1991
	WKY.SHR	Whole	+35	Turner *et al*, 1991

Table 1.7 Congenic strains for blood pressure QTLs. Recipient strain cited first on all occasions, donor strain second.

loci map closely onto potentially relevant candidate genes, the inducible nitric oxide synthase (*Nos2*) gene (*BP/SP-1a*) and the angiotensin I converting enzyme (*Ace*) gene (*BP/SP-1b*). Subsequent studies by Dukhanina *et al* (1997) described four congenic strains constructed by introgressing various segments of chromosome 10 from the Milan normotensive strains (MNS) onto the background of the Dahl salt sensitive (SS) strain. The results not only confirmed the existence of a blood pressure QTL with a strong effect (35-42mmHg) on rat chromosome 10 but the overlapping congenic strains developed in this experiment allowed the authors to exclude the locus for *Nos2* as a candidate.

All the congenic studies published so far have used the traditional breeding strategy which requires at least 8-10 backcrosses to obtain a 99% homogenous genetic background. However, theoretical calculations by Lander & Schork (1994) suggest that the production of a congenic strain can be expedited by repeated screening of microsatellite markers at multiple loci scattered throughout the recipient genome to ensure the choice of the breeder with the least donor alleles in the genetic background, concomitant with selection of the donor allele at the locus of interest. This novel "speed" congenic approach has recently proved feasible in mice (Markel *et al*, 1997), congenic strains having been constructed in only three or four generations. It has yet to be tested experimentally in the rat.

However a congenic strain is produced, the next stage in the refining and narrowing down of the QTL incorporates the concept of fine genetic substitution mapping (*Figure 1.7*) which has been used extensively in agricultural research (Paterson *et al*, 1990). The size of the new narrowed down QTL will depend on the density of microsatellite markers in the chromosomal region of interest (Dukhanina *et al*, 1997). It follows that an essential

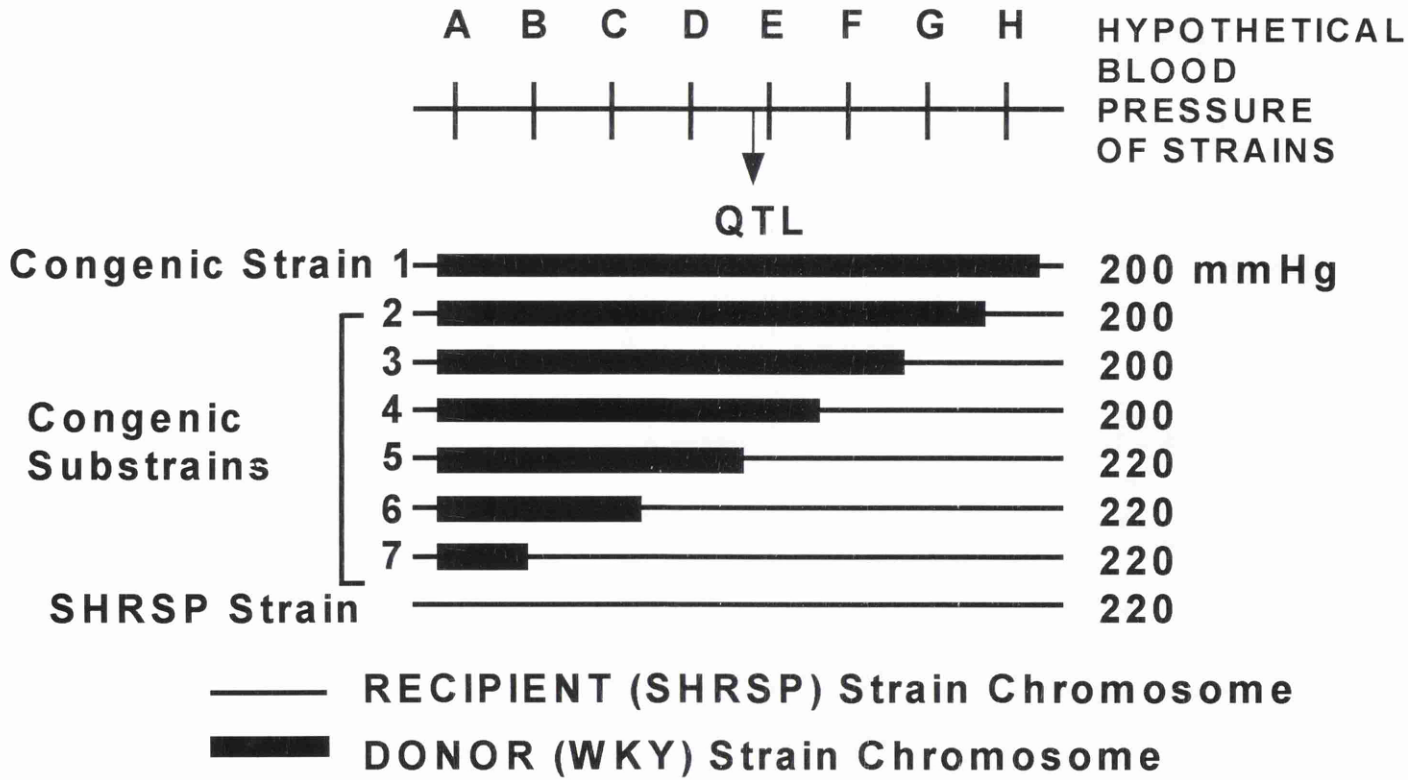

Figure 1.7 Substitution mapping of a QTL with the use of congenic strains. A relatively large QTL from the normotensive strain defined by markers A through H is substituted into the genetic background of the hypertensive strain resulting in the congenic strain 1. Strain 1 is then backcrossed to the hypertensive strain and the progeny genotyped at markers A through H to select rats with crossovers in various places throughout the region. Congenic substrains 2 to 7 are then produced and fixed in the homozygous state. The narrowed down QTL is between markers D and E as shown by a step change in blood pressure among strains (modified from Rapp & Deng, 1995).

requirement of fine mapping is a very dense and detailed map of the rat genome otherwise there will be no way of scoring for chromosomal crossovers within the particular region of interest and the size of the refined QTL will be limited. With the steady progress of the Rat Genome Project and international collaboration on new marker development, the coverage of all chromosomal regions has been vastly improved. Several rat genomic database resources are available on the Internet and are beginning to yield very dense linkage maps. For example, whilst the first rat linkage map based on microsatellite markers reported by Serikawa *et al* (1992) contained just 125 microsatellites, an integrated genetic linkage map of the laboratory rat recently released by Brown *et al* (1998) contains 1164 markers. Despite this, the smallest blood pressure QTL reported in a rat so far is 6cM in size (Kreutz *et al*, 1995; Jiang *et al*, 1997) and hence the final task of positional cloning of the genes responsible remains elusive at this time.

When eventually QTLs of interest have been saturated with new markers and narrowed down to the order of 1cM or less (approximately 1Mb of DNA), positional cloning of the causal gene(s) will be possible using yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), phage P1 clones (PACs), cosmids, and bacteriophage clones to create a physical map of the relevant region. These tools are becoming increasingly available for the rat (Cai *et al*, 1997; Woon *et al*, 1998) and can be used in parallel to generate contigs (clusters of overlapping clones representing the contiguous DNA from a genomic region), initially by screening for clones containing the closest flanking genetic markers. The limit of this approach is the distance between the two closest recombination events in the congenic substrain. Once this limit is reached, an exhaustive search of the entire region is required to find coding sequences that are transcribed to produce mRNAs. One can expect

that a region of 1 to 2cM will yield roughly 50 mRNAs. Finding these expressed sequences and assessing them as candidates for the phenotype under investigation will be a challenge and a combination of methods are currently thought possible, including exon trapping, cDNA selection, zoo blot analysis and direct sequencing (Collins, 1992). However identified, candidate genes will then have to be analysed both *in vitro* and *in vivo* to determine the presence of functional mutations which may be responsible for the phenotypes under study.

1.5 Aims

The principal aim of this study is to use a genome wide scanning strategy combined with high fidelity phenotyping to identify QTLs containing genetic determinants of hypertensive cardiovascular and cerebrovascular disease in the Glasgow colony of SHRSP, followed by the development of appropriate congenic strains with which to narrow them down. It is hoped that this will subsequently lead to the positional cloning of the genes. The steps to be taken in order to achieve this aim include:

1. The completion of a total genome scan in a F2 SHRSP x WKY cross with multiple phenotypes including systolic, diastolic and mean arterial blood pressure at baseline and after salt-loading as measured continuously by radio-telemetry, as well as left ventricular hypertrophy. This will be the first genome scan to consider autosomal male and female responses to blood pressure, to use a radio-telemetry system in an entire F2 cross, and to consider the genetic aspects of left ventricular hypertrophy in SHRSP.

2. The completion of a second total genome scan in SHRSP x WKY F2 hybrids characterised by the volume of cerebral ischaemia following acute middle cerebral artery occlusion. This will be the first genome scan to search for the QTL(s) responsible for large infarcts in both male and female SHRSP, as well as testing the contribution of hypertension to the trait.

3. The beginning of fine genetic mapping of any QTLs identified by constructing "speed" congenic strains in both directions, *i.e.*, by (A) transferring a plus allele which increases

blood pressure, left ventricular mass or cerebral ischaemia from the SHRSP to the WKY genetic background and looking for an increment in phenotype and by (B) transferring a minus allele from the WKY to the SHRSP and looking for a decrement. This will be the first study to experimentally test the applicability of the speed congenic strategy in the rat by making full use of the increasingly detailed maps of the rat genome.

4. Direct sequencing of any strong candidate genes brought to attention by either genome scan in order to detect any functional mutations which may be related to phenotype. This will only be justified by highly significant linkage to a QTL and will also be used to supplement the fine genetic mapping.

2. METHODS

2.1 General Laboratory Practice

All reagents and apparatus were of the highest quality commercially available. Appendix I provides a full list of solutions prepared, reagents used, and the sources of each. Laboratory coats and gloves (Healthline Products Ltd.) were worn and changed frequently throughout all experimental procedures. When deemed necessary by the hazard labelling of reagents laboratory spectacles, face masks and a fume cupboard/hood (Holliday, Fielding & Hocking, Ltd.) were utilised to fulfil the necessary safety requirements.

Weighing of reagents was performed on a Mettler AT250 (European Instruments Sales) balance up to five decimal places or a Mettler P2000 balance (European Instruments Sales) to three decimal places. Aqueous solutions were prepared in autoclaved glass-distilled water and mixed on a HB502 stirrer/hotplate (Bibby Sterilin Ltd.). Measurements of pH were performed using a C0720 digital pH meter (W.P.A., Cambridge) regularly calibrated with a solution of pH 7.0 prepared from buffer tablets. Volumes in the range of 1-5000µl were transferred accurately using the appropriate Gilson pipettes (Gilson Medical Electronics) and sterile tips (Alpha Laboratories Ltd.). Centrifugation of up to 14000rpm was obtained using either a Centrifuge 5402 (Eppendorf) for small samples (~1500µl) or an IEC Centra-7R Refridgerated Centrifuge (Life Sciences International (UK) Ltd.) for larger samples. All items of glassware were washed in solutions of the detergent Decon 75, rinsed with distilled water and dried in an oven at 37°C.

2.2 Rat Strains and Cross Designations

All rats were housed under controlled conditions of temperature (21°C) and light (12-hour light/dark cycle; 7 AM to 7 PM) and maintained on normal rat chow (rat and mouse No.1 maintenance diet, Special Diet Services) and water *ad libitum*. Litters were weaned, sexed and ear-tagged (National Band and Tag Co.) for identification at 3 weeks of age and housed according to sibling group and sex thereafter.

2.2.1 Rat Strains

Inbred colonies of SHRSP and WKY rats have been established at the University of Glasgow since December 1991. These result from the strain-specific brother-sister mating of thirteen SHRSP and thirteen WKY (6 males and 7 females of each) which were a gift made by Dr. D.F. Bohr in the Department of Anatomy and Cell Biology at the University of Michigan, USA, where they have been maintained as inbred colonies for more than 15 years. Their breeding stocks were originally obtained from the National Institutes of Health, Bethseda, U.S.A. Only SHRSP with adult blood pressures meeting 200-230mmHg for males and 170-190mmHg for females, and WKY of 130-150mmHg (males) and 100-130mmHg (females), are selected as breeders in order to maintain the genetic integrity of the colony and its distinct hypertensive versus normotensive characteristics. As described later, regular microsatellite screens are also performed on the colony to confirm homozygosity at all loci and thus the purity of the inbred strains.

2.2.2 F1 and F2 Generations

To yield the F1 and F2 rats utilised in both genome scans two large reciprocal genetic crosses were performed following the breeding protocol detailed in *Figure 2.1*. One male SHRSP was mated with 2 WKY females (cross 1) and 1 male WKY with 2 SHRSP females (cross 2) to produce the first filial (F1) generation. From the F1 rats of each cross, 3 males and 6 females were brother sister mated to generate the second filial (F2) generation. Reciprocal crosses ensure that a Y chromosome of both hypertensive and normotensive origin is represented in any genetic analysis of the F2 generation.

2.2.3 Congenic Crosses

As discussed in *Chapter 1.4.4* the traditional development of a congenic strain is a time consuming endeavour, requiring 8-10 generations of backcrosses. Following stages A to E outlined in *Figure 2.2* a "speed" congenic breeding procedure previously untested in the rat was utilised in this study to enable the transfer of loci of interest as indicated by the genome scans from the donor SHRSP (black shading) to the genetic background of the recipient WKY (white shading) and *vice versa* over only 4 or 5 generations of backcrosses.

Stage A (*Figure 2.2*) involved the production of two reciprocal F1 generations by mating 1 male SHRSP with 2 WKY females (cross 1) and 1 male WKY with 2 SHRSP females (cross 2). Two reciprocal crosses were necessary to allow the bi-directional congenic strains to not only possess each recipient strain's autosomal genetic background, but also the appropriate origin of sex chromosomes.

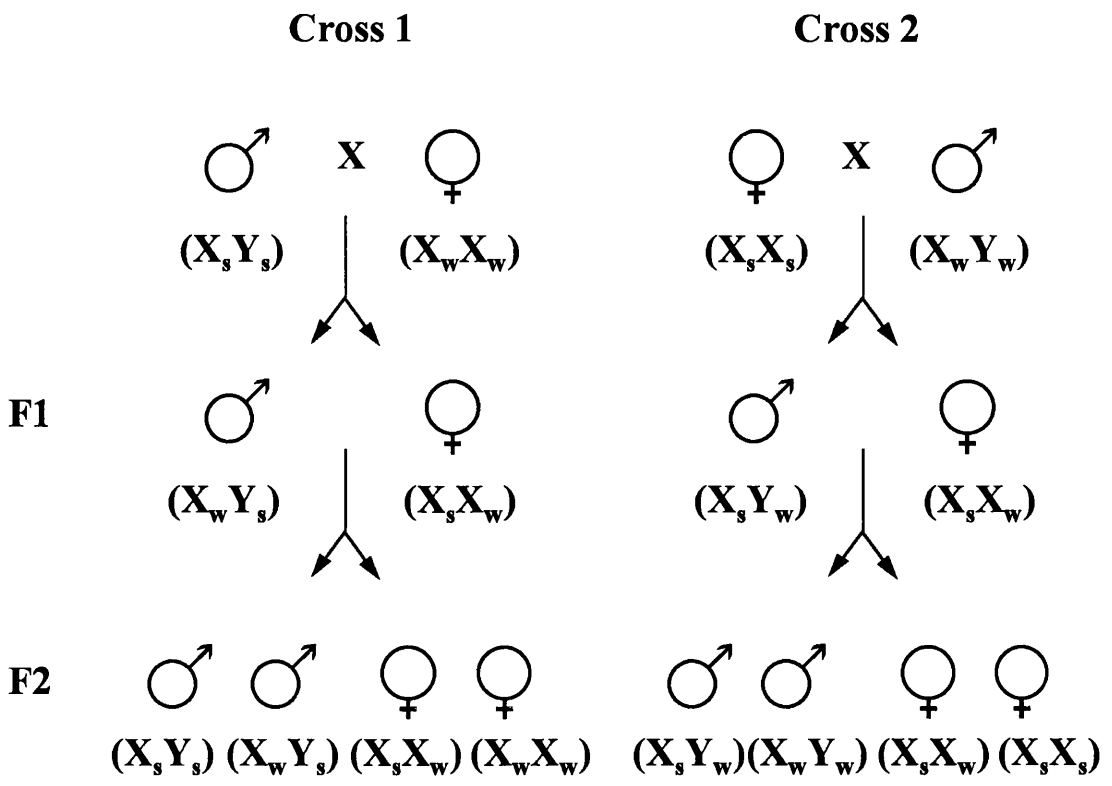

Figure 2.1 F2 reciprocal crosses. Diagram shows cross 1 and cross 2 with the origin of each sex chromosome in a subscript; X_s denotes X chromosome originating from the SHRSP, Y_s denotes Y chromosome originating from the SHRSP, X_w denotes X chromosome originating from the WKY, Y_w denotes Y chromosome originating from the WKY.

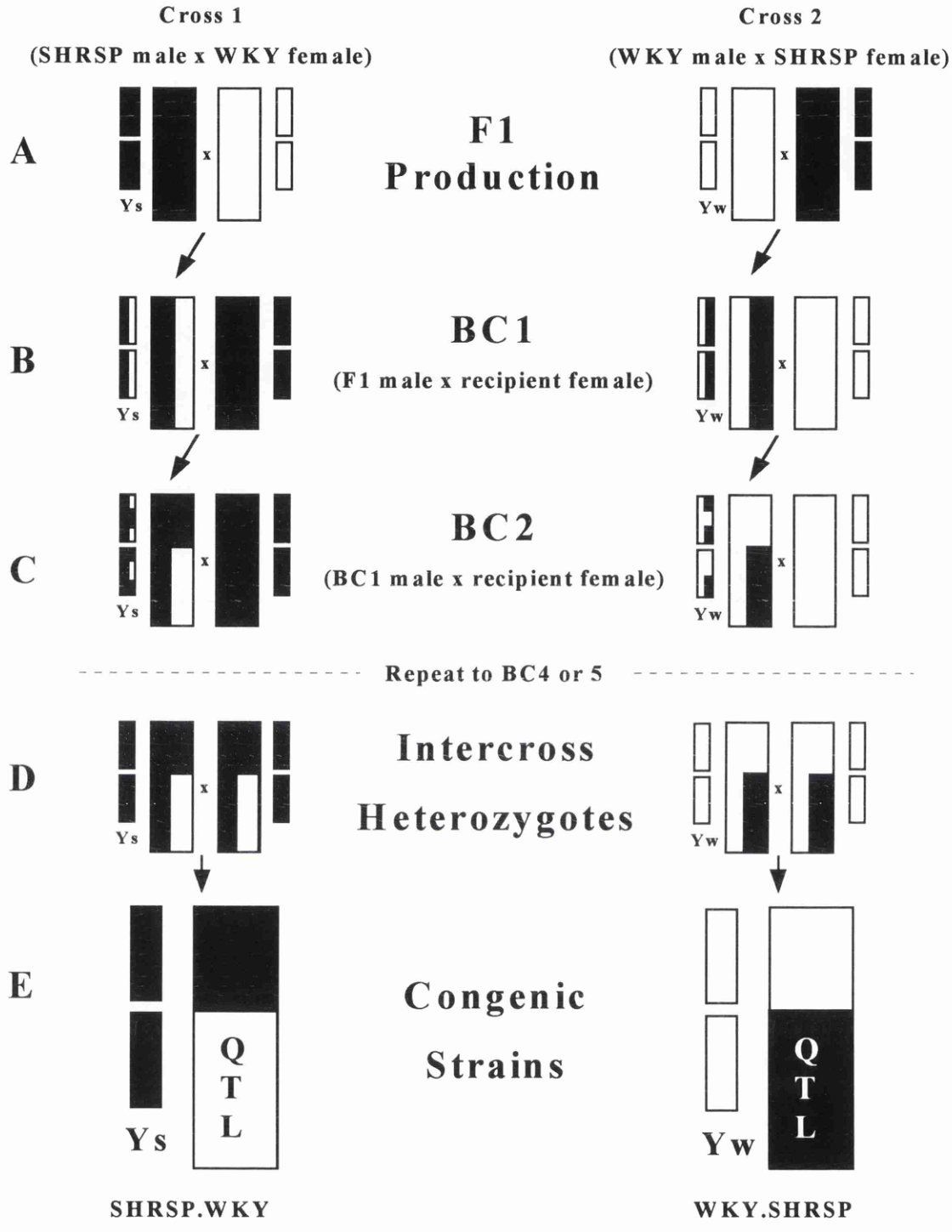

Figure 2.2 Schematic representation of the bi-directional speed congenic strategy. Dark shading = SHRSP alleles; white shading WKY. Large rectangle is the chromosome containing the QTL of interest, the smaller two representative of the genetic background. Stages A-E described in text.

Having produced a F1 generation, stage B (*Figure 2.2*) involved male F1 hybrids from cross 1 being mated with the recipient SHRSP strain (one male to two females) and male F1 hybrids from cross 2 mated to the recipient WKY strain (one male to two females). Microsatellite markers throughout the desired QTL, and an additional 53 broadly spanning the remaining genome, were genotyped in the resulting offspring. Those individuals identified as heterozygous (ws) for the marker alleles within the QTL, but mostly homozygous (ss or ww) for the recipient alleles (SHRSP and WKY respectively) everywhere else were selected as the "best" males for breeding and thus backcrossed again to the respective recipient strain to produce a second backcross (BC2 - stage C *Figure 2.2*). This procedure was repeated in all offspring after every backcross and thus allowed the number of generations necessary to eradicate the donor's genetic background to be reduced to between BC4-BC5 whilst maintaining heterozygosity in the region of interest.

Having removed the donor's background, Stage D (*Figure 2.2*) involved the intercrossing of two individuals heterozygous sw in the area of interest. As the offspring segregate 1ss:2sw:1ww following Mendel's laws, only individuals homozygous for the donor strains' alleles were selected and bred together to fix the donor allele in the homozygous state on the recipient background, thereby finally producing the congenic strain (stage E *Figure 2.2*). The number of animals required to achieve this varied greatly depending on litter size and the size of the region (and thus number of microsatellite markers) being transferred.

A possible criticism of this "speed" approach is that no matter how complete the coverage of the genetic background achieved by microsatellite markers, it is still possible that a region of heterozygosity in the background will be missed and thus fixed along with the QTL in the

final production of the congenic strain. It follows that any phenotypic change observed may not be due to the QTL, but these hidden "stowaway" loci containing residual genetic material from the donor strain. In order to control for this, those animals which were found to be homozygous at stage D (*Figure 2.2*) for the recipients' alleles in the QTL rather than for the donors' alleles were also intercrossed to provide a "congenic control". These individual's have been through the same selection processes as the true congenics and whilst not containing the donor QTL they will contain in their genetic background the same, if any, residual heterozygosity. Whether or not these animals go on to display a change in phenotype similar to that observed in the true congenics will not only determine the validity of the QTL, but also the use of a speed congenic strategy in rats.

2.3 Phenotypic Analysis

All experiments were approved by the Home Office according to regulations regarding experiments in animals in the United Kingdom.

2.3.1 Tail-Cuff Plethysmography

When the need for severe surgical procedures, such as the experimental occlusion of the middle cerebral artery (MCA), excluded the use of radio-telemetry to measure blood pressure, systolic blood pressure was measured both by the direct cannulation of the femoral artery during MCA occlusion (*Chapter 2.3.6*) and indirectly in conscious rats by tail plethysmography following the protocol of Evans *et al* (1994) overleaf.

Immediately prior to measurement rats were held within well-aerated polystyrene boxes containing familiar bedding and pre-warmed to 36°C under a low blue light intensity for 30 minutes in order to allow the vessels of the tail to become fully dilated. The rat was then wrapped in a cloth and an inflation cuff, controlled using a current/pressure transducer (Hartmann & Braun type 2), placed around the tail along with a piezoceramic transducer for pulse detection. The cuff pressure could be controlled in 1mmHg steps over a range of 0-300mmHg and the resulting pulsation signals detected by the piezoceramic transducer were amplified, filtered and then displayed on computer with the aid of IBM compatible software. The pulsation signal was visualised as a function of pressure and an estimate of the systolic pressure marked. An average of 12 readings were taken for each rat at each sitting and an average value calculated around a standard deviation of approximately 10mmHg. Visually confirmed artefactual data was removed. Special care was taken to perform measurements in both the morning and afternoon in each rat at a given age. At least three sittings were taken, and the average of all three sittings taken as the value for that age.

2.3.2 Radio-Telemetry

The Dataquest IV telemetry system (Data Sciences International) was used for the direct measurement of systolic pressure, diastolic pressure, mean arterial pressure, heart rate, and motor activity. The monitoring system consists of an implantable transmitter (radio frequency transducer model TA11PA) which senses, processes, and transmits information digitally via telemetry from within the rat; a receiver panel which converts data to a form readily accessible by the Dataquest System; and a personal computer with accompanying software for collection and observation of data. Each implant was obtained in a sterile

condition and used a maximum of 3 or 4 times following re-sterilisation in Cidex (activated glutaraldehyde solution) and 0.9% saline and re-verification that the calibrations provided by Data Sciences remained accurate to within 3mmHg.

Surgical implantation of each blood pressure transmitter within the peritoneal cavity of a rat took place under standard sterile conditions (*Figure 2.3*). Rats were anaesthetised with fluothane, and the abdominal cavity exposed by the temporary externalisation of the intestines. Sloops were placed round the aorta just below the renal arteries, and around the left and right iliac artery. On tightening of these sloops to reduce blood flow, a small hole (1mm diameter) was quickly made with a needle tip in the abdominal aorta just above the point at which it begins to branch into the iliac arteries. The flexible catheter of the transmitter was then inserted pointing upstream against the flow of blood. The catheter was surgically secured with the addition of a sterilised cellulose patch and VetBond and the sloops removed from around the arteries. The intestines were returned to the body cavity and the transmitter sutured to the abdominal wall.

On removal of the anaesthetic, rats were housed in individual cages and provided with antibiotics to prevent infection. Each cage was placed over a receiver panel connected to the personal computer for data acquisition. The rats were unrestrained and free to move within their cages. Haemodynamic data were sampled every 5 minutes for 10 seconds. Preliminary experiments showed that blood pressure and heart rate took up to 12 days to stabilise post-operatively. Experimental observations were therefore collected from day 12 after surgery as "baseline haemodynamic measurements".

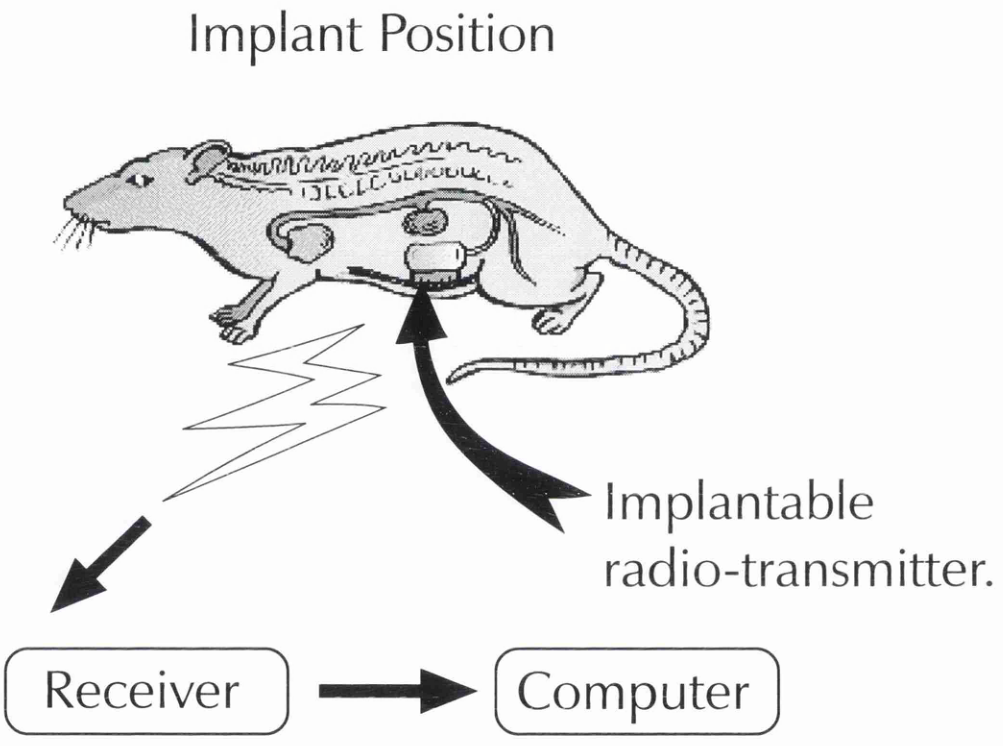

Figure 2.3 The Dataquest IV telemetry system. The radio-transmitter is implanted within the peritoneal cavity of the rat with the sensing catheter inserted into the abdominal aorta. (adapted from DataSciences International Brochure #SMD30000 REL02 1195).

2.3.3 Salt-Loading

Following baseline recording, rats on telemetry received 1% sodium chloride (NaCl) in their drinking water, and this was continued until they were euthanased. Measurements collected during this period were considered "salt-loaded haemodynamic measurements".

2.3.4 Body Weight

The body weight of each rat was obtained to within two decimal places using a CT200V portable top pan balance (Ohaus Co-operation). Where necessary, a deduction of 9g was made to take account of the telemetry probe.

2.3.5 Heart Weight and Left Ventricular Weight

Immediately after euthanasia the thorax was opened and the heart removed, blotted with tissue paper, and weighed using a Mettler AT250 balance to within three decimal places. Both atria and the right ventricle were then removed, and the left ventricle and septum weighed. The ratios of heart weight to body weight and left ventricle plus septum weight to body weight were then determined in order to correct for differences in body size.

2.3.6 Occlusion of the Middle Cerebral Artery

All surgical procedures involving the occlusion of the middle cerebral artery were kindly performed by Drs. Gratton and Carswell at the Wellcome Surgical Institute, Glasgow.

Anaesthesia was induced (5%) and maintained (1%) with fluothane in oxygen-nitrous oxide (30:70). Rats were intubated and mechanically ventilated via a small animal respirator pump and a femoral artery cannulated for chronic blood pressure recording and blood sampling. Normoxia (PaO_2 >100mmHg), normocapnia ($PaCO_2$ 34-42mmHg) and body temperature (rectal temperature 37°C) were maintained within physiological limits under anaesthesia (Blood Gas System; Ciba Diagnostics). Plasma glucose was recorded (Glucose Analyser; Beckman Instruments Inc.) and blood pressure monitored continuously under anaesthesia and again at 24hrs after ischaemia in conscious animals.

The distal portion of the left middle cerebral artery was surgically exposed via the subtemporal approach originally developed in Glasgow, with the zygomatic arch left intact (Tamura *et al*, 1981). Focal ischaemia was induced by microbipolar coagulation and division of a 2mm segment of the MCA just distal to the inferior cerebral vein (*Figure 2.4*). The temporalis muscle was then sutured, the wound closed and the anaesthetic withdrawn. The animals were maintained at 37°C until fully recovered from the anaesthesia.

Twenty-four hours after ischaemia, physiological variables were measured in the conscious animal before anaesthesia, decapitation and processing of tissue. Brains were frozen in isopentane (-42°C). Coronal sections (20μm) were cut semi-serially throughout the MCA territory on a cryostat (Bright Instrumentation Company Ltd.) and then stained with haematoxylin and eosin for histological verification of infarct volume. Areas of ischaemic damage and brain swelling were assessed, blind to rat strain or genotype, at eight pre-selected coronal levels from 10.5mm to 1.0mm to the inter-aural line (Konig & Klippel, 1963) with an MCID computer assisted image analyser (Imaging Research, Ontario,

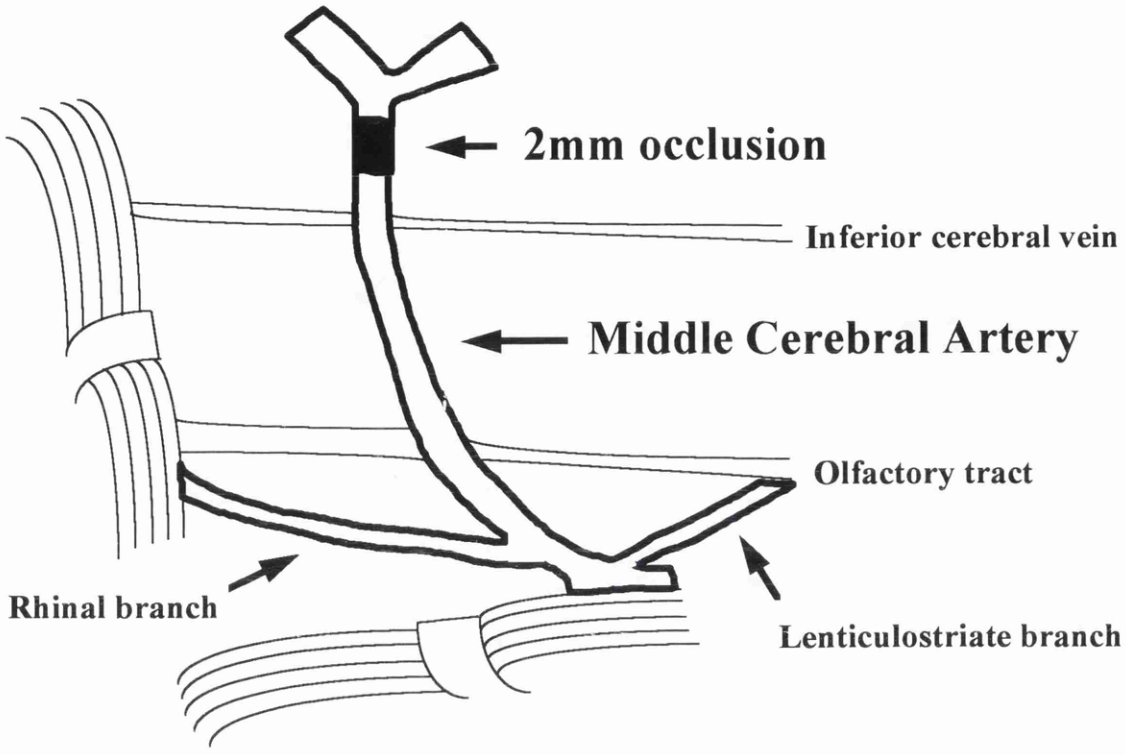

Figure 2.4 Schematic representation of the experimental occlusion of the middle cerebral artery in the rat.

Canada). The volume of infarct for each brain was then derived from integration of the areas of damage in the eight planes by use of the known stereotactic co-ordinates with endpoints for integration of 12.5mm anterior and 0.5mm posterior to the inter-aural line (Konig & Klippel, 1963; Osbourne *et al*, 1987). Comparison of the volumes of the ipsilateral and contralateral hemispheres provided a measure of brain swelling. Infarct volumes were expressed as a percentage of the ipsilateral hemisphere to account for brain swelling and differences in brain size between sexes and strains.

2.3.7 Tissue Harvest for DNA/RNA

For genotyping of F2 hybrids, livers and spleens were collected immediately after sacrifice and stored frozen at -70°C. For genotyping of congenic back-crosses, the animals were briefly anaesthetised at 4 weeks of age with fluothane and a 4mm tip from their tail removed into a 1.5ml Microfuge tube. The wound was immediately sealed with an electric cauteriser (Engel-Loter 100S) and the tails stored at -20°C.

2.4 Genetic Analysis

All experiments were undertaken following the safety guidelines set down by the Radiation Protection Service at the University of Glasgow.

2.4.1 DNA Extraction

Genomic DNA was isolated using a simplified mammalian DNA isolation procedure described by Laird *et al* (1991) which was modified slightly according to the source tissue used. This method yields high molecular weight DNA from large numbers of samples which is suitable for both PCR and Southern analysis.

In the case of the rat tail-tips for congenic genotyping, each was removed from the -20°C freezer (C & M Refridgeration Ltd.) and allowed to defrost completely in a 1.5ml microfuge tube. 700µl working tail solution (50mM Tris base pH 8.0; 0.5% SDS; 100mM EDTA, pH 8.0) was then added to each tube followed by 35µl of a 10mg/ml solution of proteinase K. This mixture was incubated at 55°C overnight in a hybridisation oven (Stuart Scientific) to allow gentle mixing until all tissue had dissolved.

Following overnight digestion, 700µl water-saturated phenol was added to each tube and the resulting solution vortexed briefly on a Fisons Whirlimixer (Fisons Scientific Equipment) followed by centrifugation at 14000rpm for 3 minutes at 4°C. The top (aqueous) layer was recovered to a new microfuge tube taking care not to disturb the interface, and 700µl of an equal volume (1:1; v/v) of water saturated phenol and a chloroform isoamylalcohol (24:1; v/v) mixture added. The tubes were again vortexed briefly followed by centrifugation at 14000rpm for 3 minutes at 4°C, the aqueous layer again recovered and 700µl of the chloroform isoamylalcohol mixture added. Centrifugation and separation of the aqueous layer was repeated as before followed by the addition of 70µl 3M sodium acetate (pH 6.0) and 700µl 100% ethanol to precipitate out the DNA on gentle shaking over 1 minute. The

mixture was then centrifuged at 14000rpm for 10 minutes to pellet the DNA at the bottom of each tube thereby allowing aspiration of the remaining supernatant. In order to wash the DNA and remove any last traces of SDS or phenol, 1ml 70% ethanol was added to each tube, vortexed vigorously and re-centrifuged for 5 minutes at the same conditions to re-pellet the DNA and aspirate the supernatant. The pellet was allowed to air-dry upside down over a tissue for 20 minutes. All samples were resuspended in 100µl TE solution (10mM Tris, pH 8.0; 0.1mM EDTA, pH 8.0) and stored at 4°C prior to further processing.

The extraction of DNA from livers and spleens for genome scan analysis differed from the above solely in terms of preparation and scale. The tissue was removed from -70°C to defrost fully and a 500mg sample removed. This was transferred to a sterile glass homogeniser with the addition of 4ml suspension buffer (100mM EDTA, pH 8.0; 50mM Tris, pH 8.0) and passed through 3 to 4 times only. The resulting solution was then removed to a 50ml Falcon polypropylene tube (Becton Dickinson & Co.) and 224µl 4M sodium chloride, 60µl of a 20mg/ml solution of proteinase K, and 1.2ml 10% SDS added, followed by gentle mixing and incubation at 37°C overnight. The resulting solution was then extracted three times with varying 6ml combinations of water saturated phenol and a chloroform isoamylalcohol mixture as described for the rat tails above, centrifuging between each stage at 2000rpm for 20 minutes at 4°C. The DNA was precipitated as a clump following the final extraction by the addition of 12ml 100% ethanol poured from a reasonable height. The DNA could then be hooked out of the polypropylene tube with a sterile heat sealed glass Pasteur pipette (Bilbate Ltd.) and transferred to a smaller tube for subsequent washing with 5ml 70% ethanol, drying, and resuspension and storage in 1ml TE at 4°C as above.

In order to quantify the concentration of DNA in each sample a 5µl aliquot of the extracted DNA was added to 995µl of sterile water (1 in 200 dilution) in a quartz cuvette. After mixing, the optical density of the sample was determined in triplicate at 260nm and 280nm in a Ultrospec 2000 UV/Visible spectrophotometer (Pharmaco-Biotech.) against a blank and the average values calculated. From the mean absorbance value at 260nm the amount of DNA in the sample (µg/µl) was calculated using the equation below:

$$[DNA]\mu g/\mu l = 200 \times (OD_{260} \times 0.05)$$

The purity of the sample was established by means of the ratio of the absorbance at 260:280nm. If this was found to approach 2.0 then UV absorption was due to residual traces of phenol. If below 1.0 other absorbing substances such as proteins probably remain. Both necessitate the discarding of that particular sample and a re-extraction. An ideal ratio to continue with was considered 1.8 and all such samples were adjusted to a working concentration of 20ng/µl and stored at -4°C.

2.4.2 Microsatellite Markers

Microsatellite markers are known di, tri, or tetra tandem nucleotide repeat elements (*Figure 2.5*) which occur at random throughout the genome and are ubiquitous in eukaryotes. All the genetic markers used in this research were microsatellite markers. Selection of these was based on the need for a thorough coverage of the entire rat genome and/or supposed location around a locus of interest as well as a requisite dimorphism between $SHRSP_{Glasgow}$ and $WKY_{Glasgow}$. Databases used to fulfil this selection included Ratmap: The Rat Genome

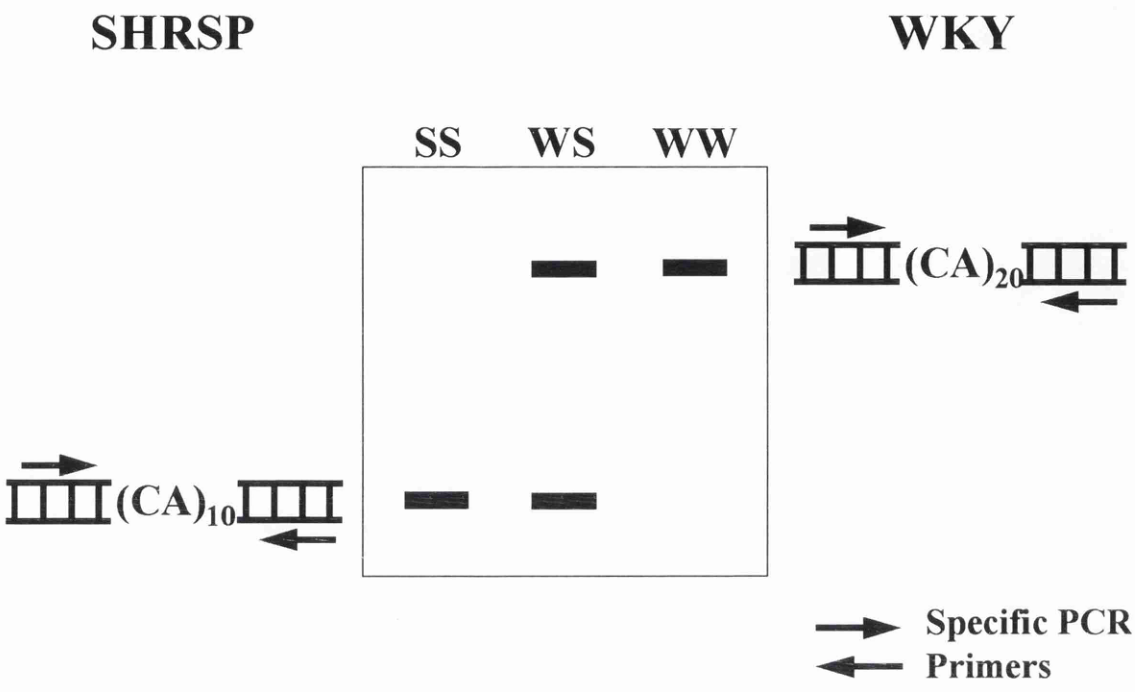

Figure 2.5 Schematic representation of genotyping microsatellite markers in the SHRSP x WKY cross by PCR, polyacrylamide gel electrophoresis and autoradiography.

Database, Goteborg University, Sweden at http://ratmap.gen.gu.se/; The Whitehead Institute Center for Genome Research Rat Mapping Project, Boston, USA at http://www.genome.wi.mit.edu/rat/public/ which contains up-dates of the original genetic map of the rat published by Jacob *et al* (1995); The Wellcome Trust Centre for Human Genetics Genetic Linkage Maps of the Rat Genome (1997), Oxford, UK at http://www.well.ox.ac.uk/~bihoreau.

2.4.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an *in vitro* method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA. Originally described by Saiki *et al* (1985), repetitive series of cycles involving template denaturation, primer annealing, and the elongation of the annealed primers by *Taq* DNA polymerase result in the exponential accumulation of a specific fragment whose termini are defined by the 5'-ends of the primers. Because the primer extension products synthesised in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR yields about a million-fold amplification.

Genotyping in both genome scans and congenic strategies was performed by PCR amplification of DNA around known dimorphic microsatellite markers from the total genomic DNA using the appropriate PCR primer pairs custom made by either Research Genetics (Huntsville, AL.), Genosys Biotechnologies (Europe) or obtained from the Wellcome Trust Centre for Human Genetics in Oxford (personal communication Drs.

Marie-Therese Bihoreau and Dominique Gauguier). Owing to the large number of samples to be typed PCR was undertaken using 96 well Falcon 3911 Microtest III titer-assay plates (Fred Baker Scientific.) in a MJ Research PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation Ltd.).

Each reaction was set up by first aliquoting 5µl of each DNA sample (20ng/µl) into the Falcon 96 well plate with the aid of a multichannel pipette, ensuring no cross contamination occurred at anytime. Once all samples had been aliquoted each was covered with a drop of mineral oil to prevent their evaporation during the PCR reaction and 5µl of a previously prepared primer solution added (contains 1.0µM of each primer and a few particles of tartrazine to aid identification). The Falcon 3913 Microtest III flexible lid (Fred Baker Scientific.) was placed on the plate, orientation of the samples marked, and loaded into the MJ Thermal Cycler to under-go a hot-start. This involved dissociation of the DNA template at 94°C for 4 minutes. Once complete, 10µl of PCR master mix (containing 2X Red, 2X recommended thermophilic buffer, 3mM magnesium chloride ($MgCl_2$), 50.0µM each dATP, dCTP, dGTP, and dTTP and 0.8 units of *Taq* polymerase) was added to each sample so that the final reaction volume in each well was 20µl containing 100ng of genomic template DNA, 45.0mM Tris, pH 8.8; 11.0mM $(NH_4)_2SO_4$, pH 8.8; 1.5mM $MgCl_2$; 6.7.0mM β-mercaptoethanol; 4.5 µM EDTA; 25.0 µM each dATP, dCTP, dGTP, and dTTP, 0.25 µM of each primer and 0.4U *Taq* polymerase. Each microsatellite marker was then amplified through 35 cycles of 94°C x 1 minute, 55°C or 60°C (annealing temperature) x 1 minute, and 72°C x 30 seconds (elongation temperature) followed by a final extension period of 72°C for 10 minutes.

To optimise the specific conditions for each pair of primers as outlined by the available databases, annealing temperatures were varied between 50°C and 68°C and concentrations of Mg^{2+} between 1.0 and 2.0mM. For some primers a modified touchdown protocol was used which involved a 0.5°C reduction in annealing temperature during the initial cycles (5°C drop). The final annealing temperature was then used for the last 30 cycles.

Following PCR the validity of the products were tested on a 1% agarose gel. Agarose (0.5g) was mixed in 50ml 1 X TBE (89mM Tris Borate, pH 8.3; 20mM ETDA, pH 8.0) in a conical flask and dissolved for 2 minutes in a microwave with care. Ethidium bromide (1µl of a 10mg/ml solution) was then added and the mixture cooled and poured into a gel tray. Once set (after 30 minutes) the gel was placed in a multi-purpose horizontal gel electrophoresis unit (Kodak International Biotechnologies, Inc.) and 8µl of several random samples from each titer plate mixed with 2 X formamide loading buffer (90% formamide; 2.5% bromophenol blue/xylene cyanol dye) and ran against an appropriate molecular weight marker for 30 minutes at 90 volts with the aid of a programmable electrophoresis power supply (Kodak International Biotechnologies, Inc.). If successful, the PCR products could be visualised on a Chromato-VUE TM-20 Transilluminator (UVP Inc.) and the actual size compared to that expected.

2.4.4 Polyacrylamide Gel Electrophoresis and Autoradiography

If the PCR amplification had been successful samples could either be stored at 4°C until required or immediately prepared for separation by polyacrylamide gel electrophoresis on standard denaturing sequencing gels, blotting to nylon membrane and visualisation of the

dimorphic differences by autoradiography (*Figure 2.5*). Preparation involved the transfer of 5µl of each sample using a multi-channel pipette (Gilson Medical Electronics) from the PCR titer plate to a Costar Thermowell thin-walled polycarbonate 96 well plate (Corning Incorporated) followed by the addition of 5µl 2X formamide loading buffer to each sample using an electronic multi-dispense pipette (Rainin edp2). Due to the large number of markers required for a total genome scan, several different PCR products were prepared in the same Thermowell plate to allow multi-loading onto the polyacrylamide gel, providing they were all of a similar size (in base-pairs).

An initial 8% polyacrylamide gel mix was made with the ready-to-use SequaGel Sequencing System (National Diagnostics). To initiate polymerisation 60µl of TEMED and 700µl of 10% ammonium persulphate (10% APS) were added to the mixture. The gel was immediately poured between two large pre-prepared vertical glass plates (Anachem Ltd.) with approximately 5ml Sigmacote wiped over the inside of just one of the plates to ease plate separation later. Once set, the gel was loaded into a vertical STS-45 standard Thermoplate Sequencer gel electrophoresis unit (Kodak International Biotechnologies, Inc.) and both reservoirs filled with 1 X TBE buffer up to the levels of the gel stands to ensure completion of the electrical circuit. A 60 well comb (Kodak International Biotechnologies, Inc.) was inserted into the gel and the gel pre-run at 2000 volts (40mA, 70W) for approximately an hour to heat it up to the optimum temperature for loading of 50-60°C.

Immediately prior to loading the PCR samples were denatured for 4 minutes at 94°C in the MJR Thermal Cycler and placed on ice. 7µl of each sample was then loaded with the aid of a duck-billed pipette tip (Sorensen Bioscience Inc.) into the gel and the orientation noted to

enable correct genotyping later. Depending on the size of the base sequence of the specific marker and their allelic differences between SHRSP and WKY, the length of time necessary to ensure complete separation of the dimorphic alleles was variable. Generally, PCR products of approximately 100 base-pairs (bp) in size were run until the slowest component of the loading dye had run down the gel 30cm, those 150bp in size 40cm, and those 200bp in size 50cm. It follows that by loading a set of markers which are large in size first, and then loading smaller markers 8cm after them, it was possible to gain the most utility out of one gel.

Once complete, the gel was cooled under running water and the plates split. A 28cm x 28cm piece of Hybond-N^+ nylon membrane previously soaked in 1 X TBE for 30 minutes was carefully placed on the gel, followed by two pieces of card, a glass plate and weights to allow blotting overnight. In the morning the membrane was carefully removed and fixed with 0.4M sodium hydroxide for 20 minutes without shaking, followed by 2 x 10 minute washes in 2 X SSC (300mM sodium chloride; 30mM sodium citrate, pH 7.0) with shaking. The membrane could now either be stored in a hybridisation tube (Stuart Scientific) at 4°C or immediately hybridised.

The membranes were hybridised with one of the primers radiolabeled with [α-^{32}P]dCTP by TdT (terminal deoxynucleotidyl transferase). Each labelling reaction contained 12.5µl of sterile distilled water, 4µl of terminal transferase buffer, 1µl of usually the forward primer (5-10µM), 1µl of [α-^{32}P]dCTP and <0.5µl TdT and required incubation at 37°C for 35 minutes followed by 10 minutes denaturation at 68°C to stop the reaction. The labelled probe was added to the pre-prepared 40ml hybridisation solution (24ml sterile water; 12ml

20 X SSC; 4ml 10% SDS; 0.1g powdered milk to prevent non-specific binding) which was in turn added to the hybridisation tube containing the appropriate membrane for hybridisation over at least 2 hours at either 55°C or 42°C in the hybridisation oven. On completion, the membrane was washed for 2 x 10 minutes in post-hybridisation wash solution (0.2% SDS; 2 X SSC), dried between two pieces of card, wrapped in Saran wrap (Dow Chemical Company) and directly exposed to autoradiography Hyperfilm (Amersham) in a Hypercassette (Amersham) for 2-14 hrs at -80 °C. Films were subsequently developed in a film processor (Kodak International Biotechnologies, Inc.) and autoradiographs independently scored by two investigators who were unaware of the rat's phenotypes. *Figure 2.6* represents an typical example of such an autoradiograph produced in this study. In order to visualise the remaining markers loaded on the same gel, the membrane was stripped by 2 x 10 minute washes in boiling 0.4% SDS followed by 2 x 10 minute washes in 2 X SSC and reprobed as before.

2.4.5 Sequencing

The enzymatic method of DNA sequencing developed by Sanger *et al* (1977) is based upon the use of specific terminators of DNA chain elongation: 2',3'dideoxynucleoside-5'-triphosphates (ddNTP). These deoxynucleoside triphosphate (dNTP) analogues can be incorporated by a DNA polymerase into a growing DNA chain through their 5'-triphosphate groups. However, because these analogues lack a hydroxyl group at the 3'-position, they cannot form phosphodiester bonds with the next incoming dNTP and the chain extension terminates whenever an analogue is incorporated. Thus when a specific ddNTP is included along with the four different dNTPs normally required for DNA

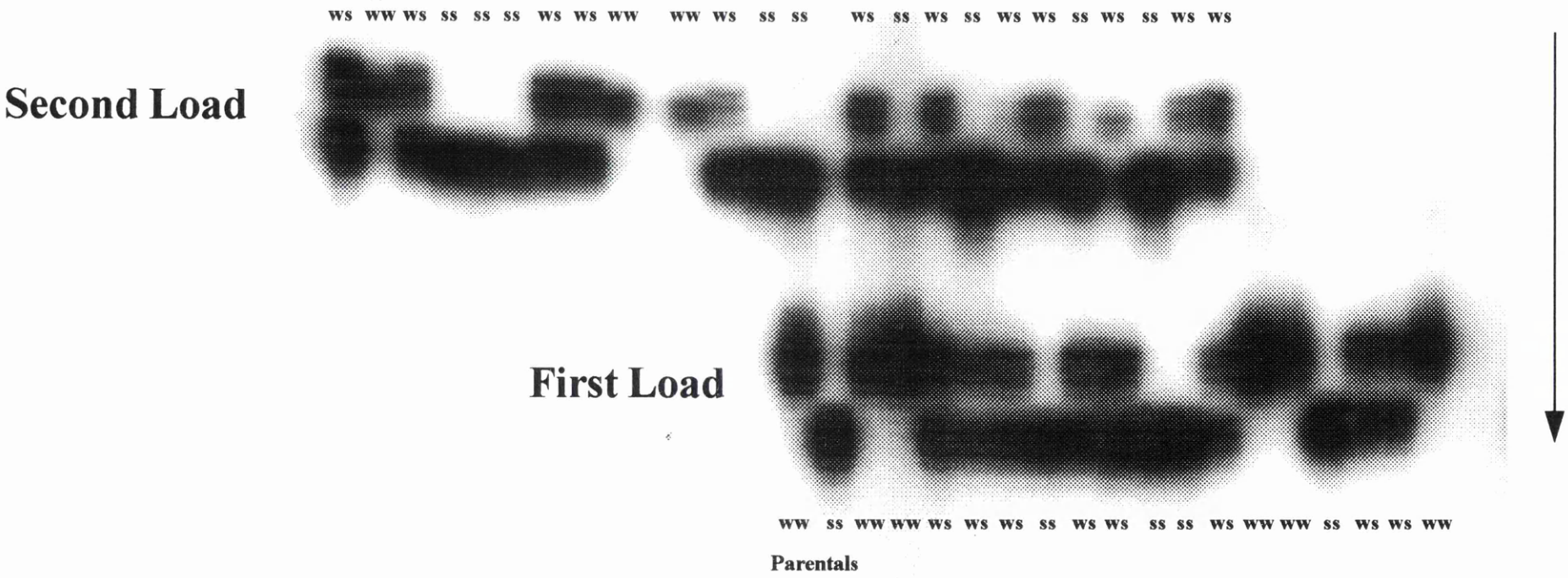

Figure 2.6 Example autoradiograph illustrating the genotyping of SHRSP x WKY F2 progeny. **ss** represents animals homozygous for SHRSP alleles, **ww** WKY homozygotes and **ws** heterozygotes. The first two samples represent parental samples, preceded by a water blank to control for contamination.

synthesis, the resulting extension products are a series of discrete length DNA chains that are specifically terminated at that dideoxy residue. To obtain sequence data, one reaction must be run for each of the four ddNTPs and when run together on a polyacrylamide gel the data provides complete sequence information.

A modified version of the Sanger method of sequencing in the form of a AmpliCycle™ Sequencing Kit (Perkin-Elmer) was used to sequence the coding regions of genes of interest in the SHRSP and WKY. This first demanded the high quality amplification by PCR of the large coding regions (130-900bp) using UITma DNA polymerase with an inherent 3' to 5' exonuclease proof-reading activity. The primers necessary for this were designed in-house from previously published sequences. PCR products were identified as such on 1.0% agarose gels and the bands cut-out with a sharp blade. DNA was extracted from the gel using a gel Nebulizer inserted into a Microcon microconcentrator (Amicon) centrifuged at 14000rpm for 20 minutes at 4°C. Once the gel had broken up, the filtrate was washed through the Microcon by adding 20µl sterile distilled water followed by centrifugation at 3000rpm for 24 minutes at 4°C. On inverting the Microcon the DNA could be collected and the concentration calculated with the aid of the DynaQuant fluorimeter (Hoefer).

Both forward and reverse sequencing primers were also designed in-house to cover between 150-200bp of the amplified coding regions. Prior to running the sequencing reaction each primer was radio-labelled with [γ-^{32}P]dATP to allow visualisation of the eventual sequence by autoradiography. Each labelling reaction contained 1.1µl of sterile distilled water, 0.6µl of 10X kinase buffer, 0.5µl of the sequencing primer (20µM), 1µl of [γ-^{32}P]dATP and 3.0µl T4 polynucleotide kinase and required incubation at 37°C for 2 hours.

For each sequencing reaction 2µl of each terminating ddNTP (G, C, A, & T) was added to separate wells in a titer-plate followed by the addition of 6µl of a sequencing master mix containing 20µl sterile water; 4.0µl cycling mix containing Ampli*Taq* DNA polymerase; 2.0µl PCR template (100fM); 1.0µl dimethyl sulfoxide (DMSO); and 1.0µl of the radio-labelled primer mix. After the PCR template was dissociated for 4 minutes at 94°C the sequencing was completed through 35 cycles of 94°C x 1 minute, the annealing temperature of the specific primer (°C) x 1 minute, and 72°C x 1 minute (elongation temperature). Each reaction was stopped by the addition of 4µl stop solution and could then be resolved on a standard 8% polyacrylamide gel after approximately 2 hours as described in *Chapter 2.4.4*. As the gel was radioactive it could be dried directly onto 3mm Whatman filter paper (Whatman International Ltd.) using a slab Gel-Vac dryer (Hybaid), put directly down to film and the sequence subsequently read by two independent observers.

2.5 Statistical Analysis

Phenotypes which were found clearly not to be normally distributed were logarithmically transformed before inclusion in any statistical procedure as dependent variables. Phenotypes which were strongly bimodal due to sex differences, but the distribution for each sex separately were acceptably normal, were analysed separately by sex. Examination of phenotypic differences between groups was accomplished with the use of standard *t*-tests. Confirmatory analysis of QTLs was performed by one-way and two-way ANOVAs with a conservative significance level of $p < 0.01$. In addition, a stepwise regression procedure was

used to assess QTL effects while controlling for possible confounding and covariate effects (Schork *et al*, 1995).

2.5.1 Genetic Mapping - MAPMAKER/EXP 3.0

Genetic markers were mapped relative to each other using the MAPMAKER/EXP 3.0 computer package with an error detection procedure (Lander *et al*, 1987; Lincoln & Lander, 1992). This is specifically designed for the construction of primary genetic linkage maps from marker genotype data from F2 intercrosses in experimental populations. MAPMAKER/EXP considers all the available raw genotypic data simultaneously in order to produce a multitude of possible maps consisting of a specific marker order and map distances as calculated from estimates of recombination fractions with the Haldane mapping function. For each map suggested by MAPMAKER/EXP the level of probability that the map would give rise to the observed data was then computed and this probability called the likelihood of the map. The "best" map was then selected as the one with the highest likelihood.

2.5.2 Linkage Analysis - MAPMAKER/QTL 1.1

QTLs affecting a given phenotype were mapped relative to the genetic markers by using the MAPMAKER/QTL 1.1 computer package obtained from Dr. Eric Lander (Whitehead Institute, Cambridge, Massachusetts). This program calculates the likely phenotypic effect of having the ss (SHRSP homozygotes) or ww (WKY homozygotes) genotype at a putative QTL. The strength of evidence for the existence of a QTL at any location is then provided

by the maximum $\log_{10}$ likelihood (LOD score) of the model at that point, and the proportion of the phenotypic variance explained by the model is also calculated (Lander & Botstein, 1989). To correct for the effects of multiple hypothesis testing stringent thresholds were utilised for mapping loci that underlie complex traits, with LOD scores of between 2 and 3.3 required to establish suggestive linkage and greater than 3.3 indicative of significant linkage as described in *Chapter 1.4.1* (Lander & Schork, 1994; Lander & Kruglyak, 1995).

3. IDENTIFICATION OF QTLs FOR BLOOD PRESSURE AND LEFT VENTRICULAR HYPERTROPHY IN SHRSP

3.1 Introduction

That a substantial fraction of the variation in blood pressure between individuals is genetically determined has been well established, with estimates of heritability of 20-40% (Annest *et al*, 1979; Mongeau, 1989). However, as discussed in *Chapter 1.2* the two major strategies developed for gene identification in human essential hypertension, linkage analyses in families segregating for rare Mendelian forms of hypertension and a candidate gene approach, both have significant limitations owing to the complex, multifactorial, and polygenic nature of the common form of the disease (Brand *et al*, 1998).

Some of the complexity inherent to the study of human subjects and families can be removed by inbred rat models of genetic hypertension. Hundreds of homogeneous progeny can be studied under controlled environmental conditions using high fidelity phenotyping. The stroke-prone spontaneously hypertensive rat (SHRSP) is one of the best such models as it is characterised by a number of vascular complications including left ventricular hypertrophy and a salt-sensitivity not dissimilar to the human disease state (Okamoto & Aoki, 1963; Yamori, 1982). Indeed it has already been utilised by several studies to confirm the polygenic inheritance of high blood pressure (Hilbert *et al*, 1991; Jacob *et al*, 1991; Nara *et al*, 1993; Davidson *et al*, 1995; Nara *et al*, 1996).

The major strategy used by these studies towards ultimate gene identification in the SHRSP has been the elucidation of quantitative trait loci (QTLs) by performing a total genome scan in F2 segregating populations obtained by crossing the SHRSP and the normotensive WKY rat. A QTL is a broad chromosomal region containing a gene or set of genes influencing a

quantitative trait, such as blood pressure. Three QTLs were identified in the first $SHRSP_{Heidelberg}$ x WKY cross: *BP/SP-1* (or *Bp1*), a locus that mapped to chromosome 10; *Bp2*, a locus on chromosome 18; and *BP/SP-2*, a locus on the X chromosome (Hilbert *et al*, 1991; Jacob *et al*, 1991). Multilocus linkage analysis performed in one of the two studies (Hilbert *et al*, 1991) showed that the rat angiotensin I converting enzyme (*Ace*) gene fell within the 10:1 odds region from *BP/SP-1* in the analysis of blood pressure after salt-loading (1% NaCl in the rat's drinking water) and within the 100:1 odds region for baseline blood pressure phenotypes. More recent data from $SHRSP_{Heidelberg}$ x WKY congenic strains revealed that *BP/SP-1* can be dissected into two loci: *BP/SP-1a*, linked to basal blood pressure and containing the inducible nitric oxide synthase (*Nos2*) gene and *BP/SP-1b*, which co-segregates with blood pressure after salt-loading and maintains linkage with the *Ace* gene (Kreutz *et al*, 1995). Additional blood pressure QTLs have also been identified in the $SHRSP_{(Izumo)}$ (Nara *et al*, 1993; Nara *et al*, 1996) on chromosome 1 (basal blood pressure only) around microsatellite markers within the genes encoding leukosianine (*Lsn)* and the myosin light chain (*Myl2*) and chromosome 3 (basal and salt-loaded) around the anonymous marker *D3Mgh16*.

Whilst these initial studies have confirmed the utility of a genome wide QTL analysis none of the above can be considered wholly comprehensive due to severe limitations in experimental design. For example they have mostly concentrated on males only, whilst it is likely that autosomal sex-specific QTLs exist (Ely & Turner, 1990). They have also all failed to fully dissect hypertension into its component factors and sub-phenotypes, including left ventricular hypertrophy, which may have different or perhaps overlapping sets of genetic components. Tanase *et al* (1982) identified that heart weight increased in proportion

to blood pressure only in hypertensive rat strains, with genetic variance accounting for 65-75% of the total variability in cardiac mass. Indeed, different hypotensive treatments that reduce blood pressure to the same degree have quite different effects on SHRSP heart size (Dominiczak *et al*, 1997) indicative of differing genetic control. This is analogous to the observation that the distribution of cardiac mass within human populations of unselected normotensive or hypertensive individuals appear to be continuous (Nunez *et al*, 1996; Weiss & Lundgren, 1978; Cutilleta *et al*, 1977; Cutilleta *et al*, 1978). Two genome scans in different rat crosses have previously revealed two QTLs on chromosomes 17 (Pravenec *et al*, 1995) and 2 (Innes *et al*, 1998) responsible for a proportion of left ventricular mass independently of blood pressure. No QTL for left ventricular hypertrophy has as yet been located in the SHRSP.

Similarly, all the genome scans on SHRSP have relied on methods of blood pressure measurement which introduce stress-related components into the study and cannot take account of diurnal variation which may possess separate genetic determinants. The development of an artefact-free direct system for monitoring blood pressure continuously over 24 hr periods in the form of radio-telemetry has already shown benefits in genetic studies. Davidson *et al* (1995) identified that male F2 hybrids with a SHRSP grandfather had significantly higher average systolic, diastolic and mean arterial pressures compared with male F2 hybrids with a WKY grandfather. This led to the suggestion that the SHRSP Y chromosome contains a locus or loci that contributes to hypertension in this strain. Its overall advantage in a total genome scan remains to be investigated.

Recently published studies have provided a large number of newly developed microsatellite markers vastly improving the density of the rat genetic linkage map (Serikawa *et al*, 1992; Jacob *et al*, 1995; Pravenec *et al*, 1996; Bihoreau *et al*, 1997; Brown *et al*, 1998). These markers, together with high fidelity phenotyping should allow a more complete mapping of hypertension in the SHRSP. It follows that the current study was designed to perform a total genome search in both male and female F2 hybrids resulting from a SHRSP x WKY cross with multiple phenotypes, including direct systolic and diastolic blood pressure at baseline and after salt-loading, heart rate, and motor activity as measured by radio-telemetry, as well as left ventricular hypertrophy.

3.2 Methods

Experimental Animals and Genetic Crosses

Inbred colonies of SHRSP and WKY rats have been established at the University of Glasgow since 1991. In order to yield two reciprocal crosses (*Chapter 2.2.2*), 1 male SHRSP was mated with 2 WKY females (cross 1) and 1 male WKY was mated with 2 SHRSP females (cross 2). From the F1 rats of each cross, 3 males and 6 females were brother-sister mated to generate F2 rats (57 in cross 1 with a male-to-female ratio of 28:29 and 83 in cross 2 with a male-to-female ratio of 37:46).

Radio-telemetry Measurements

The DATAquest IV telemetry system (Data Sciences International) was used for measurement of systolic and diastolic blood pressure, mean arterial blood pressure, heart rate and motor activity at baseline and after salt-loading following the protocol described in *Figure 3.1*. Rats at 16 weeks of age were implanted with radio-transmitters as described in *Chapter 2.3.2* and housed in individual cages after the operation, each cage being placed over a receiver panel connected to the personal computer for data acquisition. The rats were unrestrained and free to move within their cages. Previous experiments had shown that blood pressure and heart rate took up to 12 days post-operatively to stabilise (Davidson *et al*, 1995). Therefore, experimental observations collected from days 12 to 16 after surgery were designated "baseline haemodynamic measurements". On the evening of day 16 the rats received 1% (w/v) NaCl in their drinking water, and this procedure was continued until day 28 when the rats were euthanased. Measurements collected between days 25 and 28 constituted "salt-loaded haemodynamic measurements". Systolic and diastolic blood pressure, heart rate and motor activity were calculated by DATAquest software. Mean values were calculated for 60-minute intervals and exported from the DATAquest program in ASCII format.

Evaluation of Left Ventricular Hypertrophy

Immediately after exsanguination the thorax was opened and the heart removed, blotted with tissue paper, and weighed to within three decimal places. Both atria and the right ventricle were then removed, and the left ventricle and septum weighed. The ratios of heart

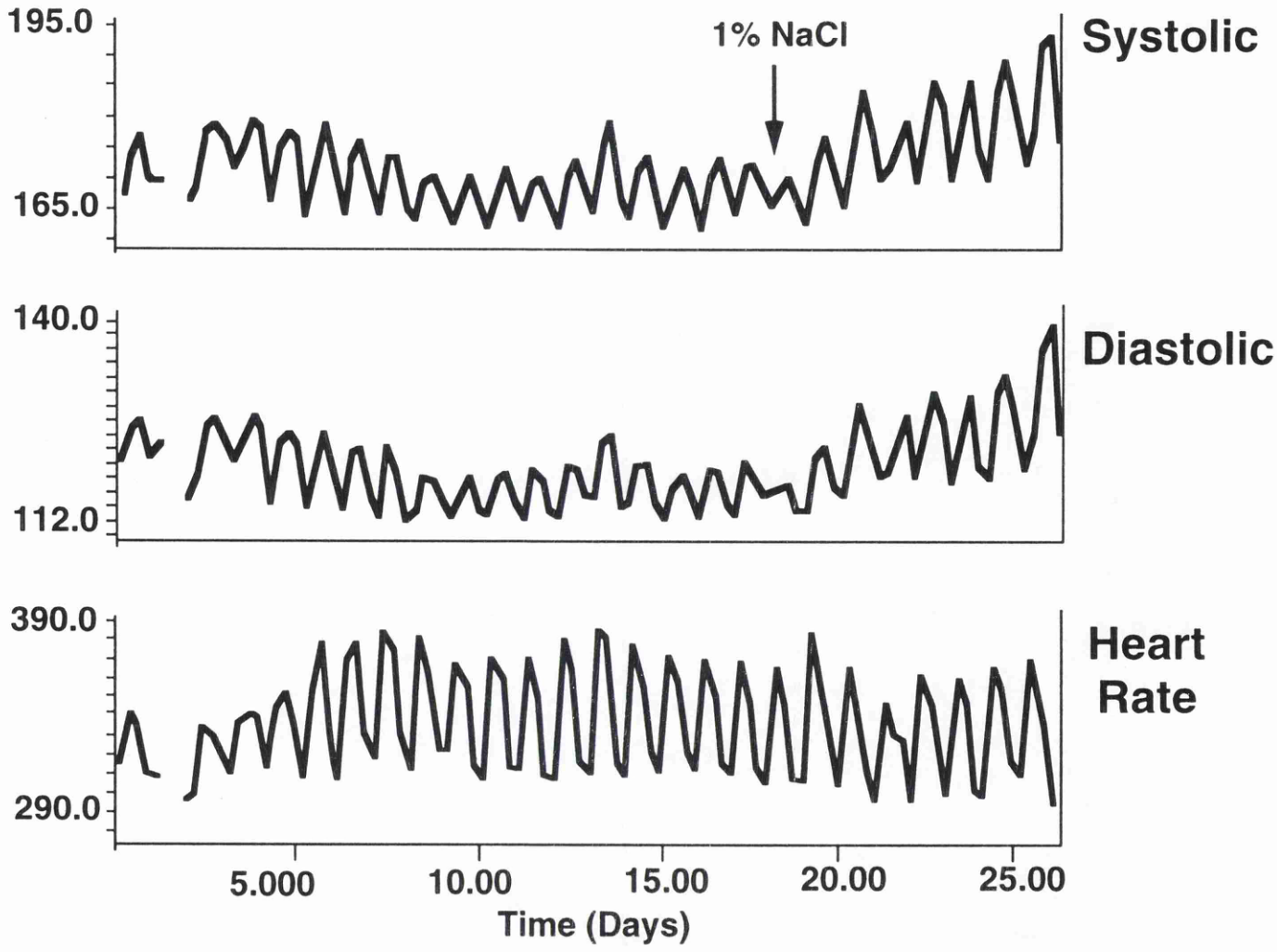

Figure 3.1 Protocol for measurement of blood pressure by radio-telemetry. Note initial time period for blood pressure measurements to stabilise following implantation of the radio-telemetry device.

weight to body weight and left ventricle plus septum weight to body weight were determined. In addition, livers and spleens were also removed as a source of DNA and stored at -70°C until required for genotypic analysis.

Genetic Markers and Genotyping

Genotyping was performed by PCR amplification of DNA around microsatellites markers as described in *Chapter 2.4.3*. The necessary PCR primers for typing the microsatellite markers were obtained from Research Genetics (Huntsville, AL.) and Genosys Biotechnologies (Europe), or obtained from The Wellcome Trust Centre for Human Genetics in Oxford (35 primer pairs from Dr. Mark Lathrop). PCR products were separated by electrophoresis on standard denaturing sequencing gels and transferred to nylon membranes as described in *Chapter 2.4.4*. The resulting membranes were hybridised with one of the primers radiolabeled with [α-^{32}P]dCTP by terminal transferase. After washing, the membranes were exposed to autoradiography film (X-OMAT AR, Eastman Kodak) for 2-14 hrs at -80 °C. Autoradiographs were independently scored by two investigators, who were unaware of the rat's phenotypes.

Genetic Linkage Analysis

As described in *Chapter 2.5* genetic markers were mapped relative to each other by using the MAPMAKER/EXP 3.0 computer package with an error detection procedure (Lander *et al*, 1987; Lincoln & Lander, 1992). Genetic distances were calculated with the Haldane mapping function. QTLs affecting a given phenotype were mapped relative to the genetic

markers by using the MAPMAKER/QTL 1.1 computer package obtained from Dr. Eric Lander (Whitehead Institute, Cambridge, Massachusetts). Compliant with the stringent statistical thresholds suggested by Lander & Kruglyak (1995, *Chapter 1.4.1*) LOD scores equal or greater than 3.3 were required to establish significant linkage and between 2 and 3.3 to establish suggestive linkage.

Phenotypic comparisons for different genotypes were performed by using a one-way ANOVA with a conservative significance level of $p < 0.01$. In addition, a stepwise regression procedure was used to assess QTL effects while controlling for possible confounding and covariate effects (Schork *et al*, 1995). Some QTLs showed possible sex specificity. In these instances a formal test was conducted for the difference in locus effects between sexes. This was accomplished by converting the LOD scores for the whole group, males, and females to likelihood ratios. The computed likelihood ratio statistic has a χ^2 distribution with one degree of freedom. A value of >3.85 suggests that the sex difference in the LOD score is significant at the 0.05 level.

Certain phenotypes were clearly not normally distributed. Values for motor activity were positively skewed and so were logarithmically transformed before inclusion as dependent variables. The distributions of body, whole heart and left ventricular weights were strongly bimodal due to sex differences, but the distribution for each sex separately were acceptably normal, so these three phenotypes were analysed separately by sex.

3.3 Results

All the significant phenotypic and genotypic data utilised in the genetic linkage analysis is given in Appendix II, as well as the primer sequences and optimum PCR conditions of each microsatellite marker used.

Radio-telemetry blood pressure measurements were obtained for 13 SHRSP (male:female = 7:6), 12 WKY (male:female = 6:6), 22 F1 (male:female = 13:9), and 140 F2 (male:female = 65:75) rats at baseline and after salt-loading. Unfortunately salt-loaded blood pressure was unrecorded in the SHRSP males due to the very high mortality in this group.

The variance in the blood pressure of parental SHRSP, WKY and F1 animals is due only to environmental factors as they are all genetically uniform. Therefore by subtracting the highest recorded blood pressure in either the parental or F1 populations from the lowest, a measure of the environmental variance is gained. By comparison, genes are segregating in the F2 rats and thus the same calculation in this population gives a measure of the total (environmental and genetic) variance. It follows that by subtracting the environmental variance from the total variance a measure of genetic variance is given. The ratio of genetic-to-total variance provides a precise estimate of the degree of genetic determination in a F2 population and, as such, is given for both baseline blood pressure and salt-loaded blood pressure phenotypes in both the male and female F2 cohorts in *Table 3.1* where mortality rates allow.

			Baseline Blood Pressure (mmHg)			Salt-Loaded Blood Pressure (mmHg)		
Cohort	**Sex**	**n**	**Systolic**	**Diastolic**	**Pulse**	**Systolic**	**Diastolic**	**Pulse**
SHRSP	M	7	250.5 (10.9)	180.9 (8.7)	69.6 (2.8)	...	...	...
SHRSP	F	6	197.9 (9.9)	140.8 (6.4)	57.1 (3.8)	214.3 (14.4)	150.5 (10.9)	63.8 (4.0)
WKY	M	6	160.3 (9.6)	110.4 (6.3)	50.0 (4.3)	168.7 (5.5)	115.8 (4.4)	52.9 (3.7)
WKY	F	6	142.1 (6.8)	99.5 (4.8)	42.6 (2.7)	147.1 (9.2)	101.2 (7.4)	45.9 (2.8)
F1	M	13	181.8 (3.6)	127.2 (3.3)	54.6 (2.7)	191.9 (6.9)	133.7 (6.1)	58.2 (2.3)
F1	F	9	164.3 (5.1)	116.8 (4.7)	47.4 (1.7)	166.5 (4.4)	115.5 (3.7)	51.0 (1.2)
F2	M	65	176.4 (16.2)	121.4 (11.5)	55.0 (6.2)	185.0 (17.8)	126.8 (13.0)	58.3 (6.1)
F2	F	75	163.9 (17.9)	113.1 (13.7)	50.8 (5.1)	171.0 (21.9)	116.9 (17.5)	54.1 (5.5)
Estimated degree of genetic determination	M		.78	.74	.75	...	...	...
	F		.84	.85	.73	.81	.82	.76

Table 3.1 Blood pressure phenotypes in SHRSP, WKY, F1 and F2 cohorts. Values are given as the mean and (standard deviation). Due to the very high mortality in the SHRSP males after salt-loading a precise estimate of the degree of genetic determination of salt-loaded blood pressure sub-phenotype was not possible in the F2 males.

A total of 603 microsatellite markers were screened and of these 181 were found to be dimorphic between $SHRSP_{Glasgow}$ and $WKY_{Glasgow}$, giving a 30% dimorphism rate. Using these markers and the MAPMAKER/QTL 1.1 computer package a QTL on rat chromosome 2 was identified with a significant LOD score of 3.3 for baseline systolic blood pressure and a LOD score of 3.4 for baseline diastolic blood pressure (*Figure 3.2A and B*). These two QTLs accounted for 12.9% and 13.8% of the variance in systolic and diastolic blood pressure respectively. The same analysis for salt-loaded blood pressures showed "suggestive" QTLs in the same chromosome region, with LOD scores of 2.9 and 2.7 accounting for 12.3% and 12.0% of the variance respectively. On analysing male F2 hybrids only, a QTL in the same region of rat chromosome 2 was again revealed with LOD scores of 3.65 and 3.5 for baseline systolic and diastolic blood pressure which accounted for 25% and 24% of the respective variance in blood pressure (*Figure 3.3A and B*). Salt-loaded systolic and diastolic blood pressures in male F2 cohorts gave LOD scores of 3.4 and 3.2, accounting for 31% and 26% of the respective variance in blood pressure (*Figure 3.4A and B*).

As *D2Mit6* is the closest marker linked to the QTL described above, the influence of this marker on a number of phenotypes was examined using a standard one-way ANOVA. *Table 3.2* shows that the locus characterised by the *D2Mit6* had a strong effect on baseline systolic and diastolic blood pressure and a lesser but still significant effect on salt-loaded systolic and diastolic blood pressure in the combined data set (males and females analysed together). The same analysis for the male F2 hybrids alone shows a significant effect of the locus characterised by the *D2Mit6* marker on all four sub-phenotypes of blood pressure. This marker does not appear to significantly influence any of the other phenotypes (*Table*

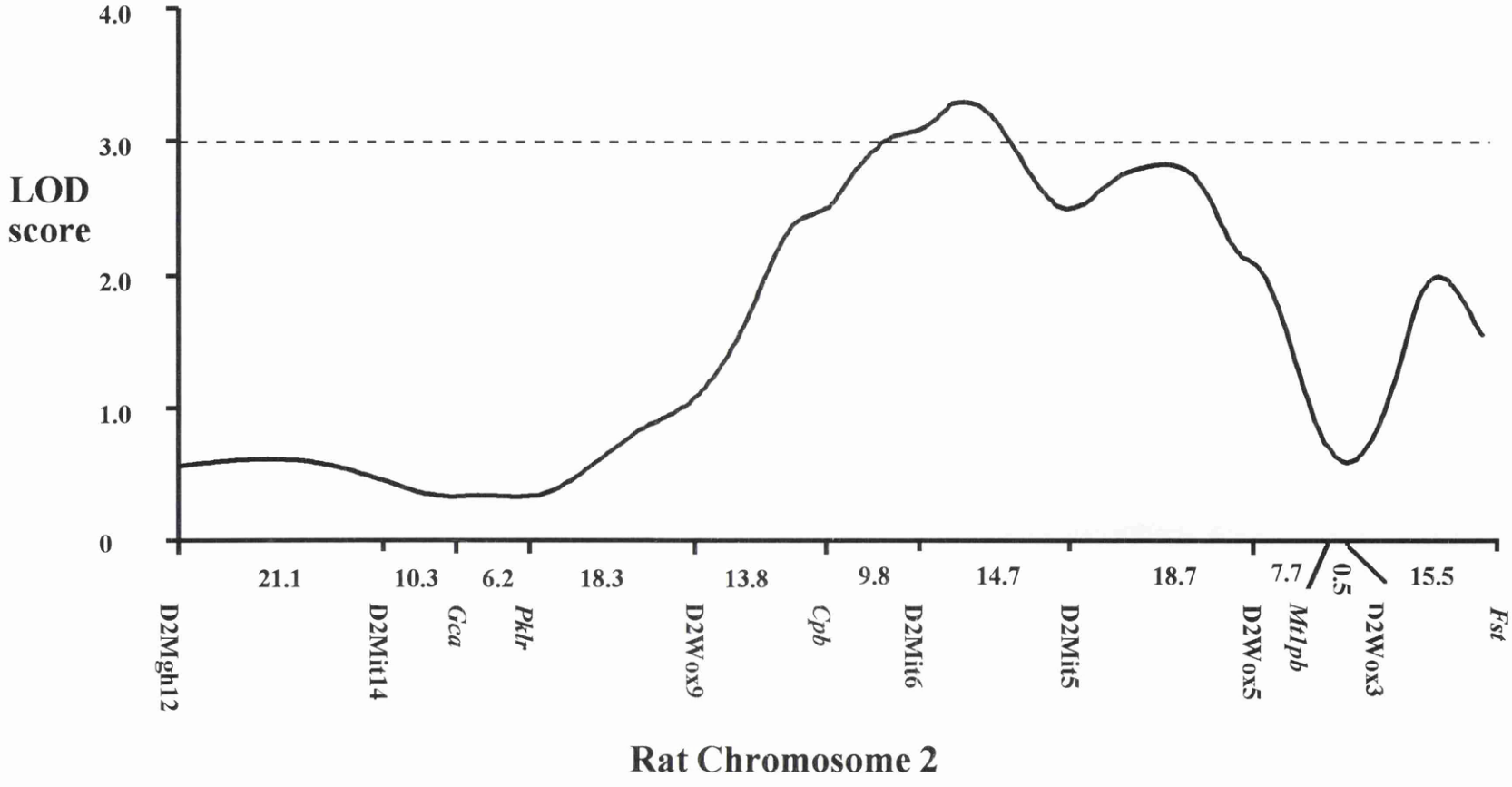

Figure 3.2A Rat chromosome 2 linkage map and baseline systolic blood pressure QTL localisation for a F2 population derived from SHRSP x WKY (males and females). Distances between markers are in centiMorgans (cM). Broken line indicates the LOD threshold for significant linkage. All markers are anonymous except *Gca*, guanylyl cyclase A; *Pklr*, pyruvate kinase L; *Cpb*, carboxypeptidase B; *Mt1pb*, metallothionein 1 pseudogene b; and *Fst*, follistatin.

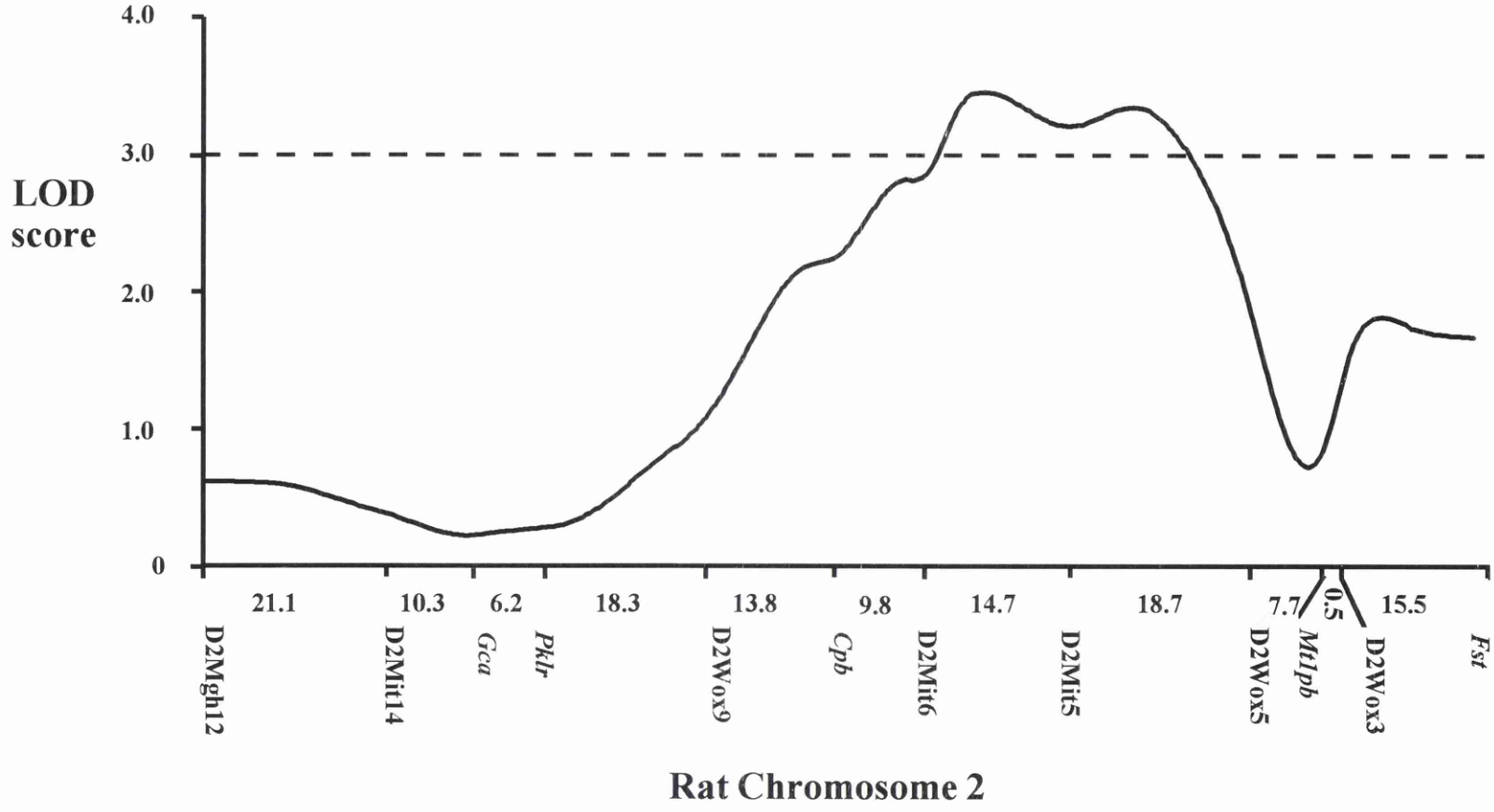

Figure 3.2B Rat chromosome 2 linkage map and baseline diastolic blood pressure QTL localisation for a F2 population derived from SHRSP x WKY (males and females). Map distances and symbols are the same as in *Figure 3.2A*.

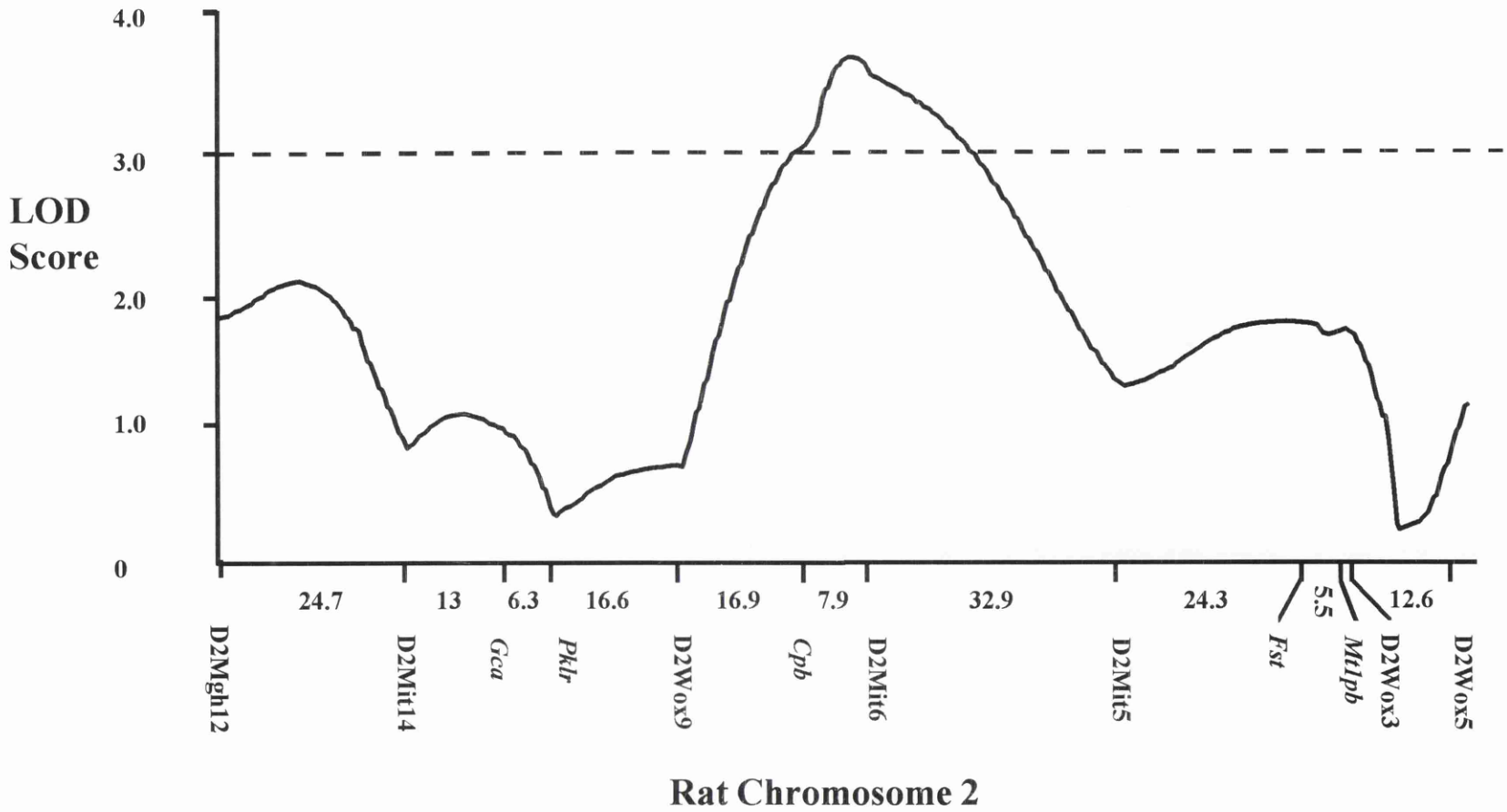

Figure 3.3A Rat chromosome 2 linkage map and baseline systolic blood pressure QTL localisation for a F2 male only population derived from SHRSP x WKY. Map distances and symbols are the same as in *Figure 3.2A*.

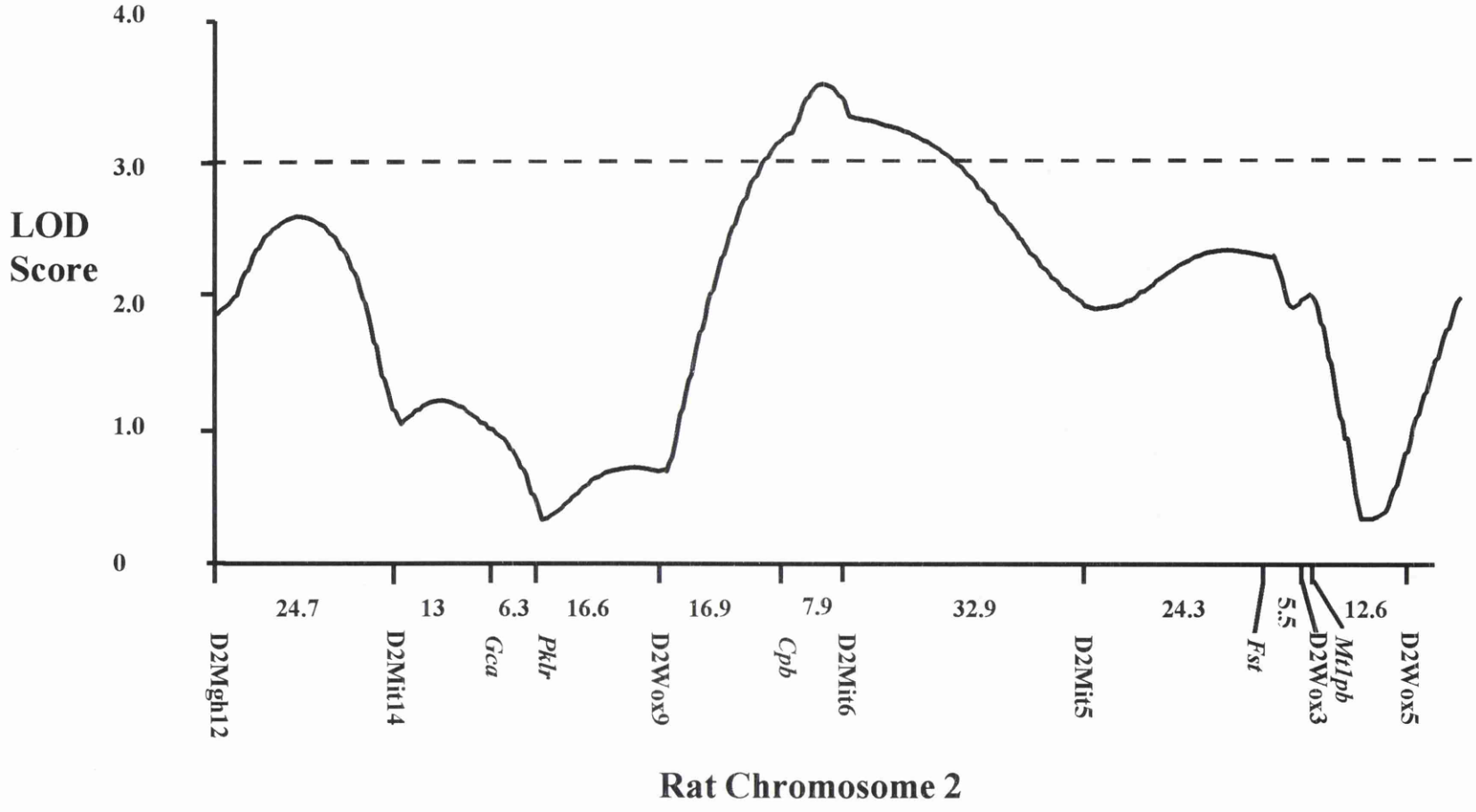

Figure 3.3B Rat chromosome 2 linkage map and baseline diastolic blood pressure QTL localisation for a F2 male only population derived from SHRSP x WKY. Map distances and symbols are the same as in *Figure 3.2A*.

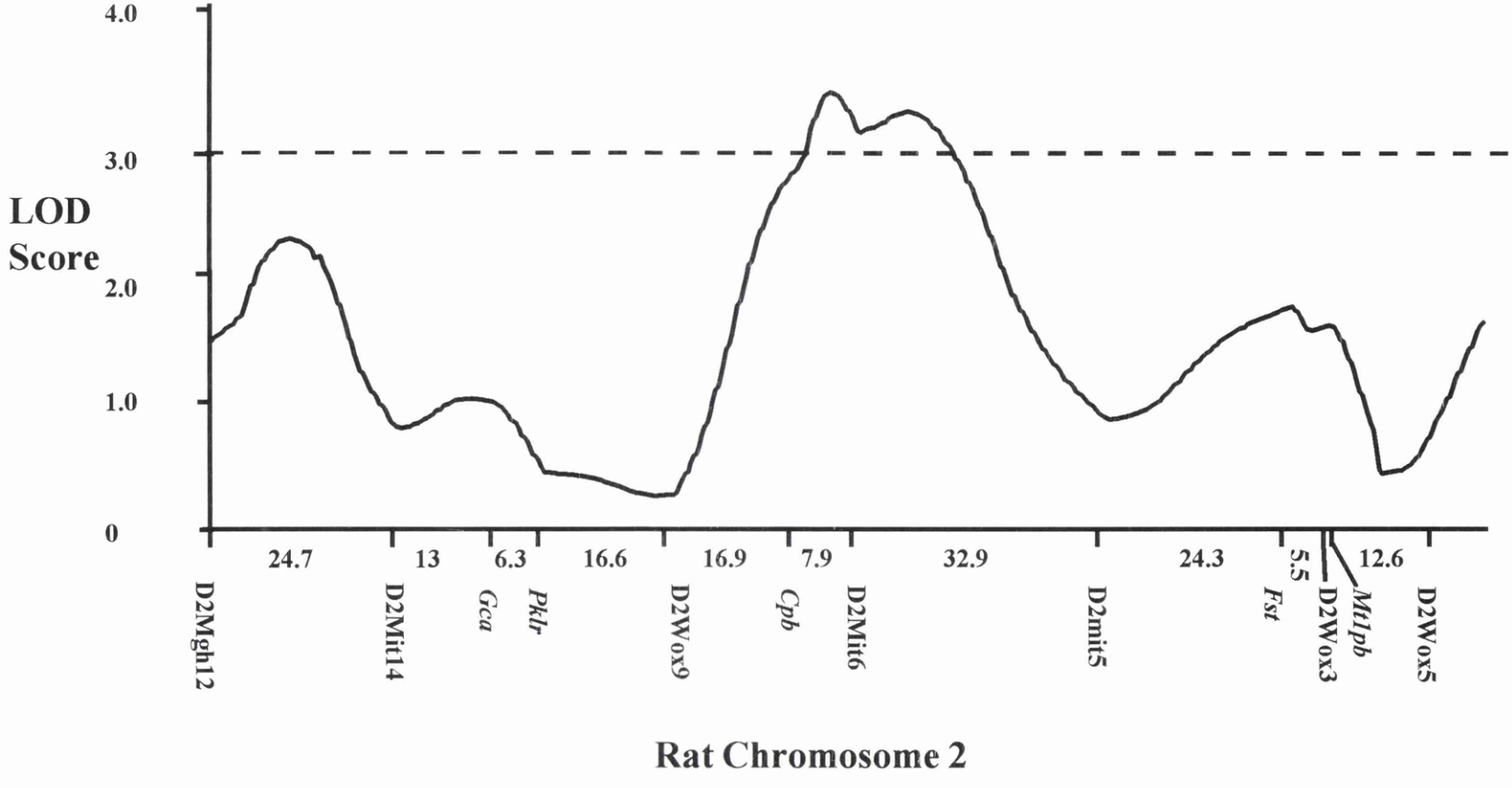

Figure 3.4A Rat chromosome 2 linkage map and salt-loaded systolic blood pressure QTL localisation for a F2 male only population derived from SHRSP x WKY. Map distances and symbols are the same as in *Figure 3.2A*.

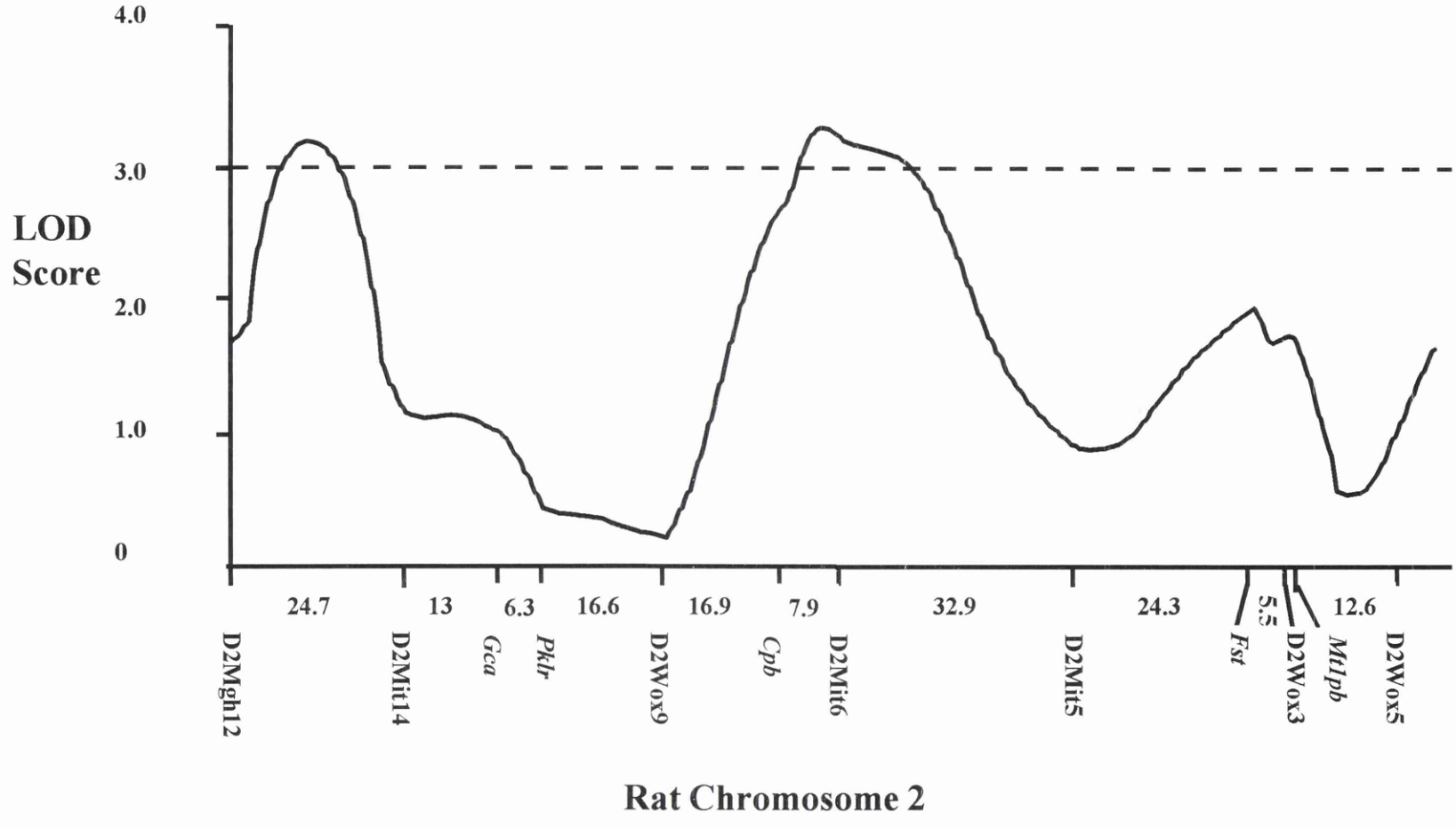

Figure 3.4B Rat chromosome 2 linkage map and salt-loaded diastolic blood pressure QTL localisation for a F2 male only population derived from SHRSP x WKY. Map distances and symbols are the same as in *Figure 3.2A*.

Phenotype	Genotype for F2 Males				Genotype for F2 Males and Females			
	WW (n = 16)	*WS* (n = 28)	*SS* (n = 18)	*p*	*WW* (n = 36)	*WS* (n = 65)	*SS* (n = 30)	*p*
Baseline SBP (mmHg)	171.4 (13.7)	171.7 (11.6)	188.7 (18.3)	$<5 \times 10^{-4}$	165.7 (16.3)	167.9 (16.5)	180.6 (20.9)	.001
Baseline DBP (mmHg)	117.0 (9.0)	118.4 (7.7)	129.8 (14.7)	.001	113.9 (11.6)	115.8 (12.2)	124.4 (16.1)	.003
Baseline Heart Rate (beats/min)	337.3 (17.8)	338.2 (21.9)	347.7 (20.2)	.235	353.9 (25.5)	356.6 (27.7)	358.2 (25.4)	.799
Baseline Motor Activity (units)	10.2 (2.1)	9.8 (2.5)	10.3 (2.4)	.785	12.9 (7.8)	13.5 (5.6)	11.6 (5.0)	.411
Salt-loaded SBP (mmHg)	177.1 (13.3)	181.3 (16.2)	197.4 (18.3)	.001	172.3 (16.7)	176.3 (21.8)	188.8 (22.2)	.004
Salt-loaded DBP (mmHg)	119.9 (8.4)	124.9 (11.7)	135.4 (14.2)	.001	117.4 (12.0)	120.8 (17.4)	129.6 (16.6)	.007
Salt-loaded Heart Rate (beats/min)	329.8 (13.9)	340.2 (19.2)	349.2 (21.6)	.014	343.5 (21.5)	352.3 (25.2)	354.8 (20.7)	.101
Salt-loaded Motor Activity (units)	10.1 (2.5)	11.1 (3.2)	10.8 (2.7)	.512	13.6 (9.0)	13.6 (4.4)	13.0 (5.3)	.882
Left Ventricular Weight to Body Weight Ratio (mg/g)	2.76 (0.34)	2.64 (0.30)	2.88 (0.30)	.045	2.83 (0.35)	2.80 (0.47)	2.83 (0.27)	.947

Table 3.2 Effects of *D2Mit6* on multiple phenotypes in F2 hybrids. Values are given as the mean and (standard deviation). Analysis was done by one-way ANOVA, with $p < 0.01$ considered significant. SBP = systolic blood pressure; DBP = diastolic blood pressure.

3.2). The *D2Mit6* locus affects blood pressure in a recessive manner, since homozygous ww and heterozygous ws rats have equivalent blood pressures, whereas homozygous ss rats have significantly elevated blood pressures (*Table 3.2*).

Further analysis of male F2 hybrids revealed a second potential QTL on rat chromosome 2, which was localised between markers *D2Mit14* and *D2Mgh12*. The LOD scores for this QTL were as follows: 2.0, 2.5, 2.3 and 3.1 for baseline systolic, baseline diastolic, salt-loaded systolic and salt-loaded diastolic blood pressures respectively (*Figures 3.3A and B and 3.4A and B*). The two dimorphic markers are on the downslope of the LOD plot and therefore a one-way ANOVA was deemed inappropriate to verify these results.

Data analysis for male F2 hybrids also revealed a QTL on rat chromosome 3, with *D3Mgh16* being the closest marker to this QTL. Blood pressure phenotypes that showed significant LOD scores in this region (*Figure 3.5A and B*) were baseline and salt-loaded pulse pressures (LOD scores of 4.2 and 5.6 which contributed 32.2% and 39.8% of the variance in pulse pressure, respectively) and salt-loaded systolic and diastolic blood pressures (LOD scores of 4.4 and 3.3 which contributed 39.6% and 36.7% of the variance in blood pressure, respectively). These results were confirmed by one-way ANOVA, with baseline pulse pressure being of borderline significance according to the stringent criteria adopted (*Table 3.3*). Similar to *D2Mit6*, the locus close to *D3Mgh16* appeared to affect the blood pressure phenotypes in a recessive manner.

The two QTLs defined by the markers *D2Mgh12* and *D3Mgh16* showed possible sex specificity by being identified in male only analyses. A formal test for the difference in locus

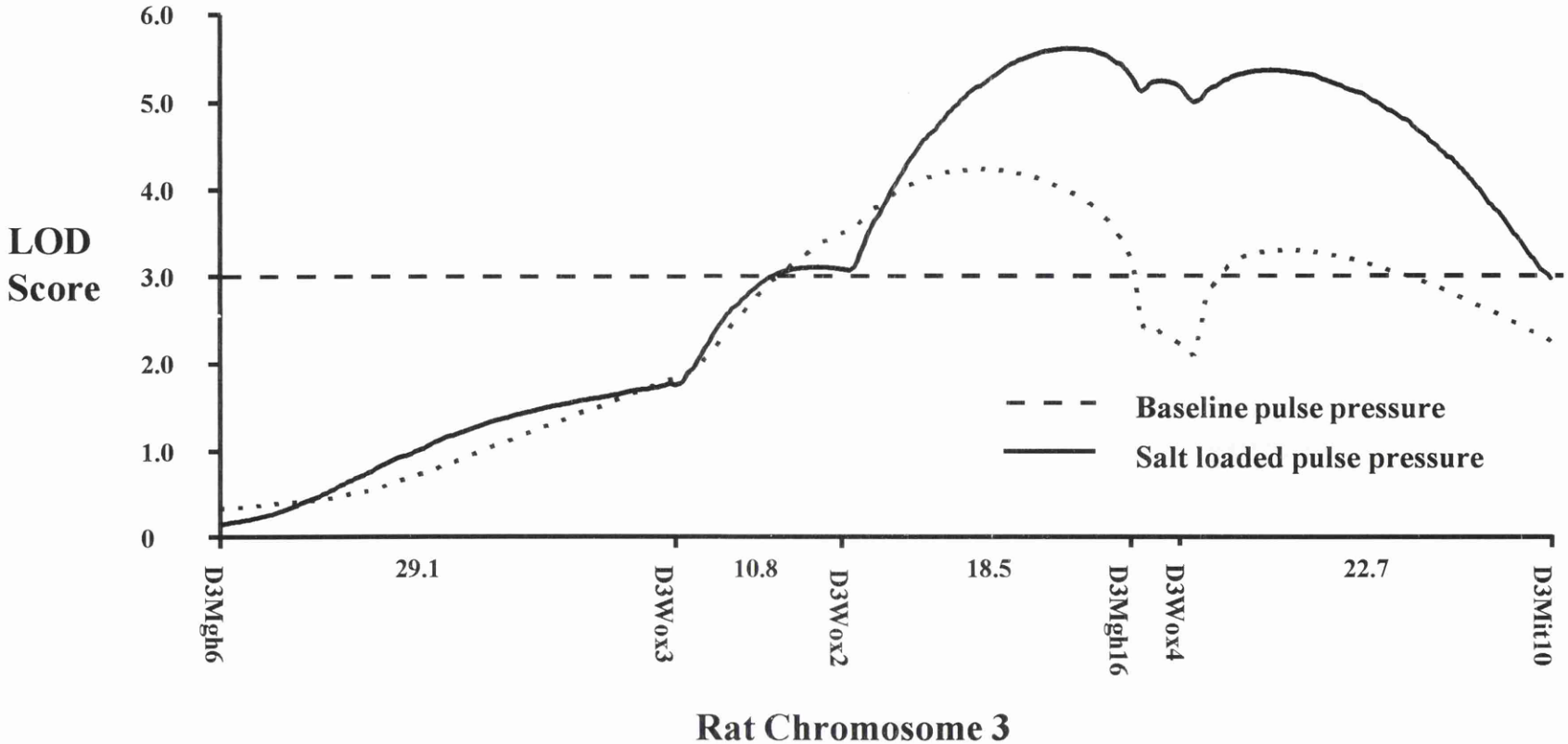

Figure 3.5A Rat chromosome 3 linkage map and baseline and salt-loaded pulse pressure QTL localisation for a male only F2 population derived from SHRSP x WKY. All markers are anonymous except *D3Wox4*, guanine nucleotide-binding protein G-s, alpha subunit.

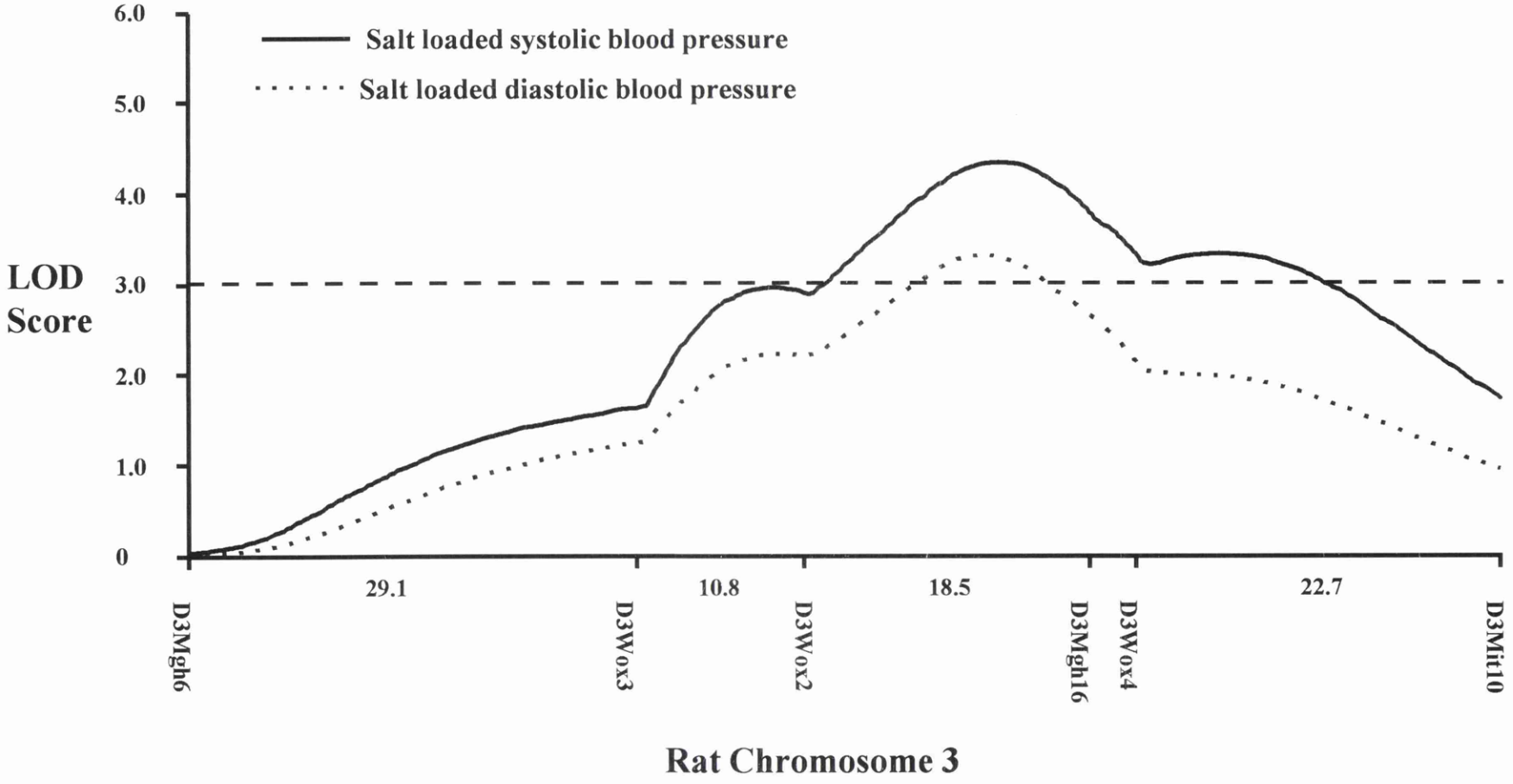

Figure 3.5B Rat chromosome 3 linkage map and baseline and salt-loaded systolic blood pressure QTL localisation for a male only F2 population derived from SHRSP x WKY. All markers are anonymous except *D3Wox4*, guanine nucleotide-binding protein G-s, alpha subunit.

Phenotype	Genotype for F2 Males			
	WW (n = 18)	*WS* (n = 28)	*SS* (n = 13)	*p*
Baseline SBP (mmHg)	169.4 (11.3)	178.0 (18.2)	185.5 (12.7)	.018
Baseline DBP (mmHg)	117.1 (7.2)	123.0 (14.1)	127.3 (7.6)	.043
Baseline Pulse Pressure (mmHg)	52.3 (4.8)	55.0 (4.8)	58.2 (6.7)	.012
Baseline Heart Rate (beats/min)	348.3 (20.2))	339.0 (19.9)	332.2 (18.5)	.080
Baseline Motor Activity (units)	10.0 (2.2)	9.7 (2.6)	10.4 (2.1)	.740
Salt-loaded SBP (mmHg)	176.1 (10.7)	184.2 (16.8)	200.4 (16.2)	$<5 \times 10^{-4}$
Salt-loaded DBP (mmHg)	120.8 (7.3)	127.0 (13.0)	137.1 (12.5)	.001
Salt-loaded Pulse Pressure (mmHg)	55.3 (4.1)	57.4 (4.7)	63.3 (4.7)	$<5 \times 10^{-4}$
Salt-loaded Heart Rate (beats/min)	345.8 (18.1)	339.5 (21.9)	337.7 (13.1)	.442
Salt-loaded Motor Activity (units)	10.7 (2.4)	10.7 (3.4)	11.3 (2.0)	.780
Left Ventricular Weight to Body Weight Ratio (mg/g)	2.69 (0.3)	2.69 (0.3)	2.93 (0.3)	.422

Table 3.3 Effects of *D3Mgh16* on multiple phenotypes in Male F2 hybrids. Values are given as the mean and (standard deviation). Analysis was done by one-way ANOVA, with $p < 0.01$ considered significant.

effect between the sexes was conducted using a simple likelihood-ratio test (*Table 3.4*). For each QTL localised close to *D2Mgh12* and *D3Mgh16*, the likelihood-ratio statistic was significant, thus confirming the sex difference in LOD scores.

A QTL on rat chromosome 14 for the ratio of left ventricular weight to body weight was also detected (*Figure 3.6*). This QTL covers a distance of 12.3cM between the markers *D14Mgh3* and *R58*. LOD scores for this phenotype were 3.7 for F2 males and 3.1 for F2 males and females analysed together, which accounted for 32.3% and 12.3% of the variance in left ventricular weight to body weight ratio respectively. Similar to the "suggestive" male-only QTL on rat chromosome 2, the two dimorphic markers are on the downslope of the LOD plot and again a one-way ANOVA was deemed inappropriate to verify these results.

No other QTLs were detected in the genome scan. Specifically no QTLs for heart rate or motor activity were detected. A stepwise regression procedure with either baseline systolic blood pressure or salt-loaded pulse pressure as the dependent variable was therefore performed in order to assess the contribution of the individual QTLs identified in this study and the putative Y chromosome locus (Davidson *et al*, 1995) to the blood pressure phenotypes as measured by radio-telemetry (*Table 3.5*). The SHRSP Y-chromosome effect, *D2Mit6* recessive effect and left ventricular weight to body weight ratio all markedly, but independently influenced baseline systolic blood pressure. Together they accounted for 34.2% of the variance in total baseline systolic blood pressure. The *D3Mgh16* recessive effect, the SHRSP Y chromosome effect, and the left ventricular weight to body weight ratio also all markedly, but independently influenced salt-loaded pulse pressure. Together they accounted for 28.9% of the total variance in salt-loaded pulse pressure.

	D2Mgh12		*D3Mgh16*	
Blood Pressure QTLs	**LR Statistic**	***p***	**LR Statistic**	***p***
Baseline Systolic Blood Pressure (mmHg)	5.50	.019	4.85	.028
Baseline Diastolic Blood Pressure (mmHg)	6.91	.009	4.50	.034
Salt-loaded Systolic Blood Pressure (mmHg)	7.50	.006	8.02	.005
Salt-loaded Diastolic Blood Pressure (mmHg)	9.31	.002	9.82	.002

Table 3.4 Likelihood-Ratio (LR) tests for the difference in locus effect between the sexes. A value of >3.85 suggests that the sex difference in the LOD score is significant at the 0.05 level.

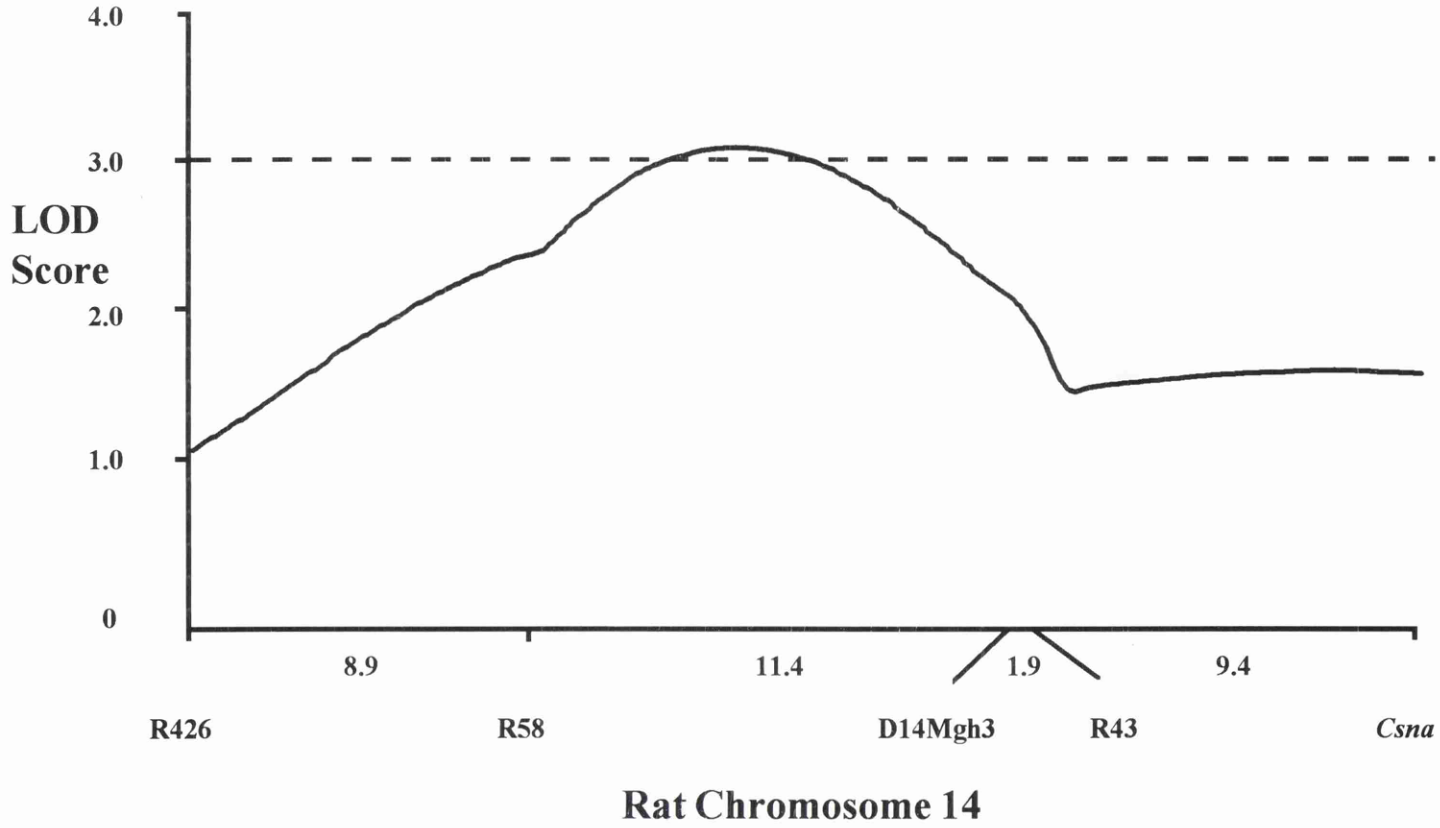

Figure 3.6 Rat chromosome 14 linkage map and localisation of QTL for the ratio of left ventricular weight to body weight in a F2 population derived from SHRSP x WKY (males and females). All markers are anonymous except *Csna*, casein alpha; *R58*, albumin; *R43*, alpha-fetoprotein; *D14Mgh3*, Vitamin D binding protein.

Dependent Variable	Explanatory Variable	Coefficient (SE)	*p*	Cumulative R^2 (%)
Baseline Systolic Blood Pressure	Y's effect	13.1 (3.3)	$<5 \times 10^{-4}$	26.1
	D2Mit6 recessive effect	8.1 (3.1)	.012	30.4
	Left Ventricular Hypertrophy	9.3 (3.8)	.017	34.2
Salt-Loaded Pulse Pressure	*D3Mgh16* recessive effect	4.1 (1.2)	.001	18.4
	Y's effect	3.0 (1.1)	.006	24.9
	Left Ventricular Hypertrophy	3.6 (1.3)	.008	28.9

Table 3.5 Stepwise regression for F2 males and females. Y's effect indicates the Y chromosome inherited from the SHRSP male progenitor (Davidson *et al*, 1995).

3.4 Discussion

The high estimated degree of genetic determination of both baseline and salt-loaded systolic, diastolic, and pulse pressure calculated in both the male and female F2 cohort suggests the existence of a gene or set of genes each with a relatively large contribution to the overall genetic variance of the blood pressure sub-phenotypes. It follows that the subsequent application of a genome wide scan approach did indeed identify two distinctly different putative blood pressure QTLs on rat chromosome 2. The most statistically significant (LOD > 3.3) of these was determined for both baseline and salt-loaded diastolic and systolic blood pressure and occurred in both male and female F2 cohorts, whether analysed separately or together. It lay ~9.8cM from the carboxypeptidase B gene (*Cpb*) with its peak close to the anonymous microsatellite marker *D2mit6*.

Other groups have previously suggested the presence of a blood pressure QTL on chromosome 2 in this region. Both systolic and pulse pressures recorded in an F2 population derived from a cross of Lyon-hypertensive and Lyon-normotensive rats were significantly correlated with the *Cpb* gene (Dubay *et al*, 1993). As *Cpb* is located very close (~0.8cM) to the angiotensin type 1B receptor (*Atr1b*) gene it is likely this QTL also corresponds to that previously identified in a Dahl salt sensitive (SS) x Milan normotensive (MNS) cross around *Atr1b* for salt-loaded blood pressure, although the statistical evidence for this QTL was merely suggestive at p = 0.014 (Deng *et al*, 1994). Most recently, Garrett *et al* (1998) identified a similarly suggestive (LOD = 2.9) QTL between *Atr1b* and D2Mit1 in a Dahl SS x Lewis rat cross. No corresponding QTL has ever been identified in a cross

involving the SHRSP before and neither of these candidate genes (*Cpb* and *Atr1b*) have been investigated further for a role in hypertension.

The suggestive (LOD <3.3) second QTL identified on rat chromosome 2 by this study was localised ~73cM away from *D2Mit6* and contributed to salt-loaded blood in the male F2 cohort only. The QTL is localised chiefly between two anonymous markers, *D2Mgh12* and *D2Mit14* with the latter being 13cM from the guanylyl cyclase A/ atrial natriuretic peptide receptor (*Gca*) gene. Jacob *et al* (1991) also provided suggestive evidence (LOD 2.47) for a baseline systolic pressure QTL around *Gca* in a $SHRSP_{(Heidelberg)}$ x WKY cross. However, as this study was conducted in males only it was unable to confirm or refute the sex-specificity of this suggestive locus identified in the current Glasgow SHRSP x WKY cross. This sex specificity has been replicated, however, by Harris *et al* (1995) finding *Gca* to cosegregate with increased blood pressure in males, but not females, from reciprocal crosses between New Zealand genetically hypertensive and Brown-Norway normotensive rats.

Gca has been considered a candidate gene for blood pressure since the finding that young Dahl SS rats are hyporesponsive to the natriuretic and diuretic effects of atrial natriuretic peptide (ANP) which implies a receptor defect (Snajdor & Rapp, 1985; Hirata *et al*, 1984). However, whilst *Gca* has been shown to co-segregate with increased blood pressure (Deng & Rapp, 1992; Harris *et al*, 1995), caution has to be exercised while interpreting these results, especially as *Gca* is at the edge of this QTL for blood pressure identified in the current study. A positive cosegregation may have arisen solely because *Gca* is localised in close proximity to the real blood pressure gene on the same chromosome. Indeed,

subsequent crosses involving both Dahl SS and SHR rats have localised this QTL to a region between the α1 isoform of the Na^+K^+ATPase (*NaKATPaseα1*) and calmodulin-dependent protein kinase II Δ (*Camk*) genes (Deng *et al*, 1994; Schork *et al*, 1995; Pravenec *et al*, 1995; Samani *et al*, 1996) with *Gca* taking a similarly peripheral position to that observed in the current study.

Various isoforms of Na^+K^+ATPase are involved not only in sodium handling by the kidney but also in regulation of vascular smooth muscle tone and cardiac contractility. It is not surprising therefore that *Na^+K^+ATPase* genes have been given serious attention as strong candidates in essential hypertension. However, *Na^+K^+ATPase* genes were found not to cosegregate with blood pressure in a Dahl SS x Dahl RR cross (Rapp & Dene, 1990) and no base changes between the two strains have been confirmed (Simonet *et al*, 1991). Lodwick *et al* (1998) also failed to find any evidence of co-segregation with blood pressure in a MNS x MHS cross despite the observation of differences in mRNA expression in the kidneys.

Camk, a major protein involved in intracellular calcium homeostasis, has received scant attention by comparison. It is interesting, however, that Vincent *et al* (1997) recently identified a similar QTL on rat chromosome 2 in a Lyon normotensive x hypertensive cross containing *Camk* which specifically influences the systolic (peak LOD score 4.4) and diastolic (peak LOD score 4.1) blood pressure responses to administration of a dihydropyridine calcium antagonist, PY108-068.

Most recently two new strong candidate genes have been physically mapped in the region of this putative QTL. Soluble guanylyl cyclases (α/β) are major constituents of the nitric oxide system which plays a prominent role in salt handling and regulation of blood pressure. The *α1* and *β1* gene loci have been mapped to rat chromosome 2 and are closely linked to *$Na^+K^+ATPase\alpha 1$* and *Camk* (Azam *et al*, 1998). Interestingly, β subunits have been shown to be altered in Dahl SS rats (Gupta *et al*, 1997), although further analysis of their potential role in blood pressure regulation remains to be done. Indeed, whilst the existence of this QTL, in Dahl SS rats at least, has been recently confirmed by congenic strains in which the chromosomal segment contained all the above mentioned candidate genes in its 41cM (Deng *et al*, 1997), further congenic strains and sub-strains will be required in both the Dahl SS and SHRSP to narrow the region significantly in order to identify the true effective allele.

The current study also identified a second sex-specific blood pressure QTL on rat chromosome 3 in the region of the anonymous marker *D3Mgh16*. Again, this QTL was present in male F2 rats only and showed a significant likelihood-ratio test of the sex difference in LOD scores. No relevant candidate genes have been identified in this area hence the particular need for congenic strains of this region.

It is of interest that several other groups have also identified blood pressure QTLs on rat chromosome 3 in a $SHRSP_{(Izumo)}$ x WKY cross. In particular, Nara *et al* (1996) found significant linkage of *D3Mgh16* with basal systolic blood pressure in both male and females, whilst Matsumoto *et al* (1995 & 1996) determined a female only QTL for both salt-loaded diastolic and systolic blood pressure with the anonymous microsatellite marker *D3Mgh12*.

The differences between these studies might be related to different methods of blood pressure measurement (radio-telemetry versus tail-cuff/femoral artery cannulation). The current study is the first to report the use of telemetry for phenotyping an entire F2 cohort in a linkage study. It is likely that such high fidelity phenotyping, in which the animals are free from catheters, tethers and stress from human contact, may affect the final results of QTL mapping. Alternatively, genetic heterogeneity between WKY controls from different sources as confirmed by both biochemical genetic markers (Matsumoto *et al*, 1991) and DNA fingerprinting techniques (Samani *et al*, 1989; Kurtz *et al*, 1989; Nabika *et al*, 1991) may be the culprit. This heterogeneity is believed to have occurred as a result of its world-wide distribution prior to complete inbreeding (Kurtz & Morris, 1987) and must be borne in mind when making any comparison of experimental results obtained using WKY from different sources as in the case above. Indeed this genetic heterogeneity between groups may also be the reason why the current study was unable to detect the blood pressure QTLs previously reported on chromosomes 1, 10 and 18 in SHRSP x WKY crosses (Hilbert *et al*, 1991; Jacob *et al*, 1991; Nara *et al*, 1993), as well as why these other groups did not record a major QTL on rat 2 in the region of *D2Mit6* as this study did.

The majority of previous studies used F2 males only for linkage analyses. The current study used male and female F2 cohorts phenotyped with high fidelity methods to enable us to identify the male sex-specific QTLs on chromosomes 2 and 3. It is possible that these male only QTLs may have resulted from epistatic interaction with the Y chromosome blood pressure raising locus previously identified by Davidson *et al* (1995) in the Glasgow SHRSP x WKY cross. However, the stepwise regression procedures carried out for baseline and salt-loaded blood pressure phenotypes did not reveal such an interaction. Instead, the

putative SHRSP Y chromosome effect and the *D2Mit6* or *D3Mgh16* recessive effects were significant but independent influences on baseline and salt-loaded blood pressure. An alternative explanation which requires further investigation may be the existence of differing hormonal interactions at the transcriptional as well as post translational level of these hypertensive alleles.

In addition to the above blood pressure QTLs, the current study is notable for identifying the first QTL for left ventricular hypertrophy in SHRSP on rat chromosome 14. However, in view of the borderline significance (LOD 3.1) it should be considered as suggestive linkage only (Lander & Kruglyak, 1995) until congenic strains can confirm otherwise. Whilst no obvious candidate genes mapped in this region in the current study, the α-adducin gene has been mapped to rat chromosome 14 (Tripodi *et al*, 1995). This codes for one of the subunits of an α/β heterodimeric protein regulating cell-signal transduction through the actin cytoskeleton which appears to be involved in the regulation of renal tubular reabsorption and extracellular fluid homeostasis (Ferrari *et al*, 1991, 1992). Initially Bianchi *et al* (1994) identified point mutations in both the α and β subunits of the adducin gene which accounted for up to 50% of the difference in blood pressure between the Milan hypertensive and normotensive rat strains. The same group went on to identify a functional mutation in the human α-adducin gene (Gly460Trp) and demonstrated significant linkage and association of the α-adducin locus with essential hypertension (Cusi *et al*, 1997). However, its relationship to left ventricular hypertrophy remains untested and examination of the α-adducin gene in the Glasgow colonies of SHRSP and WKY revealed no functional polymorphisms (G. Bianchi, personal communication).

Previous genome scans have identified two other putative QTLs for heart weight on chromosome 2 (Innes *et al*, 1998) and chromosome 17 (Pravenec *et al*, 1995). The latter showed significant linkage of the microsatellite marker within the dopamine 1a receptor with left ventricular heart weight adjusted for body weight (Pravenec *et al*, 1995). In addition, several candidate genes have been shown to co-segregate with increased heart or left ventricular weight in crosses involving inbred hypertensive rat strains, including *endothelin-3* on chromosome 3 (Cicila *et al*, 1994); *Ace* on chromosome 10 (Harris *et al*, 1995; Zhang *et al*, 1996); *Anp/Bnp* on chromosome 5 (Zhang *et al*, 1997), heat shock protein *HSP27* on chromosome 12 (Hamet *et al*, 1996) and *renin* on chromosome 13 (Rapp, 1989). Unfortunately interpretation of all these studies may be confounded by as yet unidentified cardiac modifying genes on the same chromosome and thus, as for the blood pressure QTLs above, speculation about the functional role of specific variants in these regions should remain just that.

In conclusion, this was the first study to combine the use a genome wide scanning strategy with the high fidelity phenotyping of an F2 cohort. In doing so it identified several new QTLs containing genetic determinants of hypertension in the Glasgow SHRSP x WKY cross, particularly serving to highlight the relative prevalence of sex-specific QTLs which had been previously masked by the other groups' exclusive use of males only. Both the blood pressure QTLs identified on chromosome 2 in this study have been identified separately in alternative hypertensive rat strains, indicating the important common role the gene(s) involved may play in the development of hypertension in the rat. By contrast, the QTL on chromosome 3 has only been replicated in other SHRSP x WKY crosses and is thus indicative of a SHRSP-specific hypertensive allele. This study also identified the first

putative QTL for left ventricular hypertrophy in the SHRSP, thus adding to the wealth of evidence that so-called sub-phenotypes of hypertension are, at least partially, under independent genetic control. As all these chromosomal regions of interest are large however, and mostly without any clear candidate genes, the development of congenic strains with which to narrow them down is now appropriate (*Chapter 5*).

4. IDENTIFICATION OF A QTL FOR ISCHAEMIC STROKE IN SHRSP

4.1 Introduction

In the past it was generally believed that hypertensive end-organ damage was primarily due to the effects of hypertension and environmental factors while the role played by genetics was a negligible one. Recent discoveries, however, such as the identification of QTLs for left ventricular hypertrophy independent of blood pressure (*Chapter 3*; Pravenec *et al*, 1995; Innes *et al*, 1998), along with advances in phenotyping such as radio-telemetry, have initiated the need to re-examine the role of genetics in other cardiovascular and cerebrovascular phenotypes.

Clinical and epidemiological studies reviewed by Alberts (1990) provided strong evidence for genetic influences in the development of stroke. This was supported by recent studies of Framingham offspring cohorts (Kiely *et al*, 1993) and twin pairs (Brass *et al*, 1992) which both suggested a strong familial aggregation of the disorder. In addition, several Mendelian traits featuring stroke have been described (Gunel & Lifton, 1996). Among these, the most relevant to ischaemic stroke is a syndrome of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) which has been mapped to human chromosome 19q12 (Tournier-Lasserve *et al*, 1993) and appears to result from numerous mutations in the human *Notch3* gene (Joutel *et al*, 1996 & 1997).

While the genes and pathophysiological pathways involved in Mendelian forms of stroke are currently under intensive investigation, it seems likely that their involvement will account for only a very small fraction of the most common ischaemic stroke in man (Gunel & Lifton, 1996). In this case, genetic analysis is hindered by the late onset of the clinically apparent

disease and the mode of inheritance which is complex, polygenic, and multifactorial. Moreover the genetic background is heterogeneous, similar to most other epidemiologically important diseases such as diabetes, hypertension, and coronary heart disease.

An important approach to the study of such polygenic diseases is the use of appropriate animal models in which individual contributing factors can be both recognised and analysed (Lander & Schork, 1994; Dominiczak & Lindpaintner, 1994). During the establishment of a colony of spontaneously hypertensive rats (SHR) by Okamoto and Aoki (1963) several substrains developed a tendency for spontaneous cerebral infarction or haemorrhage (Hazama *et al*, 1975). The clustering of stroke in these strains suggested a genetic basis for stroke and Okamoto *et al* (1974) selected a stroke-prone substrain, the SHRSP, in which spontaneous strokes developed in 75-80% of rats.

Spontaneous stroke in SHRSP has a number of disadvantages for genetic studies: first, it develops late in the animal's life (after 14-20 weeks of age); second, the stroke may or may not be lethal; third, it presents as multiple lesions resembling diffuse changes of malignant hypertension rather than defined ischaemic stroke (Hazama *et al*, 1975; Okamoto *et al*, 1974). Despite this, Nagoaka *et al* (1976) studied latency to stroke (or age of stroke occurrence) on a high salt diet in SHRSP, F2 hybrids and back-crosses obtained by mating SHRSP with the stroke-resistant SHR. This phenotype, which could be related to stroke susceptibility in man, was characterised by a polygenic inheritance. More recently, Rubattu *et al* (1996) performed a genome wide scan in an F2 cross obtained by mating SHRSP and SHR where latency to stroke on a Japanese diet (high salt, low potassium and protein) was yet again used as a phenotype. This study identified three major QTLs, *STR1-3*, which

together accounted for 28% of the overall phenotypic variance and were blood pressure independent. Two of these QTLs, *STR-2* and *-3*, conferred a protective effect against stroke in the presence of SHRSP alleles and *STR-2* co-localised with the genes encoding atrial and brain natriuretic peptides.

In addition to susceptibility to spontaneous stroke, SHRSP display significantly larger cerebral infarcts than their normotensive reference strain, the Wistar-Kyoto (WKY) rat (Gratton *et al*, 1998), following the experimental occlusion of their middle cerebral artery (MCA). This is a strong phenotype for genetic studies. The stringent control of physiological variables under anaesthesia, and the uniform precision in the point, origin, and length of the occlusion, increases the sensitivity of infarct volume to the influence of genetic factors and decreases variability induced by non-genetic factors. Furthermore, it is one of the most relevant animal stroke models to man as the MCA is reported to be the vessel most commonly affected in human stroke syndromes (Berkow & Fletcher, 1987; Mohr *et al*, 1986; Karpiak *et al*, 1989).

Coyle *et al* (1984) performed a co-segregation study using focal ischaemia produced by an occlusion of the middle cerebral artery (MCA) to characterise the stroke-prone phenotype. They showed a bimodal distribution of this phenotype and suggested that a single recessive gene was responsible for the pathogenesis of stroke in the SHRSP (Coyle *et al*, 1984). Subsequently Gratton *et al* (1998) observed that the distribution of infarct volumes in the first filial (F1) generation produced by crossing in-bred SHRSP and WKY rats virtually matched that in parental SHRSP, strongly suggesting a dominant mode of inheritance for this phenotype. Possible reasons for the two different conclusions may include Coyle's use

of outbred normal Wistar rats in place of inbred WKY rats, and a less severe, more distal occlusion in much younger animals (Gratton *et al*, 1998). Whatever the reason for these differences, both studies showed clear evidence for the involvement of a major genetic component above and beyond the effects of hypertension.

It follows that the aim of the current research was to identify the genetic component(s) responsible for severity of cerebral ischaemia after MCA occlusion, and thus separate it from the genes contributing to hypertension identified in *Chapter 3*, by performing a second genome wide scan in a SHRSP x WKY F2 cohort phenotyped by experimental focal ischaemia.

4.2 Methods

Experimental Animals and Genetic Crosses

The inbred colonies of SHRSP and WKY rats held at the University of Glasgow were utilised in the present study to yield the necessary reciprocal genetic crosses as previously detailed in *Chapter 2.2.2*. One male SHRSP was mated with 2 WKY females (cross 1) and 1 male WKY was mated with 2 SHRSP females (cross 2). From the F1 rats of each cross, 2 males and 4 females were brother-sister mated to generate a cohort of 59 F2 rats for phenotyping (39 in cross 1 with a male-to-female ratio of 22:17 and 20 in cross 2 with a male-to-female ratio of 9:11).

Blood Pressure Measurement

As it was not technically feasible to perform two surgical procedures on an individual rat, systolic blood pressures were measured indirectly by tail-cuff plethysmography at 12 weeks of age in parental and F2 rats rather than by radio-telemetry as described in *Chapter 2.3.1.* In addition, chronic mean arterial blood pressure was measured 24 hours after MCA occlusion by direct femoral artery cannulation as described in *Chapter 2.3.6.* Data provided by both methods of blood pressure measurement were verified by comparison with that obtained in the parental, F1 and F2 litter-mates subjected to the continuous radio-telemetry recording in *Chapter 3.*

Occlusion of the Middle Cerebral Artery

All surgical procedures involved in the occlusion of the middle cerebral artery were performed by Drs Gratton and Carswell at the Wellcome Surgical Institute, Glasgow. Male and female SHRSP (n = 8; M:F = 6:2); WKY (n = 12; M:F 8:4) and F2 (n = 59; M:F 33:26) underwent MCA occlusion following the technique of Tamura *et al* (1981) as previously described in *Chapter 2.3.6.* Infarct volumes were expressed as a percentage of the ipsilateral hemisphere to account for brain swelling and differences in brain size between sexes and strains.

Genotyping and Statistical Analysis

A total of 702 microsatellite markers were screened and of these 22% were found to be dimorphic. The microsatellite markers were obtained from both Genosys Biotechnologies (Europe) and Research Genetics (Huntsville, AL.). Genotyping was performed by PCR amplification of DNA around the microsatellites as previously described in *Chapter 2.4.3.*

Examination of phenotypes for sex differences in the parental strains was accomplished by the use of two-sample *t*-tests. Two-way ANOVA was employed to detect differences between crosses or sexes in the F2 cohort, or between genotype groups while correcting for sex.

Genetic markers were mapped relative to each other by using the MAPMAKER/EXP 3.0 computer package with an error detection procedure (Lander *et al*, 1987; Lincoln & Lander, 1992). Genetic distances were calculated with the Haldane mapping function. QTLs affecting a given phenotype were mapped relative to the genetic markers by using the MAPMAKER/QTL 1.1 computer package obtained from Dr. Eric Lander (Whitehead Institute, Cambridge, Massachusetts). A purely phenotypic model (genetic parameters set to zero) was fitted initially to assess the likely contribution of sex as the only explanatory variable, and the corresponding percentage of variance explained. The full genetic model, representing free inheritance of the trait after correction for sex, was then fitted, as were models for dominant, recessive and co-dominant inheritance.

A confirmatory analysis of the QTL near the *Anp* marker on chromosome 5 was carried out by two-way ANOVA for different phenotypes in terms of marker genotype and sex. Other possible confounding factors for size of infarct as a proportion of ipsilateral hemisphere volume were investigated by including them in a multiple regression model for the phenotype, then removing non-significant effects by backwards elimination (Rawlings, 1988) whilst retaining the correction for sex differences.

4.3 Results

All the significant phenotypic and genotypic data collected by this study is given in Appendix III, as well as the primer sequences and optimum PCR conditions of each microsatellite marker used.

Initial experiments in parental SHRSP and WKY showed that volume of infarction after MCA occlusion was bimodally distributed (*Figure 4.1*). There was no correlation between indirect blood pressures measured before the occlusion of the middle cerebral artery (MCA) and infarct volume in the F2 hybrids (*Figure 4.2)*. Moreover, there was no relationship between mean arterial blood pressure measured by direct femoral artery cannulation at 24 hours after MCA occlusion and the volume of infarction (*Figure 4.3*). Two-sample *t*-tests provided evidence (*Figure 4.1*) of a difference in the infarct volume expressed as a percentage of the ipsilateral hemisphere between males and females in the WKY strain (means ±SD were 14.2±6.2 and 7.4±3.1%; t = 2.55, P = 0.031, 95% CI 0.8 to 12.8%). The number of SHRSP females was too small for formal analysis of any sex difference. In the more relevant F2 cohort, a two-way ANOVA to examine the effect of sex and the origin of

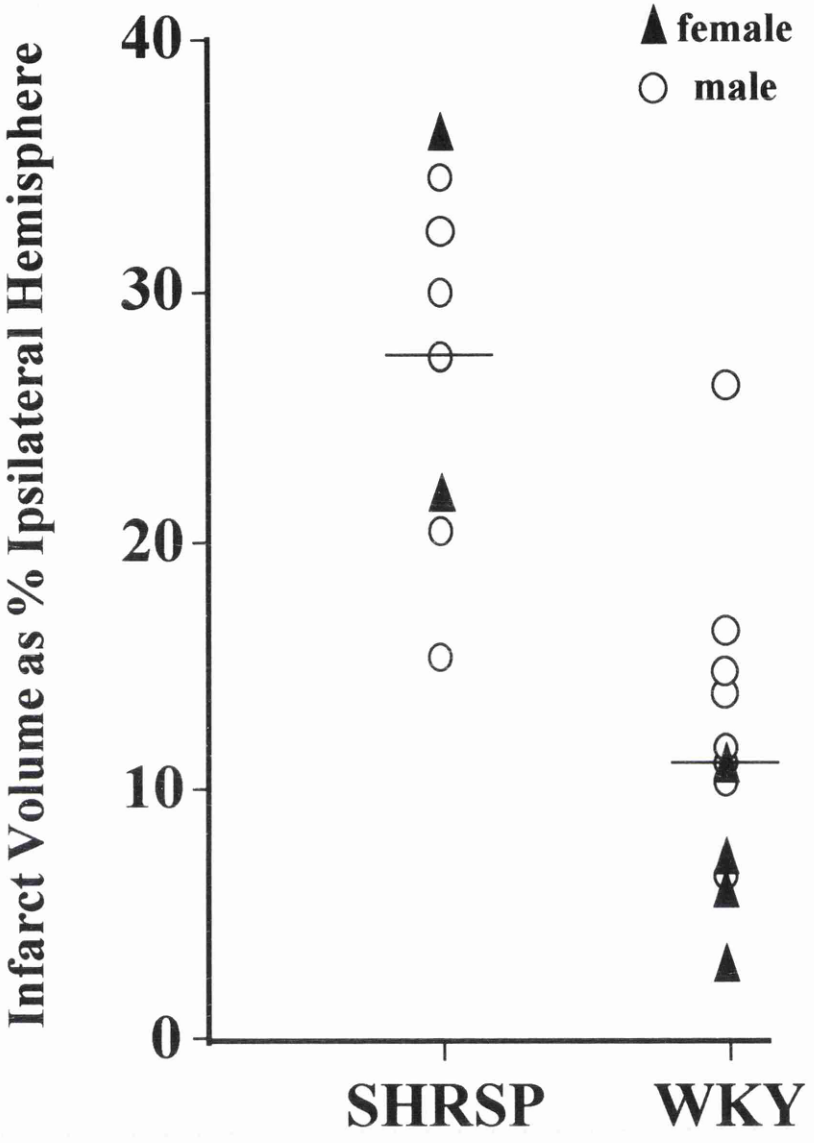

Figure 4.1 Bimodal distribution of cerebral infarction in parental SHRSP (n = 8; M:F = 6:2) and WKY (n = 12; M:F = 8:4) shown as scatter diagrams and means (p = 0.0001, 95% CI 9.9 to 22.7%).

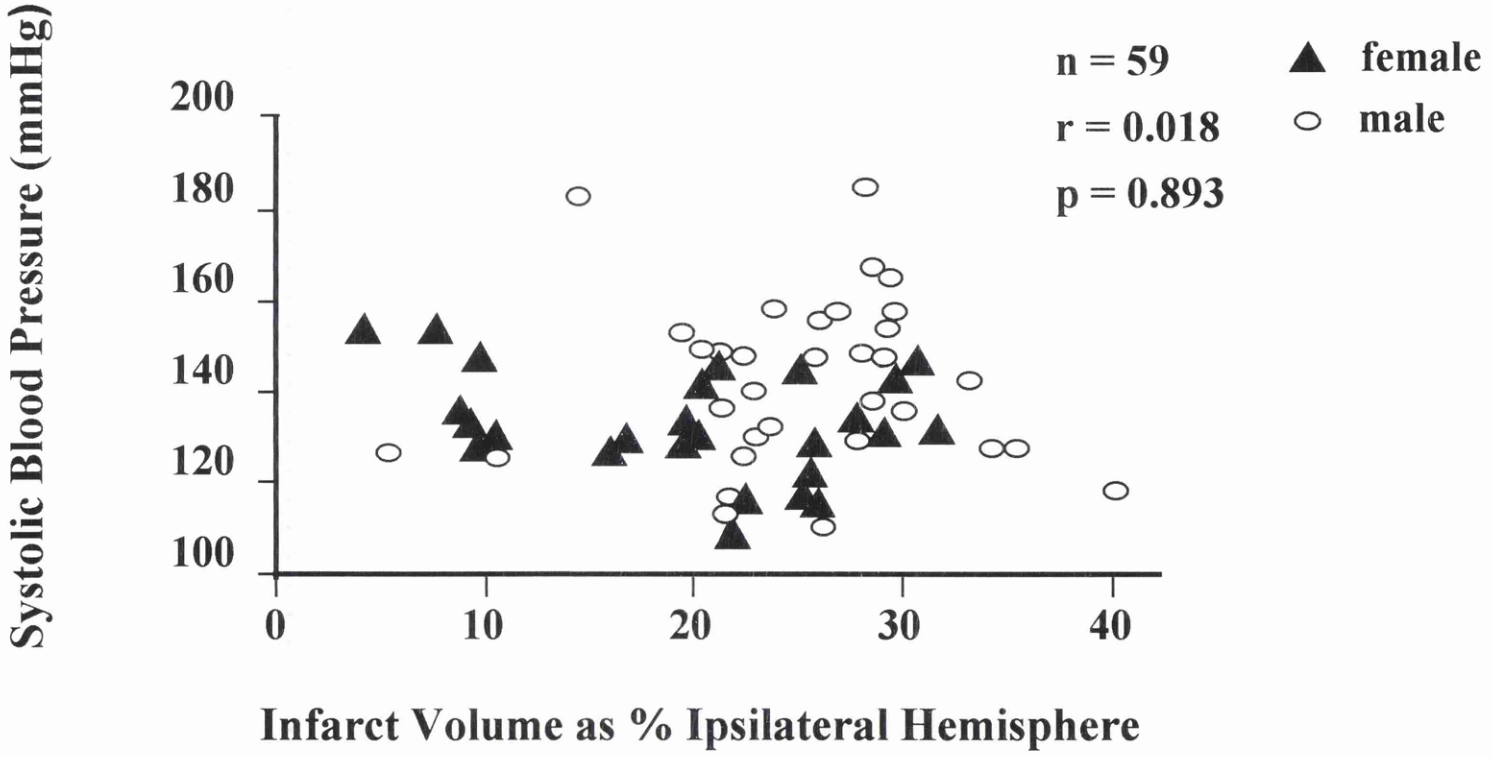

Figure 4.2 Lack of co-segregation of cerebral infarction with systolic blood pressure in the F2 SHRSP x WKY population.

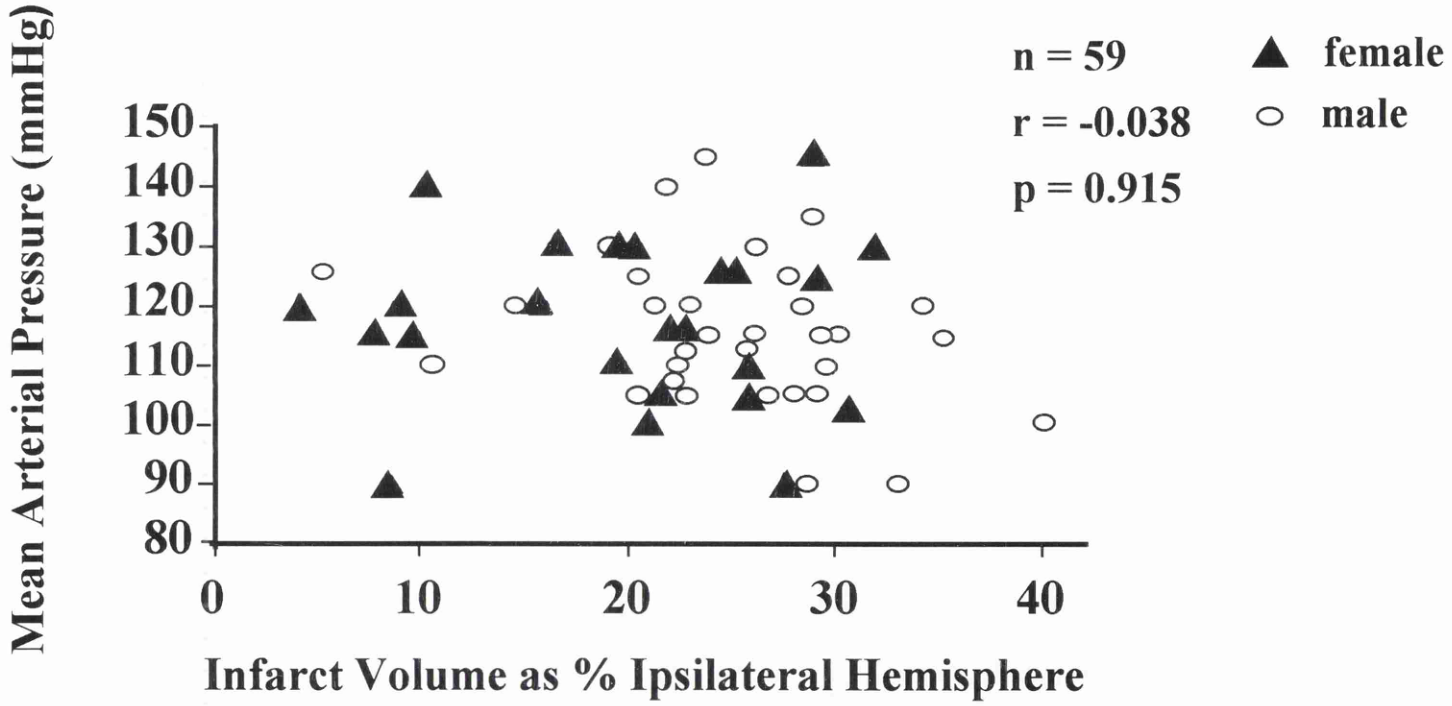

Figure 4.3 Lack of co-segregation of cerebral infarction with mean arterial blood pressure 24hrs post middle cerebral artery occlusion in the F2 SHRSP x WKY population.

the cross (SHRSP grandfather versus WKY grandfather, *Chapter 2.2.2*) again showed that sex had a significant effect with male F2 hybrids having larger infarcts than females (means ±SD were 25.2±6.8 and 20.2±7.8%; F = 6.73, P = 0.012, 95% CI 1.1 to 8.8%), whereas the origin of the cross had no effect (F = 0.19; P = 0.67).

A major QTL on rat chromosome 5 with a sex adjusted LOD score of 16.6 (corresponding $P = 1.0 \times 10^{-16}$) was identified which accounted for 67% of the infarct volume as a percentage of the ipsilateral hemisphere volume variance in the SHRSP x WKY cross (*Figure 4.4*). The likely contribution of sex as a sole explanatory factor was 1.44, which explained 10% of the phenotypic variance. The microsatellite marker in the centre of this QTL is *Anp*, a marker within the gene coding for atrial natriuretic peptide. Moreover, in the rat, mouse and man, the gene for *Anp* co-localises with the gene for brain natriuretic peptide (*Bnp*; Tamura *et al*, 1996). The influence of the *Anp* marker on a number of phenotypes was examined correcting for sex with a two-way ANOVA (*Table 4.1*). The effect of the *Anp* marker can be described as co-dominant, as the F2 hybrids homozygous for the SHRSP (ss) had the largest infarct volume with the heterozygotes (ws) falling between the F2 hybrids homozygous for the WKY allele (ww) and the ss homozygotes. Moreover, the *Anp* marker had no effect on any other phenotype tested; notably there was no significant difference in systolic blood pressure or mean arterial pressure between ss, ws and ww F2 hybrids. The co-dominant character of the QTL on rat chromosome 5 was further confirmed by comparison of QTL models that were constrained to follow different models of inheritance. As shown in *Figure 4.4*, the co-dominant model presented a better fit for the QTL than the free inheritance model.

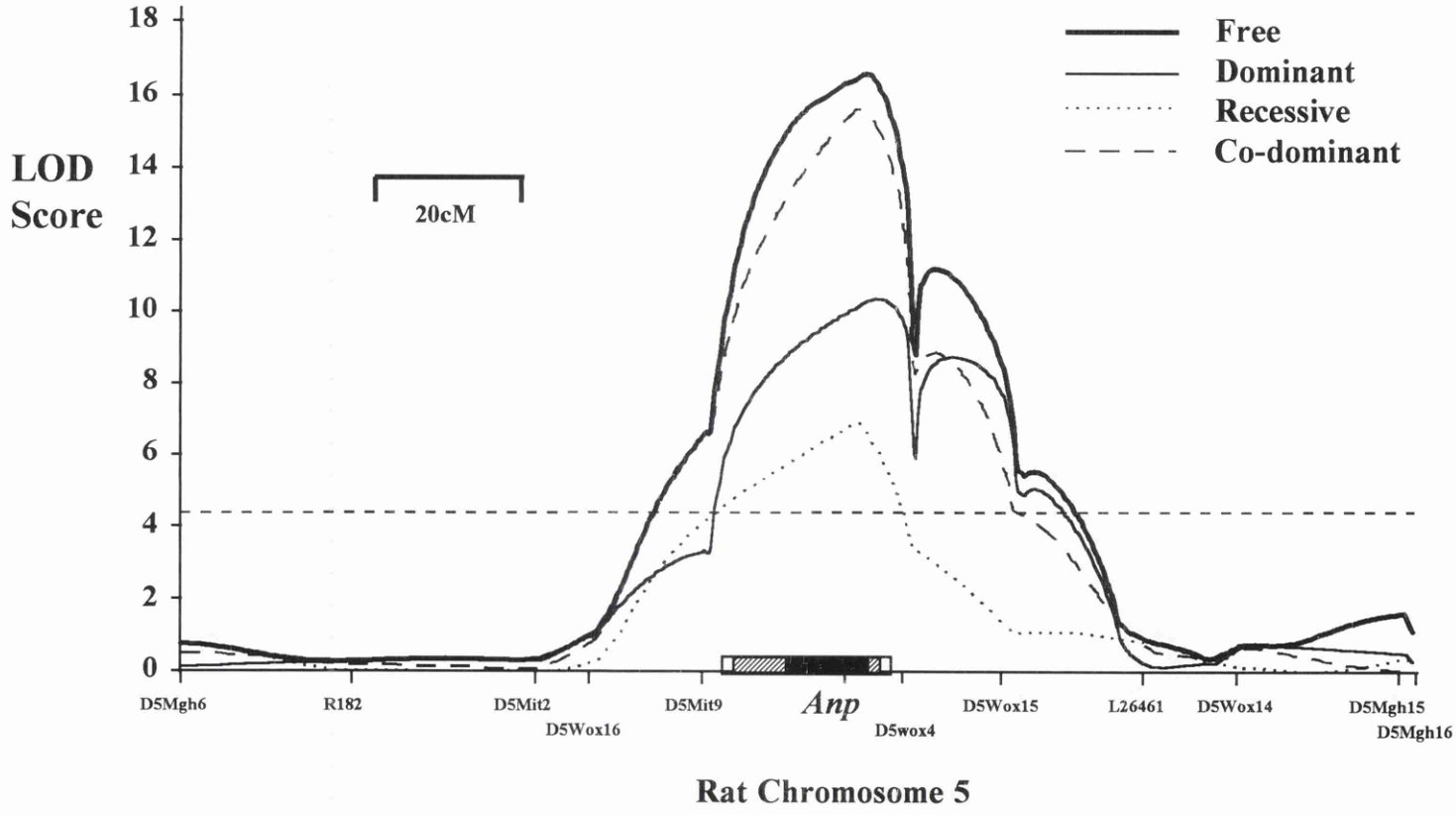

Figure 4.4 Rat chromosome 5 linkage map and infarct volume expressed as a percentage of the ipsilateral hemisphere QTL localisation for a F2 population derived from SHRSP x WKY (males and females). The broken straight line indicates the highest LOD threshold for significant linkage. All markers are anonymous except *Anp*, atrial natriuretic peptide. The 1-, 3- and 4.3-LOD support intervals are shown as black, shaded and open bars, respectively.

Phenotype	Genotype at the *Anp* Marker			
	***WW* (n = 13)**	***WS* (n = 26)**	***SS* (n = 20)**	***p***
Infarct Volume (mm^3)	79.2 (33.3)	155.5 (32.5)	208.4 (33.1)	4.7×10^{-14}
Infarct Volume/Ipsilateral Hemisphere (%)	12.2 (4.0)	23.0 (3.9)	29.9 (4.0)	4.4×10^{-16}
Systolic Blood Pressure (mmHg)	141.8 (16.4)	133.9 (16.0)	138.2 (16.3)	.35
Mean Arterial Pressure 24hrs Post Surgery (mmHg)	148.4 (15.6)	145.4 (15.3)	142.3 (15.5)	.55
Body Weight (g)	254.9 (19.4)	246.1 (19.0)	247.3 (19.3)	.41

Table 4.1 Effects of genotype at the *Anp* microsatellite marker on multiple phenotypes in F2 hybrids. Values are given as the mean and (standard deviation). Analysis was performed by a two-way ANOVA with sex and the *Anp* marker as factors; data corrected for sex.

As previous studies had revealed an important role of sex and putative loci on the Y chromosome for the blood pressure phenotype (Davidson *et al*, 1995) a backward elimination procedure was performed to confirm or refute the role of these and other possible confounders in the determination of the volume of cerebral infarction (*Table 4.2*). The possible confounders, which included systolic and mean arterial pressures, the effect of the SHRSP Y chromosome (Davidson *et al*, 1995), body weight and the grandparental origin of the cross, were all eliminated in this stepwise procedure, with the highly significant effect of the QTL centred around the *Anp* microsatellite marker being retained.

4.4 Discussion

The current study is the first to map the gene for severity of ischaemic brain damage following occlusion of the MCA in the SHRSP. The QTL on rat chromosome 5, centred around the *Anp* marker, showed highly significant linkage to size of infarction following MCA occlusion. There was no linkage between this QTL and any of the blood pressure phenotypes studied. Sex was the only additional contribution to the phenotypic variance and thus the final LOD score was sex adjusted. All other potential confounders such as body weight, the origin of the cross, or the Y chromosome effect (Davidson *et al*, 1995) were excluded. The QTL on rat chromosome 5 accounts for 67% of the infarct volume variance and no other QTLs contributing to the severity of ischaemic damage were detected.

These data are highly contrasting to those reported by Rubattu *et al* (1996). Firstly, the QTL on rat chromosome 5 detected in this study is characterised by a much higher level of significance. Secondly, the SHRSP allele at this locus contributes to a greater severity of

Response Variable	Explanatory Variable	F-Statistics	*p*
Infarct Volume/Ipsilateral Hemisphere Volume (%)	Systolic Blood Pressure	0.02	.888
	Mean Arterial Pressure 24hrs Post-Surgery	0.14	.710
	Origin of Cross	0.19	.665
	Body Weight	2.40	.128
	Anp	71.85	4.4×10^{-16}
	Sex	0.17	.678

Table 4.2 Backward elimination procedure to identify the role, if any, of several possible confounders in the genetic determination of the volume of cerebral infarction.

ischaemic damage when compared to the WKY allele, whereas the locus confers a protective effect against latency to stroke as studied by Rubattu *et al* (1996). Lastly, the current study identified only one major locus, with no significant QTLs on rat chromosomes 1 and 4. All three differences described above may be accounted for by the major contrast in the method of characterisation of the stroke-related phenotype between the two studies. The latency to stroke (or age of stroke occurrence) calculated from the time of commencement of the Japanese rat diet includes both ischaemic and haemorrhagic stroke and its analysis requires complex adjustments for age. The phenotype used for the current study is performed as a planned experimental procedure at 12 weeks of age, and is highly reproducible and pathophysiologically important. It therefore seems reasonable to suggest that the gene(s) responsible for the latency to stroke with a high salt diet may be quite independent from those which determine the size of infarct following cerebral vessel occlusion.

The discovery of one major QTL for sensitivity to experimental cerebral ischaemia in the SHRSP is in line with the previous findings of both Coyle *et al* (1984) and Gratton *et al* (1998) who, in performing purely phenotype-based co-segregation analyses, suggested a single autosomal gene was responsible for infarct susceptibility. The current study places two putative genes central to the LOD-support intervals of the QTL, *Anp* and *Bnp*, which have well established vasoactive properties including natriuresis, diuresis, vasodilatation and inhibition of the renin-angiotensin-aldosterone system (Levin *et al*, 1998) and therefore may be involved in the control of contraction, growth and remodelling in cerebral vessels. Several studies have indicated that SHR and SHRSP have elevated plasma ANP concentrations as well as alterations in the number of ANP-binding sites in the brain

(Nagase *et al*, 1997; Grove *et al*, 1997; Saavedra *et al*, 1986). It follows that further research is warranted to ascertain whether the genes encoding ANP and BNP are really likely candidates for sensitivity to experimental cerebral ischaemia (*Chapter 6*).

A previous study localised a QTL for blood pressure near the endothelin and GlutB genes on rat chromosome 5 in Dahl salt-sensitive (SS) rats (Deng et al, 1994). In addition, Zhang *et al* (1996 & 1997) found evidence for a QTL determining mean blood pressure and heart weight on chromosome 5 between *Anp* and *Bnp* in a SHR x WKY cross. Most recently, Garrett *et al* (1998) described a QTL for blood pressure on chromosome 5 in a Dahl SS x Lewis F2 cross which lowered blood pressure by 15mmHg in a subsequent congenic strain. All these studies suggest the existence of a region on rat chromosome 5 which contributes primarily to high blood pressure with any physiological effects on the brain proving secondary to the elevated blood pressure. The single locus identified for sensitivity to cerebral ischaemia in the current study, however, was independent of blood pressure. This lack of a relationship between high blood pressure and large volume of infarction in SHRSP was further strengthened by the failure to identify QTLs for cerebral ischaemia in chromosomal regions overlapping those previously identified for blood pressure phenotypes in *Chapter 3*. These observations agree with the already well-documented concept that large infarcts are not secondary to hypertension (Coyle *et al*, 1986; Gratton *et al*, 1998). For example, SHRSPs suffer larger infarcts in early life (at 5 weeks of age), before either hypertension or cerebrovascular changes become established (Coyle & Jokelainen, 1983). Secondary forms of experimental hypertension such as DOCA-salt do not increase susceptibility to cerebral infarction (Coyle, 1984), and hypotensive treatment in the SHRSP reduces infarct size following MCA occlusion only minimally and never down to WKY size

(Fujii *et al*, 1992). Indeed, elevated blood pressure during experimental stroke has been shown to reduce infarct size (Ogilvy *et al*, 1996) and vascular hypertrophy which develops alongside hypertension has been shown to reduce susceptibility to spontaneous stroke (Sadoshima *et al*, 1981).

The complete dissociation between genes for severity of ischaemic damage and blood pressure might be extrapolated to human stroke. It has been shown that similar atherosclerotic occlusion of a major cerebral artery in humans may cause a catastrophic stroke or be entirely asymptomatic with an apparently continuous distribution of neurological damage in between (Allen, 1983; Jacewicz, 1992). This may be due to a stroke susceptibility or severity gene governing collateral blood flow as the anastomosing collaterals in SHRSP have significantly smaller internal diameter and a lesser ability to dilate as compared to age-matched WKY (Coyle & Heistad, 1991). Indeed, Arribas and co-workers (1996) previously demonstrated geometric disorganisation of the medial layer of basilar arteries in the SHRSP using confocal microscopy, whereas Volpe *et al* (1996) showed impaired endothelium-dependent vasorelaxation in the aorta and basilar artery of the SHRSP.

Gender significantly influenced the outcome of ischaemia in rats, explaining 10% of the phenotypic variance seen in the F2 hybrids utilised in this study. Females displayed smaller infarcts following MCA occlusion then males, a finding which has been recently replicated by Alkayed *et al* (1998). In addition, the incidence of spontaneous stroke in SHRSP has been found to be lower in females than in males (Yamori *et al*, 1976b). Interestingly, Carswell *et al* (1998) found that female SHRSP in met-oestrus display larger experimentally

induced infarcts than those females taken at random, and also larger infarcts than males. As levels of oestrogen and progesterone fluctuate between 7pg/ml and 40-50pg/ml and between 5-10ng/ml and 45-50ng/ml, respectively, during the oestrus cycle (Smith *et al*, 1975) it appears likely that the high levels of female hormones at different stages in the 4 day cycle may be protecting against ischaemic damage and therefore clearly influencing stroke sensitivity. It is already known that ovariectomised female rats treated with oestrogen chronically before a focal cerebral insult suffer smaller infarcts than untreated ovariectomised females (Simpkins *et al*, 1997). Oestrogen has been reported to have anti-inflammatory effects including decreasing levels of cytokines such as tumour necrosis factor (TNF) alpha and acting as a free radical scavenger (Goodman *et al*, 1996; Squadrito *et al*, 1997). Oestrogen also decreases low density lipids and increases high density lipids (Crook, 1996) and promotes nitric oxide- (Pelligrino et al, 1997) and prostacyclin- (Gustafsson, 1997) mediated vasorelaxation by increasing nitric oxide synthase and prostacyclin production, respectively. Oestrogen has also been shown to inhibit endothelin-1 production thereby reducing the extent of endothelin-induced vasoconstriction (Wingrove & Stevenson, 1997). Progesterone has also been shown to exert neuroprotection (Roof *et al*, 1993) perhaps by a potent anti-oedema effect on the brain (Betz & Coester, 1990).

In conclusion, this was the first study to use the experimental occlusion of the middle cerebral artery as a stroke-related phenotype for genome wide QTL analysis. It demonstrated a highly significant QTL on rat chromosome 5 which was blood pressure independent and possessed two putative candidate genes in its centre, the *Anp* and *Bnp* genes. Whilst there is ample evidence to support further investigation of these candidate genes for a role in the pathophysiology of stroke (*Chapter 6*) the concurrent establishment

of congenic strains containing the large chromosomal region of interest remains the most positive method of ensuring progress is made towards the ultimate positional cloning of the gene(s) responsible for the severity of cerebral ischaemic damage in the SHRSP (*Chapter 5*).

5. CONSTRUCTION OF SPEED CONGENIC STRAINS

5.1 Introduction

Genome wide linkage analysis, as successfully used in *Chapters 3* and *4* to localise large chromosomal regions containing QTLs for high blood pressure, left ventricular hypertrophy and sensitivity to cerebral ischaemia in the SHRSP, is only a first step towards gene identification. Not only does the ultimate goal of positionally cloning the causal gene currently require the QTL to be narrowed to less than 2cM, but conclusions drawn from linkage analyses are prone to producing false positives (Rapp & Deng, 1995). To confirm the existence of a QTL, and to narrow down the chromosomal region of interest, a more definitive and stringent genetic test is required in the form of congenic strain construction.

A congenic strain is one in which a portion of a chromosome from one strain (the recipient) has been selectively replaced by the homologous portion of the same chromosome of another strain (the donor). If the chosen phenotype, for example blood pressure, of the congenic strain is different from the recipient strain, one can conclude that this particular chromosomal fragment harbours a QTL that contributes to a difference in blood pressure between the donor and recipient strains. Congenic strains have been traditionally developed by serially backcrossing the donor strain that harbours the genomic region of interest with the recipient inbred strain, accompanied by selection for progeny heterozygous for the desired region in each backcross generation (Wakeland *et al*, 1997). This essentially leads to serial dilution of the donor genome into the recipient genome, with continuous maintenance of the QTL. As, according to Mendel's laws, it is expected that half of the unrelated genomic material will be transmitted to a

subsequent backcross generation, 8-10 generations of backcross matings should result in a congenic strain in which more than 99% of the genome unlinked to the target gene will be of recipient strain origin (*Figure 5.1*). The introgressed region is then made homozygous by intercrossing, and the resulting congenic strain theoretically possesses a genetic background identical to that of the recipient inbred strain with the exception of the introgressed region.

Whilst the above protocol for breeding congenic strains is simple, its weaknesses are that it requires at least 3-4 years to complete and the purity of the congenic strain for recipient genome is based purely on theoretical considerations, without any experimental assessment. Recent additions to the density of a microsatellite based rat map, however, (Bihoreau *et al*, 1997; Brown *et al*, 1998) may offer the opportunity to remove both these problems by permitting the utilisation of a so-called "speed" congenic strategy origin (*Figure 5.1*).

This strategy involves the analysis of dimorphic marker loci distributed throughout the genome in order to specifically select the male progeny at each backcross generation that carries the target genomic region whilst also possessing the lowest content of heterozygosity throughout the remaining genome. Breeding with these "best" males should theoretically allow the rate of donor genomic segment elimination to be dramatically accelerated, thereby reducing the number of generations necessary to construct a congenic strain. Computer simulations have indicated that a relatively modest selection effort (60 background markers, 25cM marker spacing, 16 males per generation) will typically reduce unlinked donor genome contamination to below 1% by four

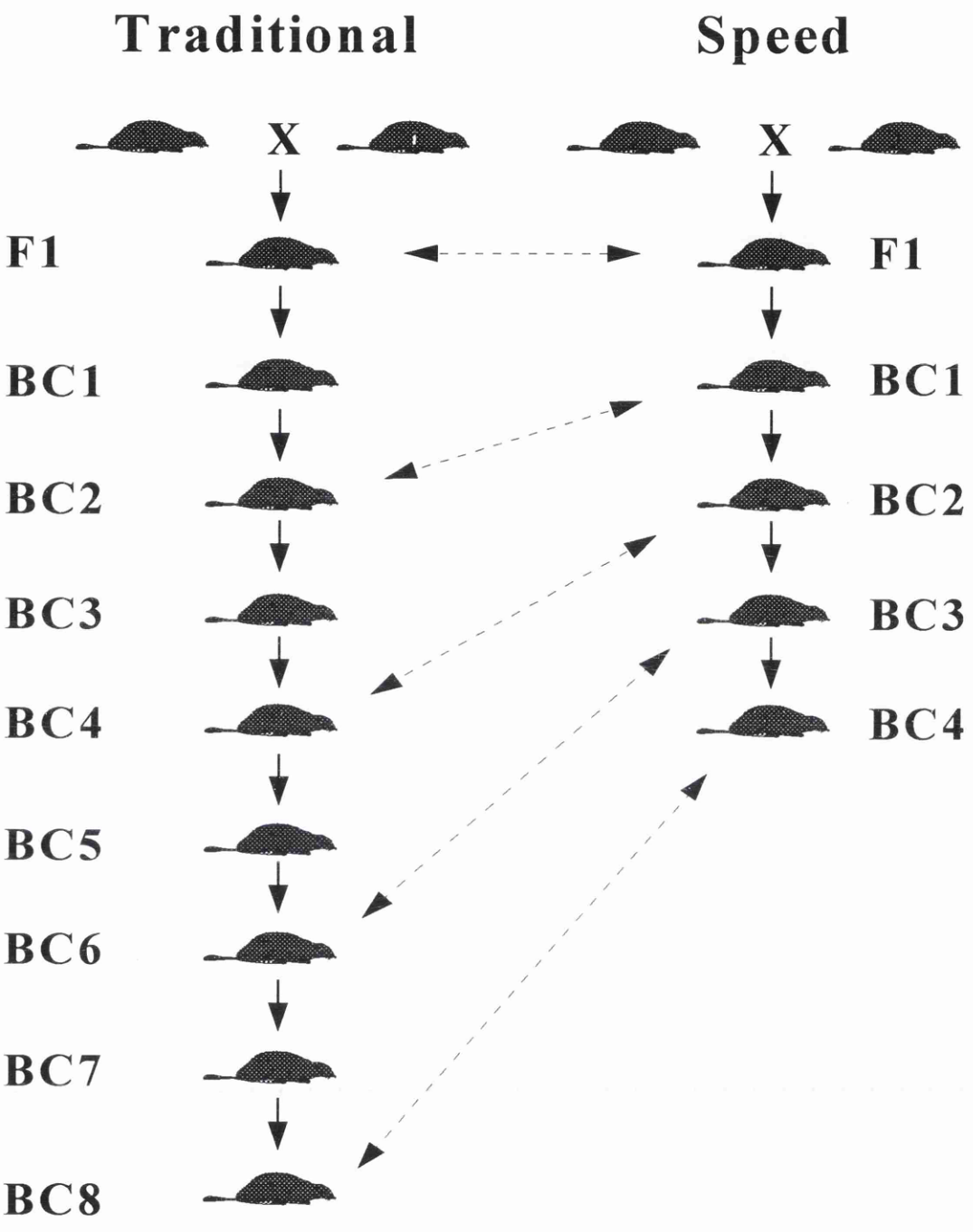

Figure 5.1 Cartoon showing the production of a congenic strain using the traditional 8 backcrosses versus the speed congenic method utilised in the mouse (Markel *et al*, 1997). The dotted arrows indicate the equivalent backcrosses at which the background heterozygosity is theoretically the same.

backcross generations (BC4), roughly equivalent to backcrossing to BC8-10 using the traditional protocol (Wakeland *et al*, 1997) Indeed, the recent development of ten "speed" congenic mice strains carrying defined genomic intervals derived from non-obese diabetic (NOD) or NZM2410 strains on the C57BL/6 genome (Yui *et al*, 1996; Morel *et al*, 1996) provided results which closely paralleled these predicted outcomes.

Whilst the feasibility of a speed congenic approach remains to be tested in the rat, several rat congenic lines have been successfully produced using the traditional breeding strategy in order to more precisely localise chromosomal regions of linkage to blood pressure regulation. Of particular relevance, Deng *et al* (1997) confirmed the existence of a blood pressure QTL on rat chromosome 2 in the region corresponding to that identified in *Chapter 3* of the current study between markers *D2Mit14* and *D2Mgh12* by constructing two congenic strains introgressing the relevant region from the WKY rat or the Milan normotensive (MNS) rat into the Dahl salt-sensitive (SS) background. These strains had blood pressure 44mmHg and 29mmHg lower, respectively, than SS rats on a 2% NaCl diet (Deng *et al*, 1997).

Although the chromosome 2 region introgressed by Deng *et al* (1997) harbours several genes that could be construed as candidates for the blood pressure QTL, speculation as to the identity of the actual QTL is premature as the substituted region was very large (41cM) and thus requires still further investigation via congenic substrains. In addition, the results of *Chapter 3* of the current study clearly indicated this appeared to be a sex-specific QTL occurring in males only, a matter Deng's study (1997) was unable to address having been performed in a population of solely male rats. Moreover, the

chromosome 2 congenic strains of Deng *et al* (1997) did not examine evidence for the second QTL on rat chromosome 2 identified in *Chapter 3* around the *D2Mit6* marker and corresponding to that also identified by several other groups in various crosses (Dubay *et al*, 1993; Deng & Rapp, 1992; Deng *et al*, 1994; Garrett *et al*, 1998).

It follows that the purpose of the current study was to begin a more comprehensive congenic analysis of the whole of rat chromosome 2 and the QTLs identified therein. This is to be achieved by the production of an improved genetic linkage map for rat chromosome 2 between SHRSP x WKY and the first application of a speed congenic strategy in the rat, introgressing the genomic regions of interest in both directions; that is from the WKY to the SHRSP and *vice versa*, and into both males and females.

5.2 Methods

Experimental Animals and Genetic Crosses

To improve the chromosome 2 genetic map achieved in *Chapter 3*, DNA from the same two F2 reciprocal crosses as described in *Chapter 3* was utilised (57 in cross 1 with a male-to-female ratio of 28:29 and 83 in cross 2 with a male-to-female ratio of 37:46). In order to simplify the PCR reaction to one 96-well plate, however, genotype analysis was restricted per microsatellite marker to a random 94 samples from this F2 set and two parentals.

Congenic strains were constructed by placing various segments of rat chromosome 2 from WKY rats on the genetic background of the SHRSP and vice versa using a previously un-tested "speed" congenic breeding procedure as previously outlined in *Chapter 2.2.3.* The number of backcrosses necessary (BC3-BC5) varied from congenic strain to congenic strain and was dependent on the rate at which heterozygosity was lost from the genetic background as well as litter size. Once a male and female were identified in which all visible background heterozygosity had been removed, they were mated to obtain rats homozygous for the donor alleles throughout the chromosome 2 region of interest. After the region was fixed, congenic strains were maintained by brother-sister mating.

Tail-Tipping and DNA Extraction

For genotyping of congenic back-crosses, the animals were briefly anaesthetised at 4 weeks of age with fluothane and a 4mm tip from their tail removed into a 1.5ml microfuge tube. The wound was immediately sealed with an electric cauteriser (Engel-Loter 100S) and the tails stored at -20°C. Genomic DNA was isolated from the tails using a simplified mammalian DNA isolation procedure involving serial phenol-chloroform extractions as described in *Chapter 2.4.1.* All samples were stored in 100µl TE solution (10mM Tris, pH 8.0; 0.1mM EDTA, pH 8.0) and stored at 4°C prior to PCR analysis.

Genotyping and Statistical Analysis

A total of 33 new microsatellite markers for rat chromosome 2 were screened and of these 79% were found to be dimorphic between $SHRSP_{(Glasgow)}$ and $WKY_{(Glasgow)}$. These markers were obtained from both Genosys Biotechnologies (Europe) and Research Genetics (Huntsville, AL) and had been recently published by the Whitehead Institute Center for Genome Research Rat Mapping Project, Boston, USA at http://www.genome.wi.mit.edu/rat/public/ within an up-date of the original rat genetic map published by Jacob *et al* (1995). Genotyping was performed by PCR amplification of DNA around the microsatellites as previously described in *Chapter 2.4.3*. The genotypic results gained from these additional genetic markers were added to those previously collected in *Chapter 3* and mapped relative to each other using the MAPMAKER/EXP 3.0 computer package with an error detection procedure (Lander *et al*, 1987; Lincoln & Lander, 1992) as previously described. On completion of the new map, the QTLs on rat chromosome 2 found to influence blood pressure phenotypes in *Chapter 3* were re-mapped relative to the new genetic map by using the MAPMAKER/QTL 1.1 computer package obtained from Dr. Eric Lander (Whitehead Institute, Cambridge, Massachusetts).

In addition to the microsatellite markers on rat chromosome 2, an additional 53 (*Table 5.1*) broadly spanning the remaining genome were used for genotyping throughout the construction of the congenic strains, and were also obtained from both Genosys and Research Genetics. The "best" males identified as heterozygous (ws) for the marker alleles within the desired genomic region on chromosome 2, but mostly homozygous (ss

Marker	Chromosome	Marker	Chromosome
D1Mit14	1	D10Wox3	10
GV30	1	D11Mgh6	11
D1Wox32	1	D12Mgh3	12
D1Mit11	1	D12Wox2	12
D1Mit1	1	D13Mgh1	13
D3Mit4	3	D13Wox4	13
D3Wox14	3	REP328	14
D3Mgh8	3	D14Wox5	14
D3Mgh16	3	D14Wox8	14
GV88	4	D15Mit2	15
D4Mgh16	4	D15Mgh3	15
D4Mgh7	4	D15Mgh6	15
D4Mit14	4	D16Wox12	16
NG56	5	R435	16
D5Wox15	5	D16Mit1	16
D5Mgh15	5	D17Wox21	17
D5Mgh16	5	D17Wox13	17
D6Wox21	6	D17Wox10	17
D6Mgh5	6	D18Wox12	18
D7Mit10	7	D18Wox16	18
D7Mit7	7	D19Wox2	19
D8Wox22	8	D19Wox8	19
D8Mgh7	8	D20Wox3	20
D8Wox13	8	D20Wox5	20
D8Mgh10	8	DXWox3	X
D9Mit1	9		
D9Wox13	9		
D9Mit4	9		

Table 5.1 Microsatellite markers (n = 53) utilised to genotype the genetic background of all chromosome 2 congenic strains established.

or ww) for the recipient alleles (SHRSP and WKY respectively) everywhere else were selected and mated with the respective recipient strain to go on to the next backcross. In order to save resources, however, a full genome scan involving the analysis of the full 53 background markers was only performed on a few animals at each backcross, followed by genotyping in subsequent backcrosses of only those markers identified as heterozygous in the previous generation.

5.3 Results

All the raw genotypic data newly collected by this study is given in Appendix IV, as well as the primer sequences and optimum PCR conditions of each microsatellite marker used. The raw phenotypic data utilised in the re-mapping of blood pressure QTLs on rat chromosome 2, and the original genotypic data used in the map construction is the same as that collected in *Chapter 3* and is given in Appendix II.

A total of 26 new microsatellite markers on chromosome 2 were genotyped using the F2 DNA and of these 9 were added to the original map obtained in *Chapter 3* using the Haldane mapping function of the MAPMAKER/EXP 3.0 computer package (*Figure 5.2*). As a result the genetic map for the Glasgow F2 SHRSP x WKY cross expanded from 12 markers spanning 136.6cM (*Chapter 3*) to 21 markers covering 179.5cM, a distance comparable to that previously reported by several other groups (Andoh *et al*, 1998; Bihoreau *et al*, 1997; the Whitehead Institute Center for Genome Research, 1998). The remaining 17 markers were excluded from the map by MAPMAKER. This is indicative of their preliminary placement in linkage groups on chromosome 2 by the

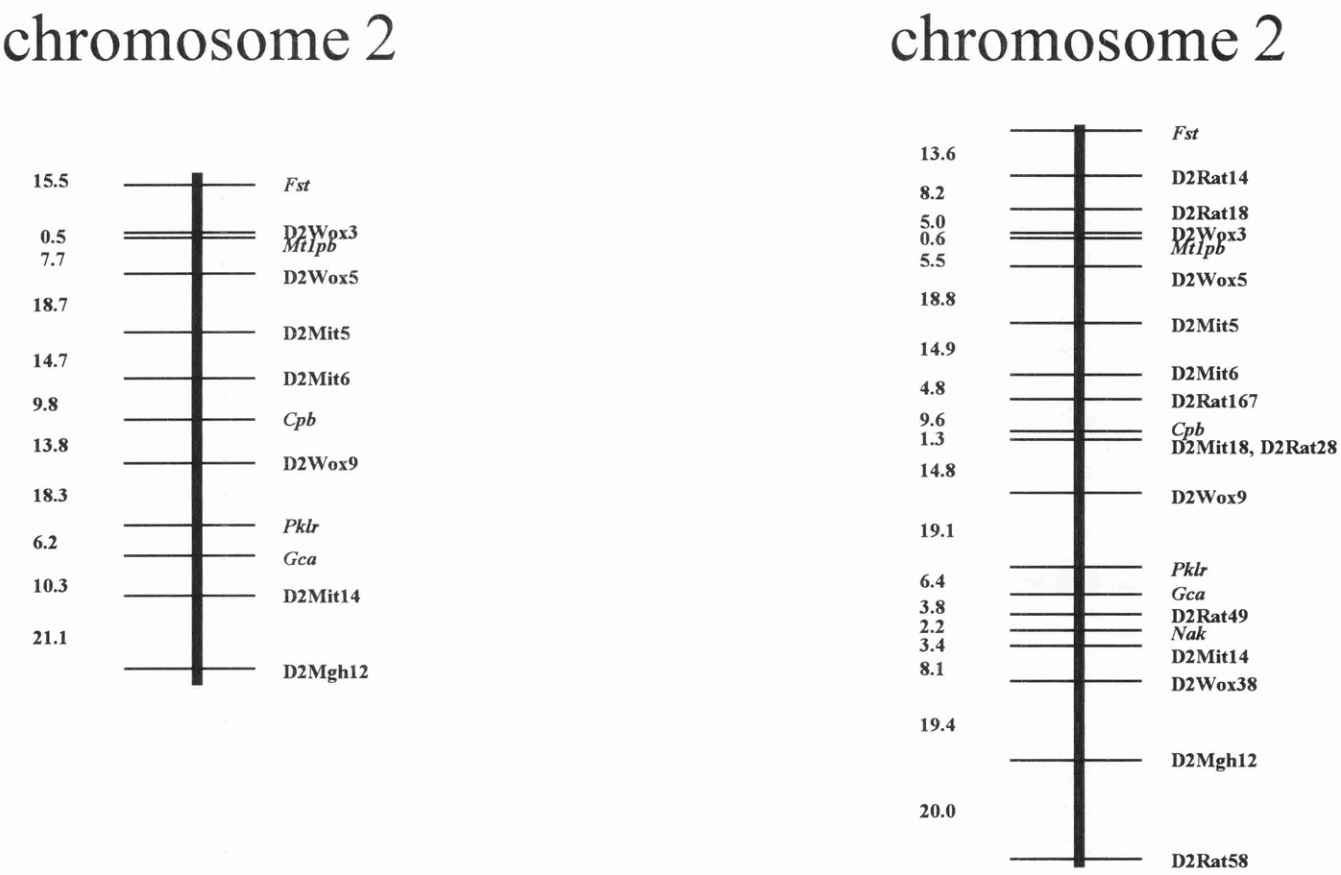

Figure 5.2 Direct comparison of genetic maps of rat chromosome 2 constructed in *Chapter 3* (left) and that achieved with the addition of new microsatellite markers (right). Numbers to the left of the map represent distances between markers in centiMorgans (cM) using the Haldane correction. All markers are anonymous except *Gca*, guanylyl cyclase A; *Pklr*, pyruvate kinase L; *Cpb*, carboxypeptidase B; *Nak*, Na^+K^+ATPase Alpha-1; *Mt1pb*, metallothionein 1 pseudogene b; and *Fst*, follistatin.

Whitehead Institute Center for Genome Research along with the warning that they may have "multiple possible placements".

Using the new map and the MAPMAKER/QTL 1.1 computer package both the significant QTL on rat chromosome 2 around the *D2Mit6* marker and the suggestive male only QTL between the markers *D2Mit14* and *D2Mgh12* were again identified. Whilst the LOD scores obtained for the *D2Mit6* QTL did not differ from those recorded in *Chapter 3*, the LOD scores for the male only QTL now reach significant, rather then suggestive, linkage levels, in particular LOD = 4.1 for salt-loaded diastolic blood pressure (*Figure 5.3*).

Five congenic strains (SP.WKYgla2a-e) were produced by introgressing regions of chromosome 2 from WKY rats into the recipient SHRSP strain (*Figure 5.4*) and four congenic strains (WKY.SPgla2a-d) were produced by introgressing regions of chromosome 2 from SHRSP rats into the recipient WKY strain (*Figure 5.5*). In the names of the congenic strains, the first abbreviation refers to the recipient, the second the donor. The numbers/letters are arbitrary designations where 2 refers to chromosome 2, and a, b, c, d and e are arbitrarily assigned to each strain. The regions transferred have been designed to cover different genomic regions of interest on chromosome 2. 2a involves the transfer of the entire map of chromosome 2, and thus both blood pressure QTLs identified. Congenic strain 2b contains no QTLs. 2c also includes both QTLs but within a smaller chromosomal region than 2a. 2d and 2e contain only the one QTL each, around *D2Mit6* and *D2Mgh12* respectively. Unfortunately, 2e has only been transferred from WKY on to the SHRSP background due to the low fecundity of the reciprocal

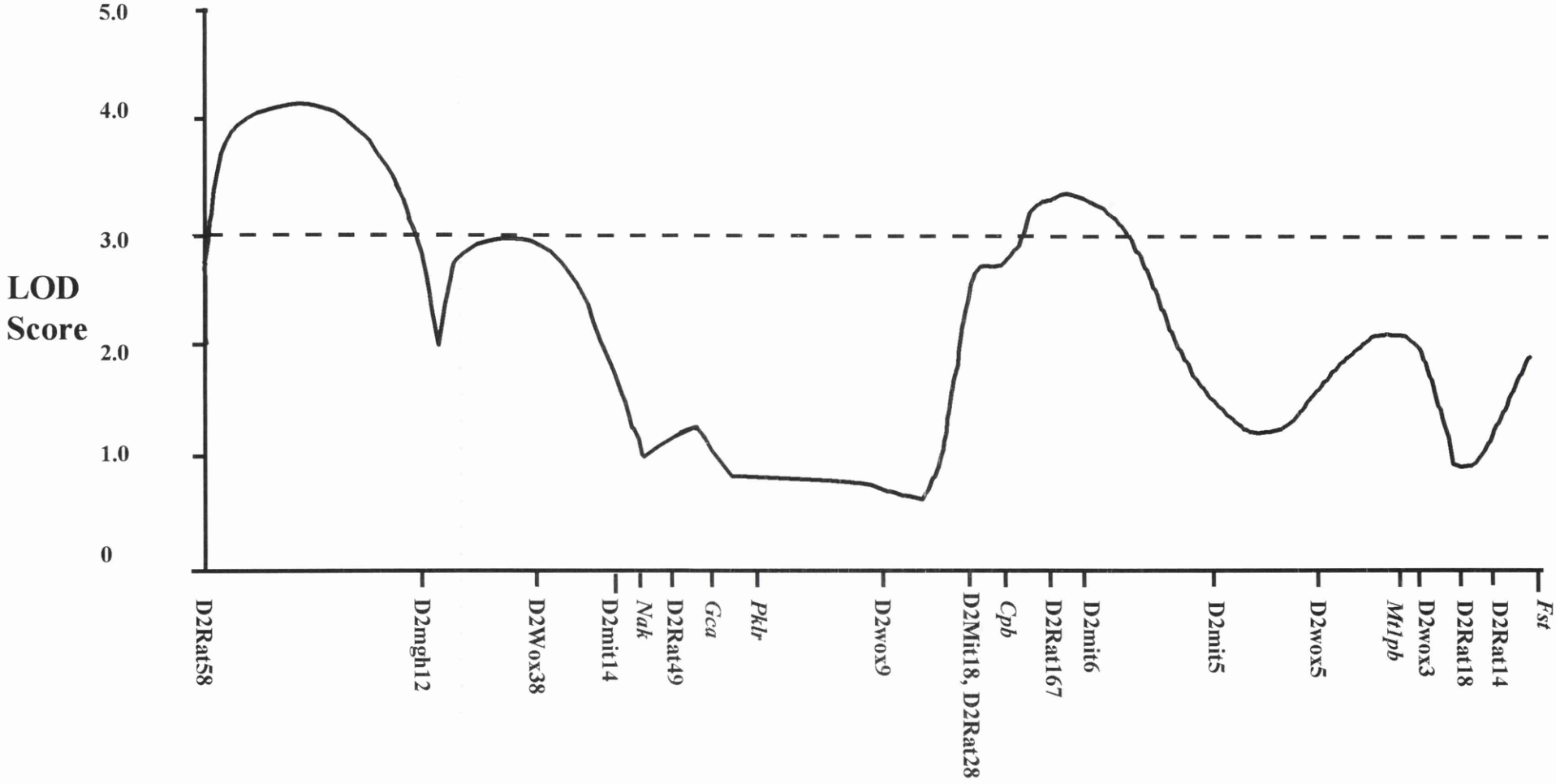

Figure 5.3 Improved rat chromosome 2 linkage map and salt-loaded diastolic blood pressure QTL localisation for a F2 male only population derived from SHRSP x WKY. Map distances and symbols are the same as in *Figure 5.2.*

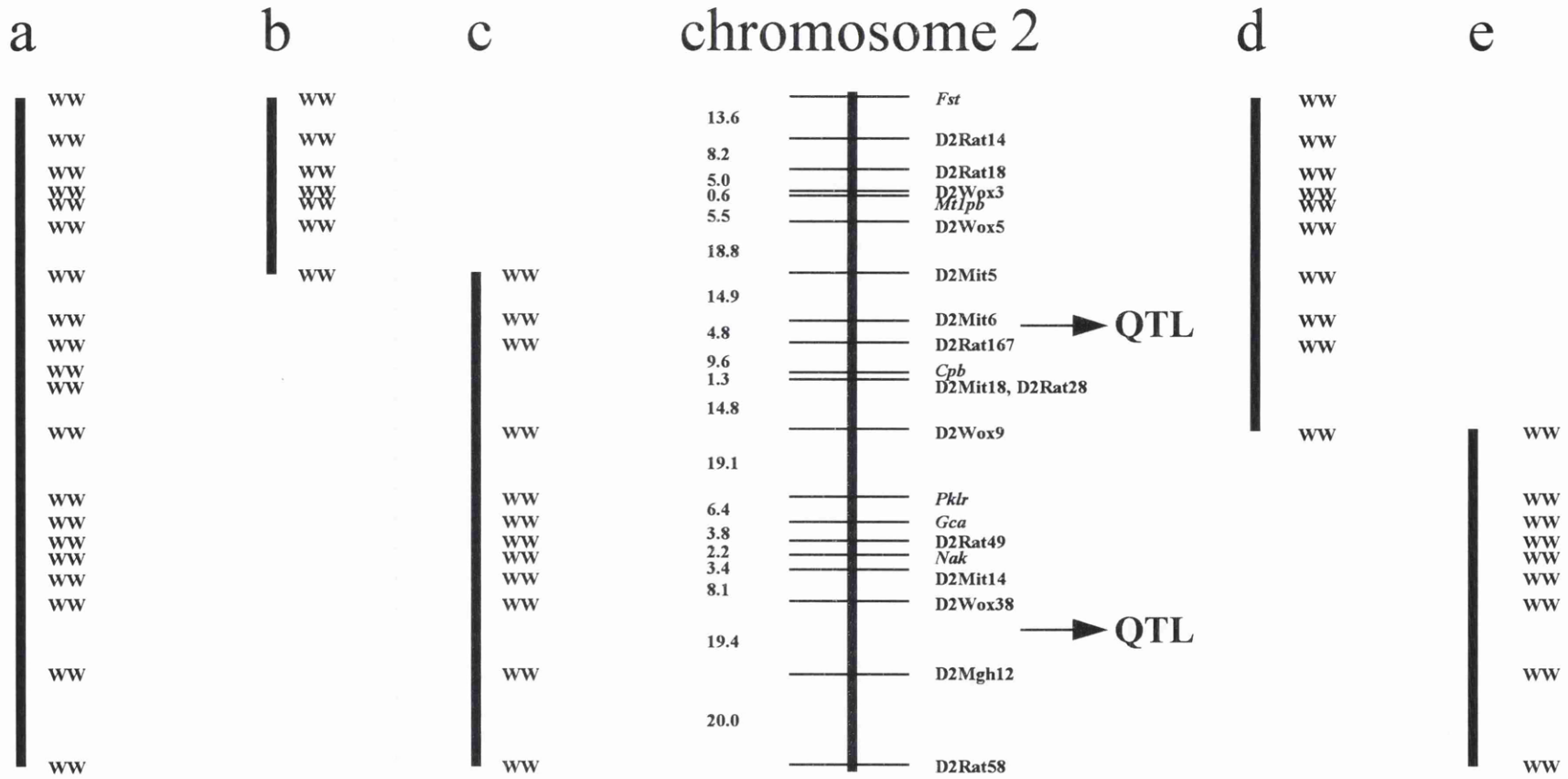

Figure 5.4 Rat chromosome 2 congenic strains SP.WKYgla2a-e where SHRSP is the recipient strain and WKY the donor strain. The solid bars a to e represent the chromosomal segment of the SHRSP being replaced by the homologous segment of the WKY. The entire region indicated by solid bars are homozygous (WW) for the WKY strain allele for all the markers listed in the corresponding positions within the solid bars on the map. Numbers to the left of the map represent distances between markers in centiMorgans (cM) using the Haldane correction and all markers are the same as listed in *Figure 5.2*. The position of both QTLs on rat chromosome 2, and their location in each congenic strain, is indicated by the arrows.

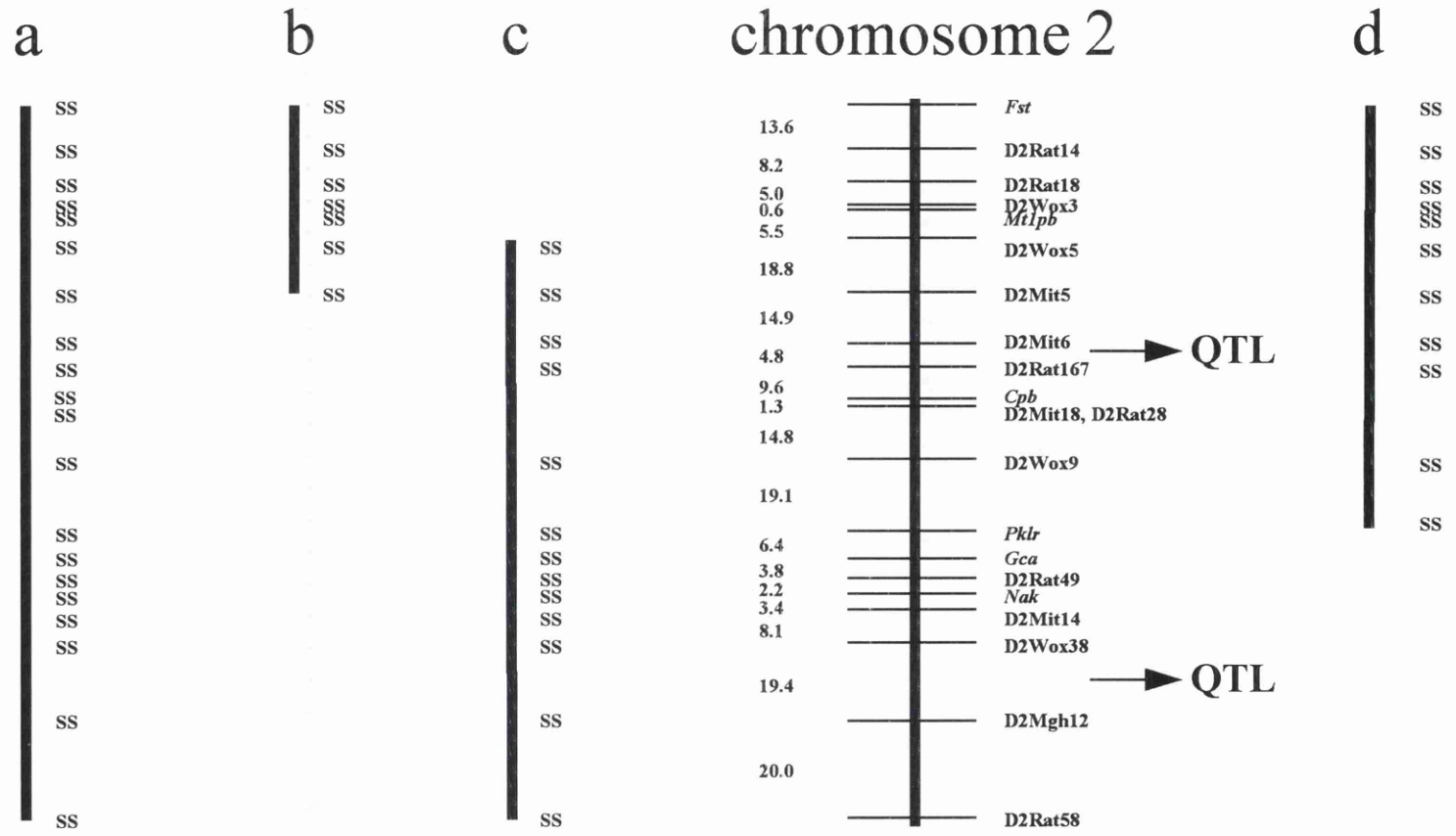

Figure 5.5 Rat chromosome 2 congenic strains WKY.SPgla2a-d where WKY is the recipient strain and SHRSP the donor strain. The solid bars a to d represent the chromosomal segment of the WKY being replaced by the homologous segment of the SHRSP. The entire region indicated by solid bars are homozygous (SS) for the SHRSP strain allele for all the markers listed in the corresponding positions within the solid bars on the map. Numbers to the left of the map represent distances between markers in centiMorgans (cM) using the Haldane correction and all markers are the same as listed in *Figure 5.2*. The position of both QTLs on rat chromosome 2, and their location in each congenic strain, is indicated by the arrows.

strain. Animals found to be homozygous for the recipients' alleles rather than for the donors' alleles in the final step of fixing the 2a strains were also intercrossed to provide congenic controls. Termed SP.SPgla and WKY.WKYgla (*Figure 5.6*) these strains have been through the same selection processes as the true 2a congenic strains but do not containing the donor QTLs. They will, however, contain in their genetic background the same, if any, residual heterozygosity missed by the total genome scan with the background markers.

Tables 5.2A and *B* illustrate the number of generations of backcrossing required for each strain to achieve complete homozygosity of the background genetic markers in a best male which could then be utilised to fix the line. As is shown, this varied between BC3 for congenic strain WKY.SPgla2b and BC5 for both WKY.SPgla2c and SP.WKYgla2e. In total 542 progeny were necessary for screening in order to produce the congenic strains, an average of 60 animals per strain, 15 animals per backcross. *Table 5.3* shows the average reduction in background marker heterozygosity through subsequent backcrosses using the speed congenic strategy, figures which mirror those theoretically expected over BC8 generations utilising the traditional approach.

5.4 Discussion

The presentation in this study of nine congenic strains derived for rat chromosome 2 clearly demonstrates the applicability of a speed congenic strategy in this species. It is hypothesised that following the future assessment of systolic and diastolic blood pressure at baseline and after salt-loading in these strains as measured by radiotelemetry they will

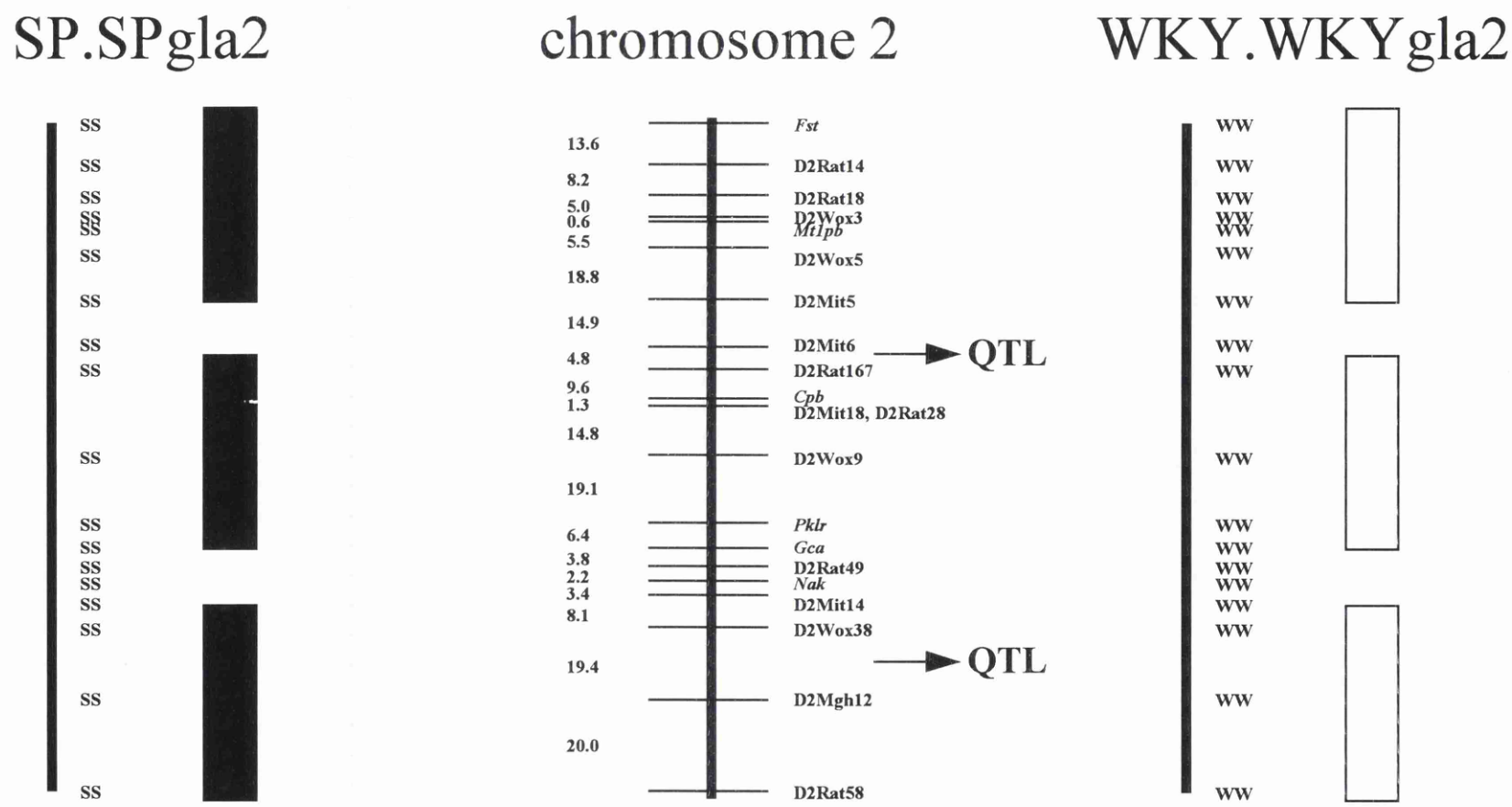

Figure 5.6 Rat chromosome 2 congenic control strains SP.SPgla2 and WKY.WKYgla2. The filled blocks represent SHRSP genetic background, the white blocks WKY genetic background. Numbers to the left of the map represent distances between markers in centiMorgans (cM) using the Haldane correction and all markers are the same as listed in *Figure 5.2*.

Strain	Generation	Progeny Screened	% Background Marker Heterozygosity of "Best" Male
SP.WKYgla2a	BC1	56	28.3
	BC2	16	13.2
	BC3	23	1.9
	BC4	34	zero
SP.WKYgla2b	BC1	56	43.4
	BC2	15	24.5
	BC3	28	9.4
	BC4	27	zero
SP.WKYgla2c	BC1	56	28.3
	BC2	16	11.3
	BC3	17	7.5
	BC4	36	zero
SP.WKYgla2d	BC1	56	28.3
	BC2	16	13.2
	BC3	23	3.8
	BC4	24	zero
SP.WKYgla2e	BC1	56	28.3
	BC2	16	13.2
	BC3	23	7.5
	BC4	31	5.7
	BC5	18	zero

Table 5.2A Rat chromosome 2 congenic strains SP.WKYgla2a-e where SHRSP is the recipient strain and WKY the donor strain. The actual number of progeny screened represents those genotyped for heterozygosity on chromosome 2 and/or residual heterozygosity.

Strain	Generation	Progeny Screened	% Background Marker Heterozygosity of "Best" Male
WKY.SPgla2a	BC1	28	17.0
	BC2	17	7.5
	BC3	14	7.5
	BC4	20	zero
WKY.SPgla2b	BC1	28	17.0
	BC2	17	7.5
	BC3	14	zero
WKY.SPgla2c	BC1	28	35.8
	BC2	6	13.2
	BC3	14	9.4
	BC4	12	5.7
	BC5	11	zero
WKY.SPgla2d	BC1	28	17.0
	BC2	18	1.9
	BC3	5	1.9
	BC4	28	zero

Table 5.2B Rat chromosome 2 congenic strains WKY.SPgla2a-d where WKY is the recipient strain and SHRSP the donor strain.

	Mean Background Marker Heterozygosity(%)			
Generation	**Theoretical**	**Recipient SHRSP**	**Recipient WKY**	**Recipient SHRSP & WKY**
F1	50.0	50.0	50.0	50.0
BC1	25.0	36.8	22.0	29.4
BC2	12.5	18.9	11.3	15.1
BC3	6.25	5.9	9.1	7.5
BC4	3.125	5.5	0.9	3.2
BC5	1.56	1.9	0.9	1.4
BC6	0.78	...	...	...
BC7	0.39	...	...	...
BC8	0.2	...	...	...

Table 5.3 Efficacy of background marker assisted congenic strain construction versus traditional theory. Data was only available from the current study up to BC5 as "best" males with no detectable background heterozygosity were identified in all strains by this stage.

further the evidence for, and the genetic dissection of, the QTLs involved. They should also allow further detailed analysis of sex specificity, epistatic and ecogenetic interactions, and candidate genes as well as the construction of sub-strains containing smaller relevant sections of chromosome.

Previous results of computer simulations comparing different speed congenic strain construction strategies in mice found that a relatively modest selection effort (60 microsatellite markers, 25cM marker spacing, 16 males per generation) will typically reduce unlinked donor genome contamination to below 1% by four backcross generations (BC4), roughly equivalent to backcrossing to BC8-10 using the traditional protocol (Wakeland *et al*, 1997). These theoretical models have been borne out by practical experience with mice speed congenic strains (Yui *et al*, 1996; Morel *et al*, 1996; Markel *et al*, 1997). The current study is the first to provide similar practical evidence in the rat. Selecting the best male at each generation, an average of only 15 rats per backcross were necessary to construct the strains within 3 to 5 backcrosses. However, it should be noted that with the exception of the "best" males which had no visible background heterozygosity by the final backcross of each strain, the average donor contamination did not fall below 1% by BC4 as observed in the mice studies. This is probably a reflection on the fact that due to time and labour restrictions a full genome scan using the 53 markers was not done in all the progeny produced at the beginning of the backcrossing program, but rather was staggered throughout the backcrosses. It follows that even better "best" males may have been overlooked in the initial generations which may have decreased the background heterozygosity at an even greater rate.

Some level of contaminating donor genome in the genetic background is unavoidable, even when utilising the traditional protocol for construction of a congenic strain. Wakeland *et al* (1997) have estimated that the levels of undetected contamination present is roughly equivalent to that at BC8 or BC10 using the traditional protocol; that is below 1%. A potential problem with this, regardless of the congenic strategy, is that during the process of fixing the chromosome region of interest for the donor strain into the homozygous state, an unlinked "stowaway" QTL may happen to be trapped in the 1% residual donor strain and thus could be carried along as a homozygote, or a heterozygote, or not at all. The presence or absence of the unknown and unlinked QTL could confound the interpretation that the blood pressure effect in the congenic strain is due to the intended QTL.

All previous studies involving the traditional construction of rat congenic strains containing blood pressure QTLs have ignored the problem of stowaway loci, citing the number of backcrosses involved as making the effect of background contamination negligible. However, the present study's speed congenic strategy has provided the first opportunity to monitor this parameter in the rat. In genotyping the genetic background at each backcross there were several chromosomal "hotspots" evident at which donor alleles appeared to be preferentially conserved, including genetic loci on chromosomes 4, 7 and 15. The utilisation of a speed congenic strategy enabled these loci to be monitored and "best" males to be selected which did not contain heterozygosity in these regions. However if, as in all the other studies utilising the traditional congenic method, an assessment of background heterozygosity had not been made, equivalent conservation may have been maintained through to generation BC8-10, which could thus have gone

on to be a possible confounder of any blood pressure changes observed in the resulting congenic strains. It follows that it might be useful in all congenic strategies, regardless of the protocol utilised, to include at least some rudimentary assessment of the genetic background of the congenic strain.

On similar lines, the use of at least one of several possible congenic controls to positively establish that a phenotype detected in a congenic strain is due to the target genomic region rather than the effect of any residual loci is probably wise, even in addition to a background scan. Examples constructed in the current study included the 2b congenic strains. Containing a chromosomal region abutting, but not overlapping, the chromosomal segments including the two putative blood pressure QTLs on chromosome 2, the 2b strains should not show a significant change in blood pressure unless stowaway loci are involved. Similarly, the congenic control strains SP.SPgla and WKY.WKYgla whilst not containing the donor QTL will contain in their genetic background the same, if any, residual heterozygosity as the true congenic strains. Whether or not these animals go on to display a change in phenotype similar to that observed in the true congenics will also determine the confounding importance of stowaway loci and hence use of a speed congenic strategy in rats. Finally, all the congenic strains were derived independently, making it very unlikely that they will all by chance contain the same genomic regions of residual donor-strain genetic backgrounds that could potentially effect blood pressure.

In constructing two congenic strains introgressing the region of rat chromosome 2 corresponding to the *D2Mgh12* marker from the WKY rat or the Milan normotensive (MNS) rat into the Dahl salt sensitive (SS) background, Deng *et al* (1997) observed that

the resulting reduction in blood pressure of the WKY congenic strain was about 15mmHg greater than that of the congenic MNS strain, despite the larger SS segment introgressed in the MNS congenic. This difference could have arisen if the QTL allele of the WKY rat was different from that of the MNS rat, if the WKY and MNS rats have the same QTL allele in the *D2Mgh12* region but the larger substitution in the MNS congenic strain contains other genes modifying its effects, and/or if there is one or more additional blood pressure QTLs located in this region of chromosome 2. It is hoped that this is one question the congenic strains developed in the current study will begin to address, as well as having the potential to confirm the putative existence of a second QTL on chromosome 2 around the *D2Mit6* marker as observed in *Chapter 3* and by several other groups using different crosses (Dubay *et al*, 1993; Deng & Rapp, 1992; Deng *et al*, 1994; Garrett *et al*, 1998). In addition, these strains should potentially begin to rule out some of the many candidate genes for blood pressure regulation on rat chromosome 2. They should also be able to confirm or refute the sex specificity of the QTL around the *D2Mgh12* marker observed in Chapter 2, a task which has not been previously possible due to other groups reliance on male only studies.

It is noticeable that whilst Deng *et al* (1997) maintained the use of tail-cuff plethysmography in phenotyping their chromosome 2 congenics, several other groups have turned to radio-telemetry in order to phenotype their congenics for chromosomes 1 (St. Lezin *et al*, 1997), 8 (Kren *et al*, 1997) and 13 (St. Lezin *et al*, 1996). By recognising the need for a more accurate and direct method capable of measuring all the components of blood pressure studies, along with a reliability in detecting small changes and diurnal variations, radio-telemetry analysis of the chromosome 2 congenics derived

in the current study should make them more suitable as a research tool with which to progress further towards identifying the gene(s) at the centre of each putative QTL.

A further utility of these congenic strains was recently highlighted by Rapp *et al* (1998b) in describing the construction of a double congenic strain containing the low blood pressure QTL alleles from both chromosomes 2 and 10 on the Dahl SS rat genetic background. Analysis of the blood pressure of this double congenic strain and its comparison with the relevant single congenic strains, and the SS strain, provides evidence for a strong epistatic interaction on blood pressure of the QTLs on chromosomes 2 and 10. This is the first described epistatic interaction on a quantitative trait in mammalian genetics. It follows that congenics hold much potential for addressing new aspects concerning the genetic regulation of blood pressure and that an ability to construct congenics, sub-strains and double congenics over fewer generations using the speed strategy can only prove advantageous.

One aspect of this research area which is currently threatening to slow progress via congenic sub-strains to positional cloning is the availability of adequately detailed maps of the rat genome. As was observed by the current study, the ability to place markers in order to improve the quality of the genetic map of chromosome 2 was limited by the paucity of precisely mapped markers. Only 9 new markers joined the existing map. Indeed, the majority of newly available markers had even yet to be precisely mapped by the Whitehead Institute Center for Genome Research themselves, and were issued on the Internet with a disclaimer reflecting doubts over their precise location.

Physical mapping methods are beginning to be developed which will provide a firmer base for future positional cloning strategies then genetic mapping. For example, radiation hybrid (RH) mapping is a somatic cell hybrid technique that was developed to construct high resolution, contiguous maps of mammalian chromosomes and which does not hold marker dimorphism as a prerequisite (Cox *et al*, 1990). Now commercially available (Research Genetics), rat whole genome RHs have been generated by taking a donor cell line (RatFR, a diploid fibroblast cell line derived from skin biopsy of a foetal Sprague-Dawley rat) and exposing it to 3000 rad of x-rays and then fusing with non-irradiated thymidine kinase deficient hamster (A23) recipient cells. Loci that are further apart on a chromosome are more likely to be broken apart by radiation and to segregate independently in the RH cells than loci that are closer together. By screening the RH panel to test for the retention or loss of specific donor chromosome markers a map can be constructed statistically. This has already provided detailed physical maps of both rat chromosome 4 (Al-Majali *et al*, 1998) and 5 (Brosnan *et al*, 1998) with their superior resolution resolving most of the ambiguities in distance and location of markers on a chromosome. This technique has also been used to physically map two new strong candidate genes to rat chromosome 2 (Azam *et al*, 1998), soluble guanylyl cyclases α and β, as discussed in *Chapter 3*, although no complete map of this region has been produced as yet.

In conclusion, the successful production of congenic strains on chromosome 2 using a "speed" strategy should promote its use in the construction of other congenics, thereby significantly increasing the pace of research towards positional cloning of the genes involved in tandem with the production of detailed physical maps. Additionally it is

hoped that following radio-telemetry analysis these strains themselves will begin to unravel the genetic basis of blood pressure regulation on rat chromosome 2 as well as bringing to attention the importance of testing the influence of stowaway loci experimentally.

6. SEQUENCING ANALYSIS OF GENES ENCODING ATRIAL AND BRAIN NATRIURETIC PEPTIDES AS CANDIDATES FOR SENSITIVITY TO CEREBRAL ISCHAEMIA IN SHRSP

6.1 Introduction

The natriuretic peptide (NP) family exerts potent natriuresis/diuresis and vasorelaxation (Levin *et al*, 1998) and consists of three peptides; Atrial-NP (ANP), Brain-NP (BNP) and C-type NP (CNP). ANP and BNP are cardiac hormones stored and secreted from the atrium and ventricle respectively (Ogawa *et al*, 1991), whereas CNP is produced and secreted from vascular endothelial cells, monocytes, and chondrocytes (Nagase *et al*, 1997). Natriuretic peptides activate the cGMP cascade through the two particulate guanylate cyclases, ANP-A (Gca) and ANP-B (Gcb) receptors (Suga *et al*, 1992). Although ANP and BNP show high affinity for the ANP-A receptor, CNP selectively binds to the ANP-B receptor.

The genes encoding ANP and BNP co-localise in human, mouse and rat on chromosomes 1, 4 and 5 respectively (Tamura *et al*, 1996) whereas CNP has a different location on mouse chromosome 1 (Ogawa *et al*, 1994) consistent with its divergent pattern of tissue specific and inducible expression. Indeed, the *Anp* and *Bnp* genes show particular sequence homology to one another with only four amino acids being different in the 17 amino acid ring structure common to both peptides (Vesely, 1992) and both have a fairly simple organisation with three exon (coding) sequences separated by two intron (intervening) sequences. (*Figure 6.1*).

The stroke-prone spontaneously hypertensive rat (SHRSP) is a good model for studying genetic determinants of stroke, being characterised by a high frequency of spontaneous strokes (Okamoto *et al*, 1974) as well as an increased sensitivity to experimentally

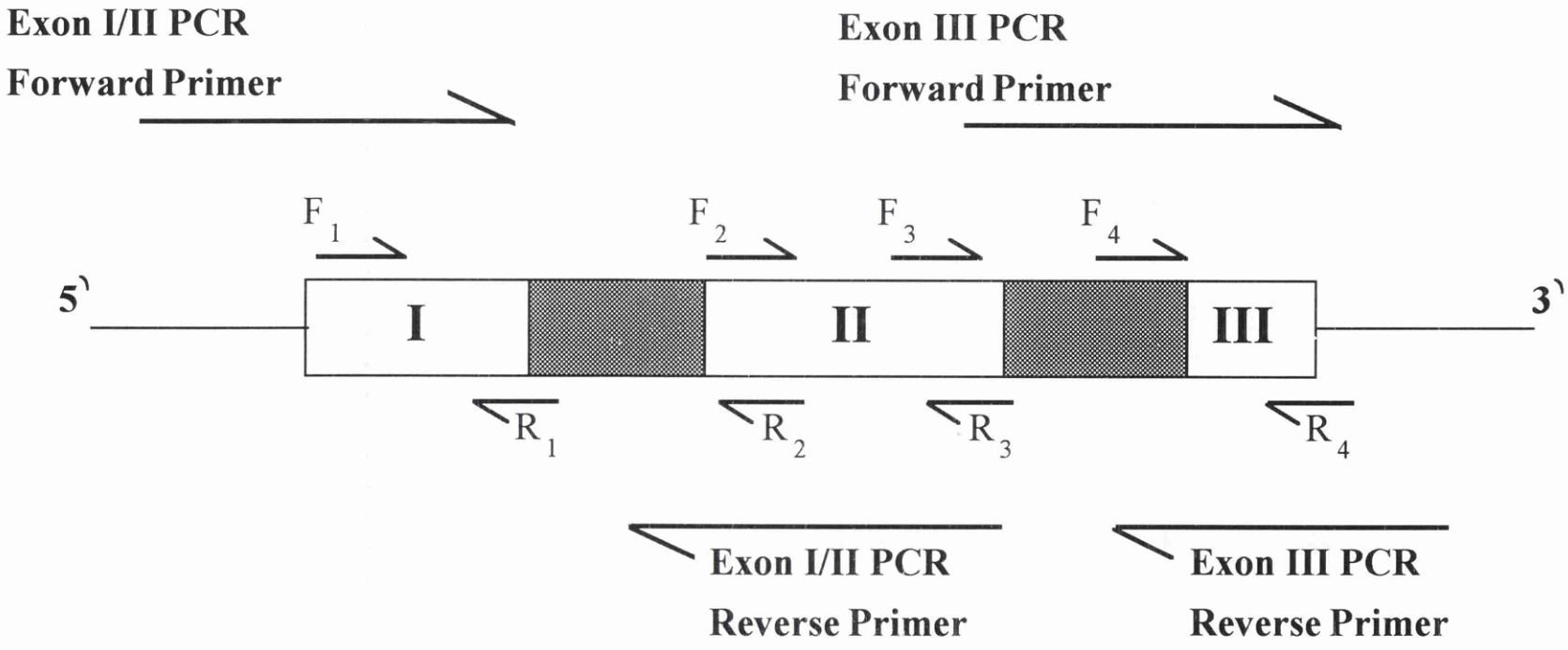

Figure 6.1 Schematic representation of the strategy utilised in the current study for the PCR amplification and cycle sequencing of the coding regions of the rat *Anp* and *Bnp* genes. The plain areas **I**, **II** and **III** represent the three exons of each gene, the chequered areas the introns. The large outer most arrows represent the primers utilised in amplifying exon I, II and III of each gene, whilst the inner most arrows represent both the forward (F) and reverse (R) primers used for sequencing.

induced focal cerebral ischaemia (Coyle & Jokelainen, 1983). Rubattu *et al* (1996) identified three QTLs for susceptibility to stroke which were localised to rat chromosomes 1, 4 and 5 in a F2 cross obtained by breeding $SHRSP_{(Heidelberg)}$ and SHR. The latter two of these conferred a protective effect against stroke in the presence of two SHRSP alleles and the QTL on chromosome 5 co-localised with the genes encoding ANP and BNP. In *Chapter 4* a highly significant QTL on rat chromosome 5 was identified for sensitivity to experimentally induced middle cerebral artery occlusion in the Glasgow SHRSP x WKY F2 cross close to that identified by Rubattu *et al* (1996). It follows that *Anp* and *Bnp* have become putative candidates for susceptibility to stroke in the SHRSP.

For either *Anp* or *Bnp* to be considered valid candidate genes for being the QTL on rat chromosome 5 there would have to be a significant nucleotide difference between either of the genes of the SHRSP and the WKY. This difference would be such that it could cause the differing genes of the SHRSP and WKY to function differently. It was therefore the aim in this chapter to ascertain whether the genes encoding ANP and BNP are likely candidates for sensitivity to cerebral ischaemia by performing sequencing analysis of the coding regions of the *Anp* and *Bnp* genes.

6.2 Methods

Sequencing

The AmpliCycle™ Sequencing Kit (Perkin-Elmer) was used to sequence the coding regions of *Anp* and *Bnp* in both SHRSP and WKY following the cartoon protocol illustrated in *Figure 6.1*. Following the extraction of DNA from tail biopsies of 4 male SHRSP and 4 male WKY rats as previously described in *Chapter 2.4.1*, high quality amplification of the exonic regions of *Anp* and *Bnp* was achieved from this DNA by PCR as described in *Chapter 2.4.5* using intronic specific primers (*Table 6.1*) designed in-house from sequences lodged at http://www.ncbi.nlm.nih.gov/Web/Search/index.html in GenBank (accession numbers K02062 and M60266 respectively). The 750 base pair amplicon containing exons 1 and 2 of *Anp* was obtained using primers 1A and 5A. Exon 3 was generated with 7A and 8A. The first two exons of *Bnp* were generated with 1B and 4B whilst the third exon used 7B and 8B. The products from the PCR amplification were electrophoresed with molecular weight markers on either 1.5 or 2% agarose gels and the bands containing the DNA was excised from the gels and electroeluted. The DNA obtained was quantified using the DynaQuant Fluorimeter (Hoefer) and used to set up sequencing reactions.

Both forward and reverse sequencing primers were also designed in-house to cover between 150-200bp of the amplified coding regions of both *Anp* and *Bnp* (*Table 6.1*). Sequencing reactions were set up and run as described previously in *Chapter 2.4.5*. Each reaction was stopped with the addition of 4µl stop solution and then 5µl of each

Primer	Sequence	Coverage
1A/F	ACAGAATGGGGAGGGTTC (561-578)	Exon 1
2A/R	TTGGACACCGCACTGTAC (805-824)	Exon 1
3A/F	GTACAGTGCGGTGTCCAA (805-824)	Exon 2
4A/F	GAGGTGCCTCCCTGGAC (1062-1078)	Exon 2
5A/R	ATCCCGTTTCACCCGCAG (1288-1305)	Exon 2
6A/R	GTCCAGGGAGGCACCTC (1062-1078)	Exon 2
7A/F	TGAAGGTAGATCATCAGACCG (1628-1648)	Exon 3
8A/R	ACCGCAAGGCTTGGGATCTT (1709-1729)	Exon 3
1B/F	AGACAAGAGAGAGCAGGACACC (1088-1110)	Exon 1
2B/R	CCAAAACCCTTCAGGCAGGCT (1300-1320)	Exon 1
3B/F	CTGAGGTCTGGGCTTCCC (1457-1474)	Exon 2
4B/R	AGACTGTCGGTAAGGTAGAGG (1817-1838)	Exon 2
5B/F	TCCAGGAGAGACTTCGAA (1611-1628)	Exon 2
6B/R	TTCGAAGTCTCTCCTGGA (1611-1628)	Exon 2
7B/F	GGAAGCTCAGCTCCTGCCTCA (2107-2127)	Exon 3
8B/R	AAGAGCCGCAGGCAGAGT (2217-2234)	Exon 3

Table 6.1 PCR and sequencing primers for *Anp* (1A-8A) and *Bnp* (1B-8B). All were designed in house from sequences lodged in GenBank (K02062 and M60266 respectively). F refers to a forward primer, R to a reverse primer.

sequencing reaction resolved on a 6-8% polyacrylamide gel and electrophoresed for approximately 2 hours. The gel was then dried onto a large Whatman filter paper and placed against film for 4-8 hours. After this the film was developed and scored by two independent observers.

6.3 Results

Sequencing the coding region of the *Anp* and *Bnp* genes revealed no difference between the SHRSP and the WKY strains. *Figure 6.2* shows a comparison between the Sprague-Dawley rat sequence of the *Anp* gene (GenBank accession number K02062) and those sequences in the SHRSP and WKY. There were four substitutions (two silent) within the coding region of the *Anp* gene as compared to the Sprague-Dawley sequence but no differences between SHRSP and WKY (*Table 6.2*). *Figure 6.3* shows a comparison between the *Rattus norvegicus* sequence of the *Bnp* gene (GenBank accession number M60266) and those sequences in the two Glasgow strains. No sequence differences for the *Bnp* gene were detected.

6.4 Discussion

As discussed in *Chapter 4,* recent evidence utilising the SHRSP has implicated both the *Anp* and *Bnp* genes as putative candidate genes for stroke. Sequencing of the coding regions of both *Anp* and *Bnp* genes in the current study revealed no significant nucleotide differences between SHRSP and the WKY strains that are potentially important to their

Exon 1 (727-846)

Sprague-Dawley ATGGGCTCCTTCTCCATCACCAAGGGCTTCTTCCTCTTCCTGGCCTTTTGGC
$WKY_{(Gla)}$ ATGGGCTCCTTCTCCATCACCAAGGGCTTCTTCCTCTTCCTGGCCTTTTGGC
$SHRSP_{(Gla)}$ ATGGGCTCCTTCTCCATCACCAAGGGCTTCTTCCTCTTCCTGGCCTTTTGGC

Sprague-Dawley TCCCAGGCCATATTGGAGCAAATCCCGTATACAGTGCGGTGTCCAACACAG
$WKY_{(Gla)}$ TCCCAGGCCATATTGGAGCAAATCCCGTATACAGTGCGGTGTCCAACACAG
$SHRSP_{(Gla)}$ TCCCAGGCCATATTGGAGCAAATCCCGTATACAGTGCGGTGTCCAACACAG

Sprague-Dawley ATCTGATGGATTTCAAG
$WKY_{(Gla)}$ ATCTGATGGATTTCAAG
$SHRSP_{(Gla)}$ ATCTGATGGATTTCAAG

Exon 2 (951-1277)

Sprague-Dawley AACCTGCTAGACCACCTGGAGGAGAAGATGCCGGTAGAAGATGAGGTCATG
$WKY_{(Gla)}$ AACCTGCTAGACCACCTGGAGGAGAAGATGCCGGTAGAAGATGAGGTCATG
$SHRSP_{(Gla)}$ AACCTGCTAGACCACCTGGAGGAGAAGATGCCGGTAGAAGATGAGGTCATG

Sprague-Dawley CCTCCG^{1007}CAGGCCCTGAGCGAGC^{1023}AGACCGATGAAGCGGGGGCGGCAC
$WKY_{(Gla)}$ CCTCC**A**^{1007}CAGGCCCTGAGCGAG**A**^{1023}AGACCGATGAAGCGGGGGCGGCAC
$SHRSP_{(Gla)}$ CCTCC**A**^{1007}CAGGCCCTGAGCGAG**A**^{1023}AGACCGATGAAGCGGGGGCGGCAC

Sprague-Dawley TTAGCTCCCTCTCTGAGGTGCCTCCCTGGACTGGGGAAGTCAACCCGTCTCA
$WKY_{(Gla)}$ TTAGCTCCCTCTCTGAGGTGCCTCCCTGGACTGGGGAAGTCAACCCGTCTCA
$SHRSP_{(Gla)}$ TTAGCTCCCTCTCTGAGGTGCCTCCCTGGACTGGGGAAGTCAACCCGTCTCA

Sprague-Dawley GAGAGATGGA^{1109}GGTGCTCTCGGGCGCG^{1125}GCCCCTGGGACCCCTCCGATA
$WKY_{(Gla)}$ GAGAGATGG**C**^{1109}GGTGCTCTCGGGCGC**A**^{1125}GCCCCTGGGACCCCTCCGATA
$SHRSP_{(Gla)}$ GAGAGATGG**C**^{1109}GGTGCTCTCGGGCGC**A**^{1125}GCCCCTGGGACCCCTCCGATA

Sprague-Dawley GATCTGCCCTCTTGAAAAGCAAACTGAGGGCTCTGCTCGCTGGCCCTCGGA
$WKY_{(Gla)}$ GATCTGCCCTCTTGAAAAGCAAACTGAGGGCTCTGCTCGCTGGCCCTCGGA
$SHRSP_{(Gla)}$ GATCTGCCCTCTTGAAAAGCAAACTGAGGGCTCTGCTCGCTGGCCCTCGGA

Sprague-Dawley GCCTGCGAAGGTCAAGCTGCTTCGGGGGTAGGATTGACAGGATTGGAGCCC
$WKY_{(Gla)}$ GCCTGCGAAGGTCAAGCTGCTTCGGGGGTAGGATTGACAGGATTGGAGCCC
$SHRSP_{(Gla)}$ GCCTGCGAAGGTCAAGCTGCTTCGGGGGTAGGATTGACAGGATTGGAGCCC

Sprague-Dawley AGAGCGGACTAGGCTGCAACAGCTTCCGG
$WKY_{(Gla)}$ AGAGCGGACTAGGCTGCAACAGCTTCCGG
$SHRSP_{(Gla)}$ AGAGCGGACTAGGCTGCAACAGCTTCCGG

Exon 3 (1669-1680)

Sprague-Dawley TACCGAAGATAA
$WKY_{(Gla)}$ TACCGAAGATAA
$SHRSP_{(Gla)}$ TACCGAAGATAA

Figure 6.2 The sequences of the coding regions of *Anp* in the Glasgow SHRSP and WKY compared with the rat Genbank sequence for the Sprague-Dawley rat (K02062). The position of substitutions are highlighted in bold.

Substitutions
$G^{1007} \rightarrow A^{1007}$ = Proline (CCG) $\rightarrow$ Proline (CCA)
$C^{1023} \rightarrow A^{1023}$ = Glutamine (CAG) $\rightarrow$ Lysine (AAG)
$A^{1109} \rightarrow C^{1109}$ = Glycine (GGA) $\rightarrow$ Glycine (GGC)
$G^{1125} \rightarrow A^{1125}$ = Glycine (GGC) $\rightarrow$ Serine (AGC)

Table 6.2 The four substitutions identified within the exon 2 of the *Anp* gene in both the Glasgow SHRSP and WKY as compared to the Sprague-Dawley sequence. The substitutions at position 1007 and 1109 are silent.

Exon 1 (1143-1268)

Rattus norvegicus ATGGATCTCCAGAAGGTGCTGCCCCAGATGATTCTGCTCCTGCTTTTCCTTA
$WKY_{(Gla)}$ ATGGATCTCCAGAAGGTGCTGCCCCAGATGATTCTGCTCCTGCTTTTCCTTA
$SHRSP_{(Gla)}$ ATGGATCTCCAGAAGGTGCTGCCCCAGATGATTCTGCTCCTGCTTTTCCTTA

Rattus norvegicus ATCTGTCGCCGCTGGGAGGTCACTCCCATCCCCTGGGAAGTCCTAGCCAGT
$WKY_{(Gla)}$ ATCTGTCGCCGCTGGGAGGTCACTCCCATCCCCTGGGAAGTCCTAGCCAGT
$SHRSP_{(Gla)}$ ATCTGTCGCCGCTGGGAGGTCACTCCCATCCCCTGGGAAGTCCTAGCCAGT

Rattus norvegicus CTCCAGAACAATCCACGATGCAG
$WKY_{(Gla)}$ CTCCAGAACAATCCACGATGCAG
$SHRSP_{(Gla)}$ CTCCAGAACAATCCACGATGCAG

Exon 2 (1487-1709)

Rattus norvegicus AAGCTGCTGGAGCTGATAAGAGAAAAGTAGAGGAAATGGCTCAGAGACAG
$WKY_{(Gla)}$ AAGCTGCTGGAGCTGATAAGAGAAAAGTAGAGGAAATGGCTCAGAGACAG
$SHRSP_{(Gla)}$ AAGCTGCTGGAGCTGATAAGAGAAAAGTAGAGGAAATGGCTCAGAGACAG

Rattus norvegicus CTCTCAAAGGACCAAGGCCCTACAAAAGAACTTCTAAAAAGAGTCCTTAGG
$WKY_{(Gla)}$ CTCTCAAAGGACCAAGGCCCTACAAAAGAACTTCTAAAAAGAGTCCTTAGG
$SHRSP_{(Gla)}$ CTCTCAAAGGACCAAGGCCCTACAAAAGAACTTCTAAAAAGAGTCCTTAGG

Rattus norvegicus TCTCTCAAAGGACCAAGGCCCTACAAAAGAACTTCTAAAAAGAGTCCTTAG
$WKY_{(Gla)}$ TCTCTCAAAGGACCAAGGCCCTACAAAAGAACTTCTAAAAAGAGTCCTTAG
$SHRSP_{(Gla)}$ TCTCTCAAAGGACCAAGGCCCTACAAAAGAACTTCTAAAAAGAGTCCTTAG

Rattus norvegicus GTCTCATAGTTCAAGCTGCTTTGGGCAGAAGATAGACCGGATCGGCGCAGT
$WKY_{(Gla)}$ GTCTCATAGTTCAAGCTGCTTTGGGCAGAAGATAGACCGGATCGGCGCAGT
$SHRSP_{(Gla)}$ GTCTCATAGTTCAAGCTGCTTTGGGCAGAAGATAGACCGGATCGGCGCAGT

Rattus norvegicus TCAGTCGCTTGGGCTGTGACGG
$WKY_{(Gla)}$ TCAGTCGCTTGGGCTGTGACGG
$SHRSP_{(Gla)}$ TCAGTCGCTTGGGCTGTGACGG

Exon 3 (2168-2184)

Rattus norvegicus GCTGAGGTTGTTTTAG
$WKY_{(Gla)}$ GCTGAGGTTGTTTTAG
$SHRSP_{(Gla)}$ GCTGAGGTTGTTTTAG

Figure 6.3 The sequences of the coding regions of *Bnp* in the Glasgow SHRSP and WKY compared with the rat Genbank sequence for *Rattus norvegicus* (M60266).

function. As a consequence the *Anp* and *Bnp* genes are not supported by the current data as candidates for the QTL causing a difference in cerebral infarct size between SHRSP and WKY.

Despite the absence of nucleotide differences between SHRSP and WKY there were four substitutions (two silent) within exon 2 of the *Anp* gene compared with the rat sequences entered into Genbank. One of these substitutions, the G → A substitution at position 1125 resulting in a serine instead of glycine has been previously reported to be present in the $SHRSP_{(Heidelberg)}$ by Rubattu *et al* (1997). They suggested that this substitution together with their observation of a 3 fold lower expression of *Anp* mRNA in the brain of $SHRSP_{(Hdl)}$ as compared to $SHR_{(Hdl)}$ was consistent with a putative role for *Anp* in the pathogenesis of cerebrovascular disease in the SHRSP. Indeed, based on this animal work Rubattu *et al* (1998) have recently presented preliminary data describing the association of a G → A transition at position 664 in exon 1 of the human *ANP* gene with a 2-fold increase in the incidence of stroke, suggesting these findings are consistent with a direct contributary role of the ANP gene to the pathogenesis of stroke in humans.

This hypothesis is not supported by the current study where coding sequences of both *Anp* and *Bnp* genes were identical between the Glasgow SHRSP and the non-infarcting WKY strain. In addition, Brosnan *et al* (1998) identified that the brain mRNA expression of both *Anp* and *Bnp* did not differ between the Glasgow SHRSP and the WKY prior to, or 24 hours after, occlusion of the middle cerebral artery.

The positive evidence for *Anp* as a candidate for stroke in both the rat and human (Rubattu *et al*, 1997 & 1998) against the present study's negative data in the rat could perhaps be most simply explained by the different nature of the stroke phenotype being studied. The major phenotype used in the current study is the volume of infarction following permanent occlusion of the middle cerebral artery, whereas Rubattu *et al* (1996, 1997 & 1998) are considering both spontaneous ischaemic and haemorrhagic stroke. It follows that the candidate genes for each phenotype might be different despite the two QTLs being localised on rat chromosome 5. Indeed it is conceivable that the relatively large QTLs identified by the genome wide scanning strategy contain more than one gene causally linked to the phenotype under study.

These discrepancies between the current study and rat data presented by Rubattu *et al* (1997) could also be explained by differences between colonies and diet. It has been well established by both biochemical genetic markers (Matsumoto *et al*, 1991) and DNA fingerprinting techniques (Samani *et al*, 1989; Kurtz *et al*, 1989; Nabika *et al*, 1991) that genetic heterogeneity between WKY and SHRSP from different sources exists, a fact which must be borne in mind when making any comparison of experimental results obtained between groups. Alternatively, the Japanese style diet (high in sodium and low in potassium and protein) utilised by Rubattu *et al* (1997) to induce spontaneous stroke in their SHRSP may provide a major contribution to endothelial dysfunction leading to spontaneous stroke. Previous studies have shown that salt-induced hypertension in rats is associated with endothelial dysfunction which is reversed by antihypertensive treatment (Luscher *et al*, 1987). Indeed, this latter difference may explain why Russo *et al* (1998) in examining vasorelaxant responses to ANP and BNP in aortae, basilar and

carotid arteries found them to be attenuated in the $SHRSP_{(Hdl)}$ as compared to the $SHR_{(Hdl)}$ and the $WKY_{(Hdl)}$, whilst Brosnan *et al* (1998) demonstrated no differences in maximum relaxation to ANP between the Glasgow SHRSP and the WKY in the middle cerebral artery.

In addition to the negative results of the sequencing, and the mRNA expression and the functional studies performed by Brosnan *et al* (1998) on the Glasgow SHRSP and the WKY, it has emerged that the position of the *Anp* marker in the genetic map obtained in *Chapter 4* is discordant with other genetic maps (Shepel *et al*, 1998; Bihoreau *et al*, 1997; Rubattu *et al*, 1996; Klina-Levan *et al*, 1997). *Chapter 4* places this marker central to the chromosome, whilst the other maps place it in the telomeric region of rat chromosome 5 in the vicinity of the anonymous *D5Mgh16* marker. This latter position has subsequently been confirmed by Brosnan *et al* (1998) by utilising two methods of physical mapping the relevant region of rat chromosome 5, radiation hybrid analysis and fluorescence *in situ* hybridisation (FISH) on cells isolated from the Glasgow SHRSP and the WKY. Whilst the physical placement of the *Anp* marker outwith the QTL influencing susceptibility to cerebral ischaemia in the Glasgow SHRSP does not alter its significance, it does serve to largely exclude both *Anp* and *Bnp* as candidates for sensitivity to cerebral ischaemia. This in turn demonstrates the lessons to be learned from concentrating on a single candidate gene within a large chromosomal region with purely statistical evidence for linkage, rather than focusing on the construction of the appropriate congenic lines and sub-lines in order to narrow down the chromosomal region of interest.

Several additional candidate genes for stroke have been identified with some relation to rat chromosome 5 which may be responsible for large cerebral infarcts in the SHRSP, including the potent vasodilator endothelin-2, the glucose transporter Glut-B, and elastase-2. However, like ANP and BNP, little evidence exists to warrant their further investigation until congenic strains suggest so. Of most interest perhaps is methylenetetrahydrofolate reductase (MTHFR) which catalyses the conversion of 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine. The *Mthfr* gene has been mapped to the chromosomal region 1p36.3 in human and the distal region of mouse chromosome 4, syntenic regions with rat chromosome 5 (Goyette *et al*; 1998). Mild MTHFR deficiency (35-50% of control values) is present at a high frequency in the general population and is associated with mild hyperhomocysteinemia, which frequently leads to ischaemic stroke in young adults (Engbersen *et al*, 1995; Frosst *et al*, 1995). Indeed, one common variant in the *Mthfr* gene has been weakly implicated in occlusive vascular disease and in patients with transient ischaemic attack or minor stroke (Frosst *et al*, 1995; Lalouschek *et al*, 1998) suggesting the need for more research in this area.

Of similar interest to *Mthfr*, the total genome scan produced by Rubattu *et al* (1996) revealed that the QTL on rat chromosome 5 showed an epistatic interaction with that on chromosome 1, the interaction term accounting for an additional 3% of the phenotypic variance. It has emerged that a strong candidate gene within this region of rat chromosome 1 is adrenomedullin (personal communication - Klaus Lindpaintner, Boston). This is a recently discovered peptide that was initially identified from human pheochromocytoma (Kitamura *et al*, 1993) and exhibits potent vasodilator activity *in*

vitro and *in vivo* (Ishiyama *et al*, 1993; Nuki *et al*, 1993; Perret *et al*, 1993). Not only does adrenomedullin produce substantial dilatation of cerebral arterioles *in vivo* (Lang *et al*, 1997), Wang *et al* (1995) have reported both the upregulation of adrenomedullin mRNA expression and peptide production following experimentally induced focal cerebral ischaemia and the exacerbation of focal ischaemic injury following intracerebroventricular administration of adrenomedullin. Whilst recent research argues the opposite finding (Dogan *et al*, 1997) both studies suggest a significant role for adrenomedullin in the evolution of stroke. Indeed, ANP has been shown to increase adrenomedullin four-fold in the circulation of healthy humans (Vesely *et al*, 1996), suggesting that effects previously attributed to ANP may actually be partially due to adrenomedullin, especially as all the described effects are similar to those of ANP (Vesely, 1992).

In conclusion, whilst there may be unidentified regulatory regions either located in the 5′-end or the 3′-end, or in the intronic regions of *Anp* and *Bnp* genes which could still be potentially important in differentially regulating the *Anp* and *Bnp* gene expression between the two rat strains, the sequencing data obtained in the present study, the functional data of Brosnan *et al* (1998), and the discordant genetic map strongly suggest that any further research into *Anp* and *Bnp* would be presumptive and only warranted if subsequent congenic strains suggest so. Indeed, despite the preliminary human data of Rubattu *et al* (1998) and the knowledge for many years that plasma levels of both BNP and ANP increase substantially with ventricular hypertrophy in malignant hypertension (Kohno *et al*, 1987 & 1994), abnormalities of genes for the natriuretic peptides or their receptors have not historically been strongly linked to cardiovascular

disease in human study (Wilkins *et al*, 1997). Furthermore, as ischaemia and the resulting hypoxia and oedema have been shown to be some of the strongest stimuli for gene induction in the brain, with more than 80 different mRNAs having been found to be induced by brain ischaemia so far, any candidate approach is potentially hazardous and time-wasting (Koistinaho & Hokfelt, 1997). It follows that the current study clearly illustrates the correct research route towards the ultimate positional cloning of the true gene(s) involved in susceptibility to experimentally induced cerebral ischaemia; namely the construction of appropriate congenic strains as in *Chapter 5*, aided by physical mapping of the specific chromosomal region.

7. GENERAL DISCUSSION

Essential hypertension and its complications in man, such as stroke and left ventricular hypertrophy, are due to the interaction of many genes and environmental factors. Previously used strategies such as linkage analysis in families segregating for rare Mendelian forms of these conditions or exploration of candidate genes have had a limited success in identifying their major genetic determinants. The methodological difficulties in directly studying genetic determinants of such complex traits have given a major impetus for the development of similar but inherently simpler paradigms in experimental animal models. In particular crosses between hypertensive and a normotensive inbred rat strains have begun to yield chromosome regions containing QTLs for blood pressure and its related phenotypes.

The research described in this thesis incorporated the use of two genome wide scans combined with high fidelity phenotyping to identify QTLs containing genetic determinants of hypertensive cardiovascular and cerebrovascular disease in F2 crosses derived from the Glasgow colonies of SHRSP and WKY. The first genome scan (*Chapter 3*) identified three blood pressure QTLs: two on rat chromosome 2 and one on rat chromosome 3. It also identified a QTL for left ventricular hypertrophy on rat chromosome 14. The second scan (*Chapter 4*) identified a highly significant QTL on rat chromosome 5 for the increased sensitivity to experimentally induced focal cerebral ischaemia.

There were several features of these QTL mapping strategies which make them particularly appropriate for congenic production, fine genetic mapping and ultimately positional cloning of the causal genes. Firstly, in contrast to previous studies which used

male F2s only, both males and females were analysed which allowed the first identification of sex specific blood pressure QTLs. Secondly, the use of the radio-telemetry system for physiological monitoring, and the experimental occlusion of the middle cerebral artery, were the first in an F2 population, thereby providing the means for the high fidelity phenotyping of subsequent congenic strains and sub-strains. Thirdly, the identification of blood pressure independent QTLs for "sub-phenotypes" of hypertension illustrated the existence of separate genetic factors, an appreciation of which will be essential if a full understanding of the genetics of hypertension and its vascular complications is to be achieved. Finally, the current study is the first to have established the applicability of a speed congenic strategy in the rat (*Chapter 5)* with which to quicken the dissection of the QTLs identified.

Given the results of the current study, the demonstrable predisposition of the rat for high fidelity phenotyping and the rapidly increasing availability of appropriate genetic resources for the rat, including dense genetic and physical maps, YACs, BACs, PACs, cosmids and bacteriophage clones, there can be little doubt that these QTLs will be completely dissected given time and a continued advancement in methodology (Rapp & Deng, 1995). However, it has been hotly debated over the last few years whether the genetic information obtained in these inbred animal models will be directly applicable to human cardiovascular disease (Dominiczak & Frohlich, 1996). For example, whilst the information on the chromosomal regions containing QTLs can be used to examine candidate genes in both rats and humans, very little success has been made in taking this approach, as demonstrated by the examination of the genes encoding *Anp* and *Bnp* in *Chapter 6.*

Fortunately, a recent strategy based on the assumption that like many other genes, QTLs are likely to be conserved between species during the course of evolution, has had some success in transferring the common or reproducible rat QTLs to candidate regions for human essential hypertension. For example, by utilising the major rat QTL on chromosome 10 (Hilbert *et al*, 1991; Jacob *et al*, 1991) to investigate the homologous region on human chromosome 17 in familial essential hypertension (518 affected sib-pairs) Julier *et al* (1997) found strong evidence for linkage with two microsatellite markers (*D17S183* and *D17S934*) which are 18cM proximal to the *ACE* gene (*Figure 7.1*). Whilst the final identification of the gene or genes responsible will require a systematic investigation of the region in both species, this study illustrates the potential of using reproducible rat QTLs as candidate regions for polygenic human disorders. It also serves to validate the performance of genetic studies in experimental models of hypertensive cardiovascular disease. Indeed, combining the use of all the complementary genetic resources available for the human, mouse and rat can only enhance the productivity of this strategy, and will become even more productive on the completion of The Human Genome Project.

A further inter-relationship between human and experimental genetics is likely to include pharmacogenetic approaches. It has been recognised for several years that the heterogeneity of essential hypertension extends to the blood pressure response to hypotensive medication. Moreover, at least part of this variability is genetic as documented by the resistance of some racial sub-groups to particular classes of hypotensive drugs. Several studies showed that compared to white subjects, black hypertensives show a much poorer response to treatment with angiotensin converting

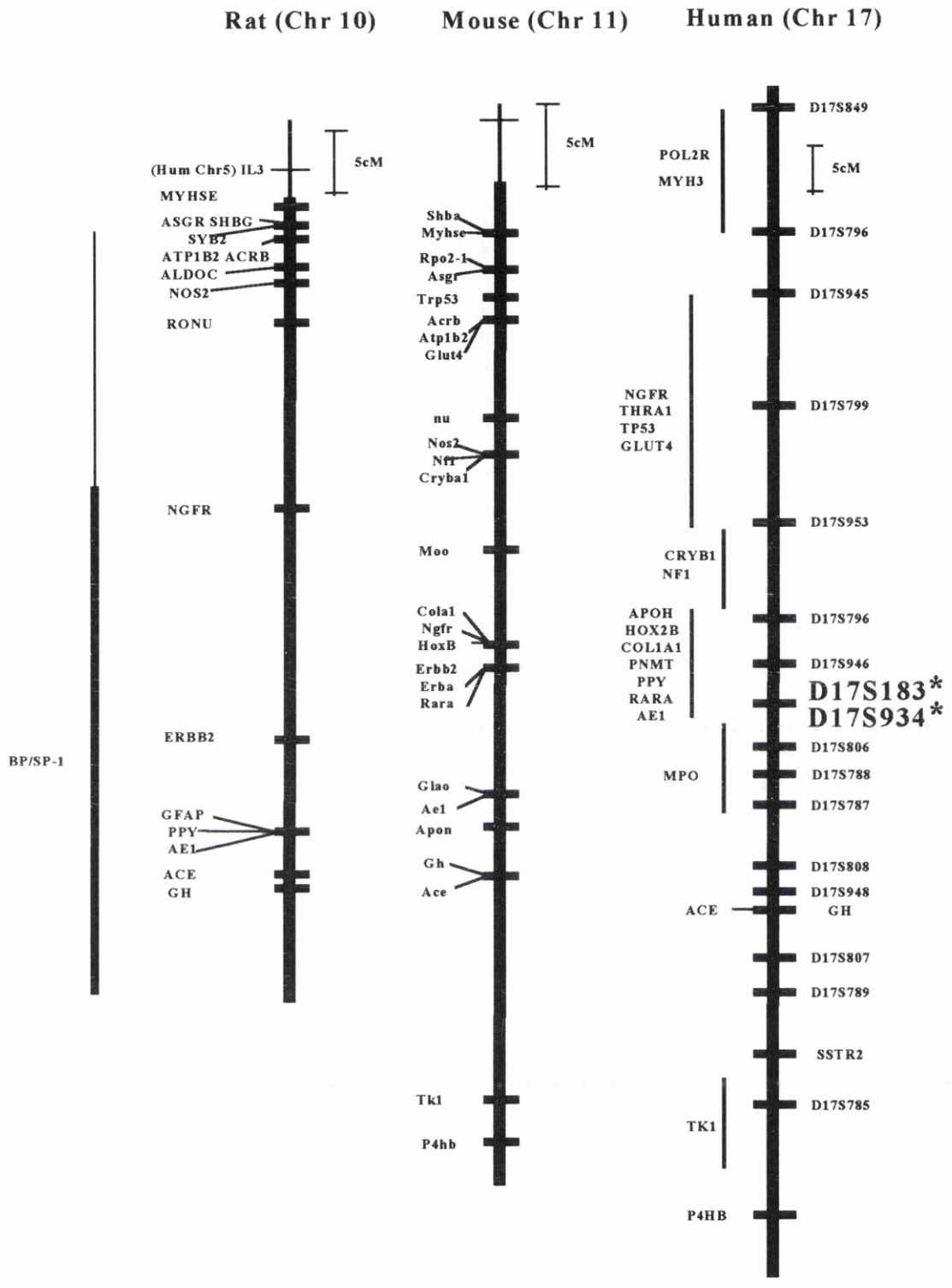

Figure 7.1 Comparative mapping of the blood pressure QTL on rat chromosome 10 (adapted from Julier *et al*, 1997). Markers *D17S183** and *D17S934** on human chromosome 17 indicate the significant area of linkage to essential hypertension.

enzyme inhibitors or beta blockers (Materson *et al*, 1993). Vincent *et al* (1997) studied the cosegregation of genetic loci with acute cardiovascular responses to drugs involved in the renin-angiotensin system, the sympathetic nervous system and calcium metabolism in the Lyon model of genetic hypertension. A QTL was identified and mapped on rat chromosome 2 that influenced systolic and diastolic blood pressure responses to the administration of a dihydropyridine calcium antagonist. These findings provide strong support for the contention that genetic factors may influence the response to hypotensive drugs. This may ultimately lead to the successful treatment of patients with drugs that will control blood pressure at very low doses and with great tolerability by being specifically targeted to the genetic defect, or its pathway.

In conclusion, the successful dissection of the QTLs identified in this study as involved in the pathogenesis of hypertension, left ventricular hypertrophy and the severity of cerebral ischaemia in the SHRSP should allow, via congenic strains, positional cloning and comparative mapping to human chromosomes, the translation of this information from the experimental model to human cardiovascular and cerebrovascular disease, as well as an increased understanding of the pathological pathways involved. This will in turn hopefully improve prospects for the prevention and/or improved treatment of these complex traits which are the major causes of morbidity and mortality in the UK.

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APPENDIX I

Reagents	Source
Agarose	Gibco BRL
Ammonium persulphate	BDH
Ampli*Taq* DNA polymerase	Perkin-Elmer
Bromophenol blue	Sigma
Buffer tablets	BDH
Cellulose patchs	Data Sciences Int.
Chloroform	Fisher Scientific
Cidex	Johnson & Johnson
Concentrated hydrochloric acid	BDH
Cresol Red	Aldrich
Decon 75	Decon Laboratories Ltd.
dNTPs/deoxynucleoside triphosphates	Promega
ddNTPs/dideoxynucleoside triphosphates	Perkin-Elmer
Dimethyl sulfoxide	Sigma
EDTA	Bio-Rad
100% Ethanol	Joesph Hills
Ethidium bromide	Sigma
Formamide	BDH
Fluothane	Zeneca Ltd.
Glacial acetic acid	Fisher Scientific
Haematoxylin-eosin stain	Surgipath (Europe) Ltd.
Hybond-N^+ membrane	Amersham
Isoamylalcohol	BDH
Isopentane	Fisher Scientific
10X kinase buffer	Promega
Magnesium chloride/$MgCl_2$	Promega
Mineral Oil	Sigma
[α-^{32}P]dCTP	Amersham
[γ-^{32}P]dATP	Amersham
Phenol, water-saturated in Tris buffer	Life Sciences
Powered milk	Premier Beverages
Proteinase K	Sigma
0.9% Saline	Baxter
20X SSC	National Diagnostics
SDS (sodium dodecyl sulphate) powder	Bio-Rad
20% SDS	Bio-Rad
SequaGel Sequencing System	National Diagnostics
Sigmacote	Sigma
Sodium acetate	Sigma
Sodium chloride	Fisher Scientific
Sodium hydroxide pellets	Fisher Scientific
Stop solution	Perkin-Elmer
Sucrose	Fisher Scientific
T4 polynucleotide kinase	Perkin-Elmer

Taq DNA polymerase	Promega
Tartrazine	Sigma
10X TBE	National Diagnostics
TEMED	Sigma
Terminal deoxynucleotidyl transferase/TdT	Promega
TdT buffer	Promega
10X Thermophilic buffer	Promega
Tris base	Bio-Rad
UITma DNA polymerase	Perkin-Elmer
VetBond	3M Animal Care Products
Xylene cyanol	Sigma

Solutions

1. 10% Ammonium Persulphate (10ml)

1g APS dissolved in 10ml sterile distilled water. Store at 4°C.

2. Chloroform Isoamylalcohol mixture (250ml)

240ml chloroform mixed with 10ml isoamylalcohol. Store at 4°C wrapped in light resistant bottle.

3. 2.5mM dNTPs (200µ)

5µl each of dGTP, dATP, dTTP and dCTP (all provided in 100mM stock solutions) added to 180µl sterile distilled water. Store frozen.

4. 0.5M EDTA, pH 8.0 (1L)

186.1g EDTA dissolved in 1L sterile distilled water. pH adjusted with sodium hydroxide pellets. Sterilised by autoclave.

5. 70% Ethanol (100ml)

70ml 100% ethanol mixed with 30ml sterile distilled water.

6. 2X Formamide Loading Buffer (10ml)

9ml formamide, 160µl bromophenol blue, 160µl xylene cyanol, 680µl sterile distilled water. Store at 4°C.

7. PCR Master Mix (10µl, n=1)

Contains 4µl 5X Red, 2µl 10X recommended thermophilic buffer, 1.2µl 25mM $MgCl_2$, 1.1µl 2.5mM dNTPs, 1.6µl sterile distilled water and and 0.08µl *Taq* polymerase. The *Taq* was added immediately prior to the PCR reaction to avoid denaturation. Can be stored frozen until required.

8. Phenol choroform isoamylalcohol (200ml)

100ml water saturated phenol mixed with 100ml chloroform isoamylalcohol mixture. Store at 4°C in light resistant bottle.

9. 8% Polyacrylamide Gel Mix (100ml)

32ml SequaGel concentrate, 58ml SequaGel diluent, and 10ml SequaGel buffer mixed together.

10. Post-Hybridisation Wash Solution (1L)

10ml 20% SDS, 20 X SSC and 890ml sterile distilled water mixed together.

11. Primer Solution (5µl, n=1)

0.8µl of each 6 µM primer (A and B) mixed with 3.4µl sterile distilled water. Final addition of a few particles of tartrazine to aid identification. Can be stored frozen until required.

12. 5X Red (10ml)

6.5g sucrose dissolved in 10ml sterile distilled water. Addition of 1µl cresol red for colour. Store at 4°C.

13. 0.4% SDS (500ml)

2g SDS powder dissolved in 500ml sterile distilled water by heating to 68°C in microwave.

14. 10% SDS (1L)

500ml 20% SDS mixed with 500ml sterile distilled water.

15. 3M Sodium Acetate, pH 6.0 (1L)

408.1g sodium acetate dissolved in 1L of sterile distilled water. pH adjusted with glacial acetic acid. Sterilised by autoclave.

16. 1% Sodium Chloride (1L)

10g sodium chloride dissolved in 1L sterile distilled water.

17. 4M Sodium Chloride

117g sodium chloride dissolved in 500ml sterile distilled water. Sterilised by autoclave.

18. 0.4M Sodium Hydroxide (1L)

16g sodium hydroxide pellets dissolved with stirring in 1L sterile distilled water.

19. 2 X SSC (1L)

100ml 20 X SSC mixed with 900ml sterile distilled water.

20. Suspension Buffer (500ml)

25ml 1M Tris (pH 8.0), 100ml 0.5M EDTA (pH 8.0), and 375ml sterile distilled water mixed together.

21. 1X TBE (1L)

100ml 10X TBE mixed with 900ml sterile distilled water.

22. TE (20ml)

200µl 1M Tris (pH 8.0), 40µl 0.5M EDTA (pH 8.0), and 19.76ml sterile distilled water mixed together.

23. 1M Tris, pH 8.0 (1L)

121.1g Tris base dissolved in 1L sterile distilled water. pH adjusted with concentrated hydrochloric acid. Sterilised by autoclave.

24. Working Tail Solution (20ml)

1ml 1M Tris (pH 8.0), 100ml 0.5M EDTA (pH 8.0), 1ml 10% SDS and 14ml sterile distilled water mixed together.

APPENDIX II

Chromosome 2 Primers

Marker	Primer Sequences	Tm (°C)	Size (bp)	Mg^{2+} (mM)
D2Mgh12	L - AATAACCAATAAAggACATgCTCC R - AggAggAAAAgAgTTgAgTCCC	60-55	156	1.5
D2Wox5, RCA51.03	L - CACTgCTTTTCTCACCAAACC R - CTCTTCTgTTCTCTgTgAgCg	60-55	160	1.5
D2Wox15, Cpb, R132	L - ggTgCTAgTAgACAATAAgATAgAT R - TTCATgAgTTTTCACTgTTTgC	55	145	1.0
D2Wox3, RCA12.43A	L - gCCAgCAgggTTAgAgAgA R - CTAAgAAAgAATATgTgAAggTTg	55	92	1.5
D2Wox9, RCA54.06	L - CTgAggACCAATCATgTTCAC R - CCAggTTACAgTgAgTTCC	63-58	146	1.75
D2Mit14, Mitr360	L - ATgAgAggTCAAAgCTTCTCA R - AgACCTgggACAgggTCCT	63-58	102	1.5
D2Mit6, Mit155	L - TgTCAAAggCAggAATCAAC R- ACCCCTTTTgAgATAgCgCT	55-50	190	1.5
D2Mit5, Mit382	L - CAgCAggTggAAACAAgTCA R - gggAgggATTTgATggAgAT	55-50	184	1.5
D2Mit21, Gca	L - gTTgACTTgATCCTCTggCTg R - gggAgggAgTgTCTgTCCA	55 X2	156	2.0
D2wox13, Fst, R239	L - CCTCCTCCAgAgCCTTCA R - gAggAACATCCACTTCAgTCC	55 X2	182	1.75
D2Mit3, R308, Mt1pb	L - ACAgACAgACAAgCAAggAC R - CCAAAATgAggCTTCTgCAA	60	200	1.5
D2Wox19, R34, Pklr	L - CCTTTCTATgAggATgTTCCC R - CACCCCCAgTACAgAggAg	55 X2	133	1.0

Chromosome 3 Primers

Marker	Primer Sequences	Tm (°C)	Size (bp)	Mg^{2+} (mM)
D3Mgh6	L - gCAgACAAATgTTCAATCAAgg R - CCTTTACTTCATCTCCATTCAAA	55-50	107	1.5
D3Wox3, RCA 07.11	L - TCAAGGATCAACTgggAgATTAg R - CACTTCAAgAATgTgTgCAgg	55-50	100	1.5
D3Wox2, RCA 16.11	L - CTAAgTAAATTTCCCTgTCCC R - gTgggCAATgTTCATgTg	58-53	152	1.5
D3Mgh16	L - AgTCAgggCTATgTATTgAgAACC R - CCTCTgACCCCTACATggg	55	120	1.5
D3Wox4, Gnas	L - gTTAggCTTCTgTggAgTTTgT R - TgggAggTCggAATgACTATg	55-50	177	1.5
D3Mit10, Mit546	L - CATACACAggCAgggCTTCT R - CCTgAAACTgAgCgTgAACA	60-55	209	1.5

Chromosome 14 Primers

Marker	Primer Sequences	Tm (°C)	Size (bp)	Mg^{2+} (mM)
D14Wox12, R426	L - CCAACTgCTATgACACCAgg R - AACTgggAgTAgTAggTgACTTgg	60	194	1.0
D14Wox10, R58, Alb	L - TCCTTTggTgATgATTAATATCAC R - ATAAggATTCTCAgATgCAAATg	55	90	1.5
D14Mgh3, Dbp	L - CAAgCACACCgTAgTAgAggg R - AATggCTAgCTATCTATTgTgCg	55-50	120	1.5
D14Wox8, R43, Afp	L - AAgCATAgCAgTgAATTggTg R - TTCATCATCCTTTCATAAAggC	60	151	1.0
D14Wox14, Csna, R101	L - ACTTgATTACACACACAAACACAgA R - CTTTgCTTTCTTTTAgCCATTT	55	126	1.0

Raw Phenotypic Data

Codes are as follows: Cross G refers to those F2 hybrids (n = 140) with a SHRSP grandfather; Cross H a WKY grandfather. LV/Bod is the left ventricular weight to body weight ratio; mean SBP, mean baseline systolic blood pressure; mean DBP, mean baseline diastolic blood pressure; mean PP, mean baseline pulse pressure.

Cross	Sex	LV/Bod	meanSBP (mmHg)	meanDBP (mmHg)	meanPP (mmHg)
G1	F	4.8	140.529	91.24	49.289
G2	F	2.66	169.981	119.743	50.238
G3	F	2.79	164.097	113.548	50.549
G4	F	2.81	152.583	105.167	47.416
G5	F	2.71	165.155	113.537	51.618
G6	M	2.72	175.056	121.365	53.691
G7	F	3.12	203.779	142.876	60.903
G8	F	2.82	165.54	115.223	50.317
G9	F	2.73	150.992	103.305	47.687
G10	M	2.37	163.951	112.611	51.34
G11	M	2.61	178.064	124.031	54.033
G12	M	3.33	188.875	129.279	59.596
G13	M	3.16	195.794	137.513	58.281
G14	M	3.3	189.094	127.583	61.511
G15	M	3.22	200.162	137.854	62.308
G16	M	2.79	194.039	134.614	59.425
G17	F	3.3	161.892	110.885	51.007
G18	F	3.5	171.188	117.245	53.943
G21	M	3.22	167.955	116.322	51.633
G22	M	2.76	177.665	122.825	54.84
G43	F	3.68	148.515	97.573	50.942
G44	F	3.07	223.01	159.222	63.788
G26	F	3.69	177.619	130.556	47.063
G27	F	2.8	148.149	96.428	51.721

G28	F	4.05	193.332	136.594	56.738
G30	F	2.74	165.606	115.688	49.918
G32	F	2.92	164.57	113.713	50.857
G33	F	3.51	171.58	115.293	56.287
G34	M	2.7	183.223	127.571	55.652
G35	M	2.69	171.942	116.903	55.039
G36	M	3.22	220.776	154.033	66.743
G37	M	3	196.762	135.377	61.385
G39	M	2.77	176.501	120.947	55.554
G40	M	2.6	170.627	115.756	54.871
G41	M	2.65	200.02	140.754	59.266
G45	F	3.66	164.143	111.548	52.595
G46	F	3.33	178.954	122.833	56.121
G48	M	3.14	182.102	122.334	59.768
G49	M	3.08	193.839	129.9	63.939
G50	M	2.58	172.842	120.126	52.716
G51	F	3.4	162.15	109.812	52.338
G52	F	3.43	176.358	117.459	58.899
G53	F	2.73	151.384	104.639	46.745
G54	M	3.27	196.231	134.634	61.597
G55	M	2.79	181.365	122.2	59.165
G57	F	3.06	156.847	108.152	48.695
G58	F	2.91	162.218	110.997	51.221
H1	M	2.4	175.138	116.155	58.983
H2	M	2.66	163.485	109.363	54.122
H3	F	3.1	164.929	112.311	52.618
H4	M	2.42	166.223	116.655	49.568
H5	M	2.64	162.11	111.837	50.273
H6	M	2.99	169.667	115.429	54.238
H7	M	2.7	177.056	124.311	52.745
H8	M	2.52	168.499	117.469	51.03
H9	F	3	163.641	117.987	45.654
H10	M	2.62	170.924	119.264	51.66
H11	M	2.83	194.4	132.619	61.781
H12	M	3.28	174.857	114.641	60.216
H13	F	2.83	153.607	102.79	50.817
H14	F	2.82	162.77	110.228	52.542
H41	M	2.57	166.125	112.044	54.081
H42	M	2.57	175.795	119.43	56.365
H17	F	2.79	163.417	111.344	52.073
H18	F	2.77	161.924	112.928	48.996
H19	F	2.4	146.758	102.109	44.649
H20	M	2.31	161.779	112.84	48.939
H21	M	2.68	189.375	127.667	61.708
H22	F	2.58	152.44	106.008	46.432
H23	F	2.33	139.103	99.83	39.273
H24	F	2.46	153.123	107.029	46.094
H25	F	2.59	149.674	101.538	48.136
H28	M	2.68	172.812	116.788	56.024
H29	M	1.97	168.912	114.672	54.24
H30	M	2.7	179.686	118.398	61.288
H31	M	2.31	167.444	114.588	52.856
H32	F	2.29	165.352	110.368	54.984
H33	F	2.43	151.852	105.494	46.358
H34	M	2.34	165.26	114.598	50.662

H35	F	2.7	153.97	108.864	45.106
H36	F	2.83	172.308	120.128	52.18
H37	F	2.36	162.784	113.349	49.435
H38	F	2.57	154.069	104.574	49.495
H39	F	2.76	158.429	108.678	49.751
H40	F	2.81	175.422	118.955	56.467
H43	M	2.22	156.564	109.059	47.505
H44	F	3.04	165.143	114.003	51.14
H45	F	2.67	161.449	110.438	51.011
H46	F	2.32	160.098	112.843	47.255
H47	F	2.48	166.739	122.047	44.692
H48	M	2.79	142.924	101.536	41.388
H49	M	2.73	182.634	109.728	72.906
H50	F	2.36	143.913	95.709	48.204
H51	F	2.42	157.125	108.221	48.904
G59	F	*	248.267	176.407	71.86
G60	F	3.1	153.734	105.29	48.444
G61	F	3.313	130.495	82.905	47.59
G62	F	2.866	168.27	113.923	54.347
G64	M	2.906	169.076	117.183	51.893
G65	M	3.208	232.824	169.005	63.819
G66	M	2.775	182.906	125.76	57.146
G67	M	2.938	191.575	131.605	59.97
G68	M	2.927	184.447	127.404	57.043
G71	M	3.448	195.156	132.841	62.315
H52	M	2.25	169.574	115.77	53.804
H53	M	2.48	178.854	122.496	56.358
H55	F	2.39	154.118	107.185	46.933
H56	F	2.95	162.815	111.437	51.378
H57	F	2.66	159.821	109.215	50.606
H58	F	2.74	150.137	102.08	48.057
H59	F	2.77	176.087	123.598	52.489
H60	M	2.42	167.897	116.801	51.096
H61	M	2.61	164.32	111.959	52.361
H62	M	2.48	182.776	129.292	53.484
H63	F	2.62	183.297	130.656	52.641
H64	F	2.93	184.825	130.322	54.503
H65	M	2.4	162.673	112.652	50.021
H66	F	2.33	149.872	104.099	45.773
H67	F	2.71	163.227	113.364	49.863
H68	M	2.73	178.693	124.238	54.455
H69	M	3.15	169.048	121.784	47.264
H70	M	2.52	161.442	114.849	46.593
H71	M	2.85	150.024	102.825	47.199
H72	M	2.43	142.993	101.079	41.914
H73	M	2.51	153.985	106.843	47.142
H80	F	2.69	181.118	128.443	52.675
H81	F	3.04	191.857	129.553	62.304
H82	F	2.82	179.667	119.43	60.237
H88	F	2.62	177.049	123.127	53.922
H74	M	2.79	185.923	126.973	58.95
H75	F	2.86	157.866	109.264	48.602
H76	F	2.52	144.867	102.647	42.22
H77	F	2.59	147.605	102.333	45.272
H78	F	2.9	165.105	113.988	51.117

H79	F	2.8	166.289	113.328	52.961
H84	F	2.66	137.874	96.994	40.88
H86	F	2.52	153.019	107.574	45.445
H91	F	1.94873	151.7	104.55	47.15
H89	M	2.60888	163.74	112.19	51.55
H90	M	2.59057	151.7	114.84	36.86

Codes are as follows: Cross G refers to those F2 hybrids (n = 140) with a SHRSP grandfather; Cross H a WKY grandfather; saltMSBP, mean salt-loaded systolic blood pressure; saltMDBP, mean salt-loaded diastolic blood pressure; saltMPP, mean salt-loaded pulse pressure.

Cross	Sex	saltMSBP (mmHg)	saltMDBP (mmHg)	saltMPP (mmHg)
G1	F	147.93	100.07	47.86
G2	F	173.3	120	53.3
G3	F	168.56	115.73	52.83
G4	F	163.95	112.8	51.15
G5	F	171.57	117.94	53.63
G6	M	181.96	126.13	55.83
G7	F	211.33	146.46	64.87
G8	F	173.61	119.66	53.95
G9	F	154.98	105.26	49.72
G10	M	168.69	116.4	52.29
G11	M	182.11	125.52	56.59
G12	M	194.9	132.74	62.16
G13	M	209.57	146.95	62.62
G14	M	193.91	130.44	63.47
G15	M	196.82	132.08	64.74
G16	M	204.7	141.06	63.64
G17	F	181.66	126	55.66
G18	F	203	147.03	55.97
G21	M	182.6	128.11	54.49
G22	M	183.01	126.24	56.77
G43	F	159.54	104.61	54.93
G44	F	235.43	166.98	68.45
G26	F	178.47	134.1	44.37
G27	F	153.41	105.07	48.34
G28	F	229.25	162.76	66.49
G30	F	165.65	112.18	53.47
G32	F	167.62	112.33	55.29
G33	F	175.99	115.19	60.8
G34	M	193.36	134.83	58.53
G35	M	177.07	120.62	56.45
G36	M	223.34	155.43	67.91
G37	M	199.14	132.27	66.87
G39	M	177.33	119.71	57.62
G40	M	173.83	117.53	56.3
G41	M	206.23	143.5	62.73
G45	F	170.2	114.64	55.56

G46	F	181.56	122.72	58.84
G48	M	199.53	134.46	65.07
G49	M	218.61	147.9	70.71
G50	M	170.99	115.65	55.34
G51	F	173.22	116.54	56.68
G52	F	194.44	131.1	63.34
G53	F	159.68	110.63	49.05
G54	M	244.01	170.6	73.41
G55	M	189.85	127.71	62.14
G57	F	166.2	114.01	52.19
G58	F	162.63	108.55	54.08
H1	M	182.448	121.36	61.088
H2	M	173.9	116.38	57.52
H3	F	170.983	114.61	56.373
H4	M	167.894	116.83	51.064
H5	M	171.023	118.01	53.013
H6	M	174.969	118.2	56.769
H7	M	180.502	125.24	55.262
H8	M	177.21	123.59	*
H9	F	166.44	115	*
H10	M	*	*	*
H11	M	197.24	131.1	66.14
H12	M	180.05	118.27	61.78
H13	F	154.6	99.64	54.96
H14	F	170.43	115.4	55.03
H41	M	171.41	115.38	56.03
H42	M	184.29	126.14	58.15
H17	F	171.58	116.29	55.29
H18	F	171.92	116.88	55.04
H19	F	151.35	105.22	46.13
H20	M	167	116.39	50.61
H21	M	199.74	135.84	63.9
H22	F	160.85	109.01	51.84
H23	F	142.83	99.82	43.01
H24	F	161.88	111.93	49.95
H25	F	158.58	106.15	52.43
H28	M	189.71	129.97	59.74
H29	M	183.31	124.1	59.21
H30	M	196.18	132.32	63.86
H31	M	181.19	125.53	55.66
H32	F	169.3	112.14	57.16
H33	F	163.61	112.33	51.28
H34	M	170.37	117.78	52.59
H35	F	164.09	114.46	49.63
H36	F	180.34	124.34	56
H37	F	168.41	117.22	51.19
H38	F	159.35	108.25	51.1
H39	F	163.24	109.41	53.83
H40	F	187.25	126.51	60.74
H43	M	171.48	116.49	54.99
H44	F	170.51	115.89	54.62
H45	F	169.07	115.24	53.83
H46	F	157.02	107.12	49.9
H47	F	168.8	120.5	48.3
H48	M	148.13	103.54	44.59

H49	M	189.18	113.11	76.07
H50	F	147.89	99.01	48.88
H51	F	157.84	107.68	50.16
G59	F	279.44	208.54	70.9
G60	F	165.24	113.75	51.49
G61	F	140.8	88.89	51.91
G62	F	169.88	113.32	56.56
G64	M	176.35	121.12	55.23
G65	M	234.68	169.95	64.73
G66	M	187.52	130.55	56.97
G67	M	205.54	143.4	62.14
G68	M	192.62	135.62	57
G71	M	209.08	143.05	66.03
H52	M	170.12	115.17	54.95
H53	M	183.13	123.7	59.43
H55	F	157.99	106.39	51.6
H56	F	160.72	105.85	54.87
H57	F	161.68	109.37	52.31
H58	F	149.04	98.2	50.84
H59	F	170.84	115.6	55.24
H60	M	172.21	118.8	53.41
H61	M	174.57	119.1	55.47
H62	M	185.04	130.09	54.95
H63	F	195.45	136.94	58.51
H64	F	180.21	123.68	56.53
H65	M	172.51	119.77	52.74
H66	F	154.33	106.87	47.46
H67	F	162.85	111.92	50.93
H68	M	187.56	130.49	57.07
H69	M	174.53	123.98	50.55
H70	M	163.64	113.47	50.17
H71	M	159.64	108.61	51.03
H72	M	160.68	114.12	46.56
H73	M	160.14	108.87	51.27
H80	F	188.28	131.87	56.41
H81	F	208.65	140.74	67.91
H82	F	185.9	120.91	64.99
H88	F	194.02	134.96	59.06
H74	M	197.82	136.73	61.09
H75	F	165.66	113.89	51.77
H76	F	148.57	99.68	48.89
H77	F	150.9	101.94	48.96
H78	F	166.16	111.04	55.12
H79	F	183.02	124.58	58.44
H84	F	141.48	96.49	44.99
H86	F	156.51	106.03	50.48
H91	F	156.05	106.43	49.62
H89	M	172.27	118.99	53.28
H90	M	172.92	118.58	54.34

Raw Genotypic Data

Chromosome 2 (first 1-50 animals) Animals are displayed in same order as for phenotypes. H is a heterozygote, B a SS homozygote and A a WW homozygote. - indicates no genotype recorded for that animal.

D2Mgh12		HBAABHAHAHBHBABHAHAAHBHBAABBHHBHH-BHABBA-BBHAHHHAH
	21.1 cM	
D2Mit14		BAABHAHABBHBHBHAHAAHBHBAABBHABHHHBHABBAHBHHABHHHH
	10.3 cM	
Gca		HBAABHAHABBHBABHABAABHBBAABBHHBHHHBHHBBAHBHHABHHHH
	6.2 cM	
Pklr		HBAHBHAHABBHBABHABHAB-HBBHABBHHBHHHBHBBHBBBHHBHHHH
	18.3 cM	
D2Wox9		HBHAHHAHABBABABBABAABBBAAHBHHBHHABHHBHAHHHHHHBHBHH
	13.8 cM	
Cpb		HBBAHHAHHHHABABBAHAHBBABHHH-HHBHBABHHB--------HBAH
	9.8 cM	
D2Mit6		HBBAHHAAHH-ABABBAHAHHBABHHHHHBBAHABHHBBAHAAHBBHBAH
	14.7 cM	
D2Mit5		HBBAHHAA-HBABABBAHAAA-BABHHHHHBBAHABHHHBHHAAHBHHAH
	18.7 cM	
D2Wox5		HBHAHHAHHHHHBHBBAHHAHHAHHHHHHBHAHABHHHBHHHAHBBH-HH
	7.7 cM	
D2Mit3		ABH-H-A-ABBABHHBAHAAH-AHHHAHHBHAHAHHHHBHHHAHBBHAAH
	0.5 cM	
D2Wox3		ABHAHHAHABBABHHBAHAAHHAHH-AHHBHAHAHHH-BHHHAHBBHAAH
	15.5 cM	
Fst		HBHAHHAHHBBABHBBAHAHHHAHHHAHHBHHHABHH-BHHHAHBBHAAH

Chromosome 2 (animals 51-100)

D2Mgh12		HBHA-HHHAHBHBHHHHAHB-H-HAABAAB---HHHHHHBHHHHHBHHHH
	21.1 cM	
D2Mit14		HHAHHHHHHHBH-BHHHAHB-HHHAABAABBBHHHHH-HHHHAHAAAABH
	10.3 cM	
Gca		HHAHHHHHHHBHBBHHHAHHAHHHBABAABB-HHHHHHHH-HHHAAAHBH
	6.2 cM	
Pklr		HHAHH-HHHHBHBHHHHAHHAHHHBABAABBB-HHH--HH--HHAAAHBH
	18.3 cM	
D2Wox9		HHAHHHHBHHBHBHAHHH-AAHHHBABHHHBBHHHHBHHHHHHAAAAHB-
	13.8 cM	
Cpb		HHAAHHHBHHBHBHAHH-HAAHHHBABHHHBBHHHB--HH--AAHAAHBB
	9.8 cM	
D2Mit6		AHAAHHHBHHHHBHHHHHHAAHHHBAHHHHBBAHHH---HAA--HAABHB
	14.7 cM	
D2Mit5		ABAHHH-BHHH-BH-HHAHAAH--BAH-HHBB-HHB-HHH-HAAHAABBB
	18.7 cM	
D2Wox5		HHHBHHABHBHHHHHHHAHHHHBHBAHAH-BBAHHBHHHHAHAHHAAHBB
	7.7 cM	
D2Mit3		ABABHHABHHHHHHHHHAHHHHBHBHAHHHBBAHHB-HHHAHAHHAAH-H
	0.5 cM	
D2Wox3		ABABHHABHHH-HHHHHAHHHHBHBHAHHHBBAHHBHHHHAHAHHAAH-B
	15.5 cM	
Fst		ABABHHABHHHHAHHHBAHBBBH-HHAHHABAHHB--HH-AAHHAAHB-

Chromosome 2 (animals 101-140)

D2Mgh12		HB-AH-AHB-HBH-BHHHA---AAAHHHHBHAHBHHBAAA
	21.1 cM	
D2Mit14		BBBAHHAHHAAAAAHHHHHHBHHBHHHHHHHAAHB-HHHH
	10.3 cM	
Gca		HHBBAHHAHAAAA-AHH-HHHBAHBHHHHHHAAHBA-HHH
	6.2 cM	
Pklr		HHBBAHHAHAHAA-HHHHHHHBHHBHHHHHHAAHBA-HHH
	18.3 cM	
D2Wox9		HHHBAHHHHAAAAHAHHHHBHBHHHHHAHHAAAABABHHH
	13.8 cM	
Cpb		HHHBAHHHBAHAA-AHH-ABHBAHHHHAHHAA-ABA-HHH
	9.8 cM	
D2Mit6		H-HBAHAABHHAHHAHH-ABHBAHHHHABHAHHABABHB-
	14.7 cM	
D2Mit5		HHHB-B-ABHH----AA-ABHH--HBAABHAHHABABHBB
	18.7 cM	
D2Wox5		HHHBHBAABHAHHBAAAHABHHHHHBHABHHHHAHABABH
	7.7 cM	
D2Mit3		HHHB-BAABHA-H-AAA-ABHBHHHBHABHHHBAHA-ABH
	0.5 cM	
D2Wox3		HHHBABAABHAHHBAAAHHBHBHAHBHABHHHBAHABABA
	15.5 cM	
Fst		HHHB-BAABHAHHBAAAHABABHAHAHA-HHAHHAHABAB

Chromosome 3 (first 1-50 animals)

D3Mgh6		HHHBAAHAHHHAHHBHHHHAAHHHHHHHHHHHBAAAHAHHHBHBHBBABAA
	32.7 cM	
D3Wox3		HHHBAAAAHHHABHHHHHHHHHAHHHHBHHHB---HHHBHBHBBBAABAA
	11.6 cM	
D3Wox2		HHHBAAAAHHHABHHHHH--HHAHHHHBHHHBAHAHHHBAHBABBAABAA
	20.2 cM	
D3Mgh16		BHHBAAAAHHHABHHH-HHHHHAHHHHBHAHBAHHHHB-ABA-BBAHHHA
	3.5 cM	
D3Wox4		HHHB-AAAHHHABHH---HHHHAHHHHBHAHBAHHHHBHABAHBBAHHHA
	16.8 cM	
D3Mit10		HAHBAAAAHHHABAHHHHHHHHA-HHHBHAHBAAAHH-HABAH---HHHA

Chromosome 3 (animals 51-100)

D3Mgh6		HHAAHHABHHAHAHHHHBAH-HBHHHBHHH-HBBBHAB-B-AABHHAAHA
	32.7 cM	
D3Wox3		AHH-HAABHAAHHHBHHHBHHAHBHBHHHHHBABBBAAHHBHHH-HAAHH
	11.6 cM	
D3Wox2		AHH-HAABHBBHHHBHHBHHAHBHBHHHHHBABBBAHHHBHHH-HAAHHH
	20.2 cM	
D3Mgh16		AA-AHAABAAAHHHBHHBBA-AB-BHH--ABABBAA--------HAAHAH
	3.5 cM	
D3Wox4		AAHAHAABAAAHHHBHHBHAAABABHHHH-BH--HHHAHBHBH-H-AHAH
	16.8 cM	
D3Mit10		AAHAHAABAAAHHHBHABHAAA-HBHHHHAB-BBHHBA-BHBHH--HAHA

Chromosome 3 (animals 101-140)

D3Mgh6		BHBHHBBHBABBHHHHHHB-BHHAHHAHHHHHH-AHAA--
	32.7 cM	
D3Wox3		HBBBHBBBAABHAHBHHHHAHAHAAAHAHHAHHHHHAHHA
	11.6 cM	
D3Wox2		BBBHBBBAH-BAAHBHHHHAHAHAAAHAHHAHHHHHAHHA
	20.2 cM	
D3Mgh16		HBBBHHBBAABHAHHBHHAAHAHAAHABAAHHHHBHHHHB
	3.5 cM	
D3Wox4		HBH-HHHBABBHAHH-HHHAHHHAAHABAAHHHHBH-HAB
	16.8 cM	
D3Mit10		HHBB-AH-BAHBHAH-BHHHAHA-AAHABA-HHHBHHHHA

Chromosome 14 (first 1-50 animals)

R426		BBBHAABHAHHHBBHHABBHAAAHHBABBAB---BAHHBHHABHHAHHHB
	8.9 cM	
R58		BHBHAHBHAHHBBBBHABBHAAAHHBABBABHABBABHBHHAHHHHAHBB
	11.4 cM	
D14Mgh3		BHBHAHHHAHHBBBBHAHBHAAAHABABBA-HABBABHBHHHBHHAAHHA
	1.9 cM	
R43		BHBHAHHHAHHBBBBHAHBHAAAHABABBABHABBABHBHBHBHHAAHHA
	9.4 cM	
Csna		BHB---HHHHHBBBBB---BAAAHABABBABHA----HBHHHHHAHAHHA

Chromosome 14 (animals 51-100)

R426		HBABHHHAAHHH--B--AB-HAHHBHHHHBHAHBB-HBBBHA--HBAAAH
	8.9 cM	
R58		HBAABHHAAHHHHABHHABBHAAHBHHHHBHAHABBHHHBAHAAHBAAAH
	11.4 cM	
D14Mgh3		HAABAHHAHBHHHABHHABB--AHBHHHHH-----HHHHBAHAAABAAAB
	1.9 cM	
R43		AAABAHHAHBHHHABHHABBHA--------HAHABBHHHBAHAAABAAHH
	9.4 cM	
Csna		HAABAHHA-HHB-A--HHBBHHHHBHHHHHHAHABBB--BAHAAABAABH

Chromosome 14 (animals 101-140)

R426		AHABH--HB-ABHHHH--AHHBAHB----BHAHHBHHA--
	8.9 cM	
R58		AHABHHHABHHBHHHHHHAHBBAHBHABHBHAHHBH-AA-
	11.4 cM	
D14Mgh3		HHAHHHBHBHAHBHHHHHAAHBAHHHABH-HHHHBHHAAH
	1.9 cM	
R43		HHAH-HB-BHAHBB-HH-AHHBAHHAABHB---HBHHA--
	9.4 cM	
Csna		--HHHHBBB-A-H-HHHHAAHBAHH----------

APPENDIX III

Chromosome 5 Primers

Marker	Primer Sequences	Tm (°C)	Size (bp)	Mg^{2+} (mM)
D5Wox7, R182	L - gACCCTgACTTggAAAgTgAA R - TgTgCACggTCTTCAAggTA	50	169	1.5
D5Mgh6	L - CTCACAAAACTTgACTgAAATTgC R - TCAAgATgCTATggAAAAgCC	60-55	155	1.5
D5Mit2, Mitr980	L - TCTgCATTgAAACTAATCTAAATCC R - TTgCACACATTggAATgTCA	63-58	201	1.5
D5Wox16, Rep198	L - CAgCCTTCATTCTCACAC R - TgACTTCTgTgAgCTCCTAC	55-50	160	1.5
D5Mit9, Mit735	L - CTATgTgCAAAAACgCATgC R - TCACAggTAATggTAgAAACgTT	50	127	1.5
D5Wox4, RCA14.12	L - CCTgCTgTTTCTgACTCCC R - AgCATCCAAgACTgggTgC	60-55	157	1.5
D5Wox15, Rep142	L - ggTAgAggTgAgTggAATgAA R - CCTCCTCAgCTCTgCTAgTC	60-55	118	1.5
D5Wox10, Anp, R241	L - gggACTgATACATgTgggTATg R - ggTCTCTgTACCTCAATgTTgC	55	161	1.5
D5Wox14, Rep56	L - TgggTAggTCgTgTCTTCTC R - CCTggTTAgAggAgggAgTC	55-50	227	1.5
D5Mgh15	L - CACCTCgACCAACACCAAC R - TTAATCCCAACTgTgACATTCg	60-55	159	1.5
D5Mgh16	L - gCATACAgCTTTACAgTgCTgC R - AgACAAgggACATgCTCgAg	60-55	133	1.5

Raw Phenotypic Data

Codes are as follows: Cross G refers to those F2 hybrids (n = 140) with a SHRSP grandfather; Cross H a WKY grandfather. IHVol is the ipsilateral hemisphere volume; CHVol is the contralateral hemisphere volume; InfarctV is the infarct volume; IF/IH is the infarct volume expressed as a percentage of the ipsilateral hemisphere volume.

Cross	Sex	Weight (g)	IHVol (mm^3)	CHVol (mm^3)	InfarctV (mm^3)	IF/IH (%)
G77	F	200	589.3	547.2	60.5	10.27
G78	F	202	581.6	542.1	55.7	9.58
G79	F	214	680.5	580.7	130.3	19.15
G80	F	193	667.9	558.2	141	21.11
G81	M	318	598.8	507.7	115.7	19.32
G82	M	314	730.9	599.1	188.6	25.8
G83	M	294	653.6	538.6	139.6	21.36
G84	M	294	701.7	585.6	149.1	21.25

G85	M	283	681.1	554.8	225.3	33.08
G86	M	309	697.6	571.9	199.2	28.56
G87	M	322	719	570.6	201.5	28.03
G88	F	213	644.2	535.7	126.5	19.64
G89	F	216	699.3	561.9	142.6	20.39
G90	F	188	640.6	533.5	177	27.63
G91	M	340	777.7	665.1	159.9	20.56
G108	F	187	619.7	552.7	139.4	22.49
G109	F	198	468.9	438.4	40.2	8.57
G112	F	182	746.2	654.3	216	28.95
G113	M	312	705.5	617.4	161.3	22.86
G114	M	287	703.6	584.9	195.6	27.8
G115	M	303	668.2	567.7	148.8	22.27
G116	M	330	676.3	586	98.2	14.52
G117	F	195	701.8	558.1	177.7	25.32
G118	F	184	665.8	562.9	171.5	25.76
G119	F	197	742.4	640	161.6	21.77
G120	F	202	608.2	521.4	138.5	22.77
G121	F	201	733.7	629.2	188.7	25.72
G122	F	184	723	632	120.1	16.61
G123	M	290	767.8	656.9	165.6	21.57
G125	M	290	597.2	518.1	62.7	10.5
G126	M	276	828.2	608.7	332.9	40.2
G127	M	281	654.6	542.4	147.8	22.58
G128	M	300	725.7	543.7	190.6	26.26
G129	M	280	663.3	589.8	152.9	23.05
G130	M	320	620	576.9	32.6	5.26
G131	M	308	621.1	547	147.1	23.72
G132	F	183	657	520.3	208.9	31.8
G133	M	282	683.5	547	240.5	35.19
G134	M	307	661.5	523.9	226.5	34.24
H115	F	198	747.3	627	192.3	25.73
H116	F	197	647.1	539.1	162.2	25.07
H117	F	197	589.7	539.8	53.2	9.02
H118	F	197	574.1	547.4	23.6	4.11
H119	F	215	638.1	576.1	48.5	7.6
H121	F	190	652.8	578.9	103.2	15.81
H122	M	303	764.1	602.2	218.5	28.6
H123	M	308	726.2	585	213.2	29.36
H124	M	308	703.4	604.8	168.2	23.91
H125	M	386	677.8	562.4	197.3	29.11
H126	F	189	644.9	532	186.9	28.98
H131	M	279	614	483.4	165.1	26.89
H132	M	279	649.7	547.5	182.9	28.15
H134	M	288	712.9	599.1	214	30.02
H138	M	276	650.6	560.5	142.3	21.87
H139	M	290	643.1	544.4	190.5	29.62
H140	F	182	684.2	586.3	141.3	20.65
H141	F	215	658.3	530.2	202	30.69
H142	M	271	726.4	595.9	210.1	28.92
H143	M	282	648.1	562.4	169.4	26.14

Raw Genotypic Data

Chromosome 5 (n=59) Animals are displayed in same order as for phenotypes. H is a heterozygote, B a SS homozygote and A a WW homozygote. - indicates no genotype recorded for that animal.

D5Mgh6		HAHAHHBA-HHAABHHHHABHAHHHHAHHHBBAHHBBBBBHABBAAHHAHBHAH-ABAA
	25.5 cM	
R182		HAHAHHBABHAAAHBHHHABHHHHBBHHHHHBHH-BHBHBH-BHHHH-HHHHHHHA--H
	27.3 cM	
D5Mit2		HAHAHHH-BHAAHHBHAHAHHBHBABH-HHHAHHHHHBHHAHBABB-A-H-HAHAHHHB
	8.1 cM	
D5Wox16		AAHAHHHHBHAAHBBHAHAHHBHBBBHHHHHAHHHHHBBHHHBABBHHHHH-HHAHBHH
	17.1 cM	
D5Mit9		AAHHHHHHBHHAABHHABAHHHHBBHBHHHBHHHAH-BBHHHHABBBHHH----HHBHH
	21.6 cM	
Anp		AAHHHBHHBBHAABHHABHBHAHHHHBAHABHBHAHBBBHHAAAHBBHBBHBBHBABHH
	8.1 cM	
D5Wox4		AAHHHBHHBBHAABHHABHBHAHHHHBAHABHBHHHHBHHHAAHAHBHBBHBHHBHHHH
	14.4 cM	
D5Wox15		AAHHBBHHHHBAABBHABHBHHHH-HBAHABHHH-HHBHHHAHAAHHHBHHHHHHH--H
	22.7 cM	
L26461		AAHHB-HHHHBAHBAH-BAHHHHHAHBAAABHHHHBAHA--HHHAAA---HHAHHHAHH
	11.9 cM	
D5Wox14		AAHHBHHAHHBHHHHHABABHHHHAHBAAHHAHHHBAH-HAHAAAH-HAHHHA-HHHHH
	23.2 cM	
D5Mgh15		AHHHBHAAHABHBBAHABAHAHHHAHBAABHAHABBAHHHHHABHAABAHHHAAHHABA
	1.2 cM	
D5Mgh16		AHHHBHAAHABHBBAHABAHAHHHAHBAABHAHABBAHHHHHABHAABAHHHAAHHABA

APPENDIX IV

New Chromosome 2 Primers

Marker	Primer Sequences	Tm (°C)	Size (bp)	Mg^{2+} (mM)
D2Rat167	L - ggTAACCTgACAgATCACCTCC R - TCCAAATTTTCCTTTTggTTTT	55-50	215	1.5
D2Rat49	L - ATCCCTggTgAgggAAAAgT R - CTTATTgTATTTACATgTgCACgC	55-50	125	1.5
D2Mit18, Mit380	L - ggggATgTTCTgCTggTAgA R - TTCCAATTCTggAggACCTg	55-50	198	1.5
D2Rat14	L - gCTAAATgggACACCTggAA R - AAACCAAAgTCTgTggCTCC	55	139	1.5
D2Rat18	L - ggAgTgATCTgTTTCgTATAAATgC R - CTTCTCTTCCTCACCCTCCC	55	129	1.5
D2Rat28	L - gAgTgCAAAgCCCAgTCTTC R - CCACATgCCTTTCAgTTTCC	55-50	230	1.5
D2Rat58	L - ACTgTATTCCACCACCACCC R - TgAAgACCTTTCggTgTgTg	55	125	1.5
D2Wox26, R405, Nkaa1b	L - TggCTgTAATCCCTCATgg R - CAgCACCTgggACTCAAg	60	194	1.0
D2Wox38, R487	L - TCAgAggCAgCCATCAAC R - TCTAATACATCAgCACAggAgC	55-50	200	1.0

Background Primers

Marker	Primer Sequences	Tm (°C)	Size (bp)	Mg^{2+} (mM)
D1Mit14	L - TCTgCCTTCTCACATgAACA R - TTCCATCTACTgCTgTTTAggg	55-50	112	1.5
GV30	L - TCTTCCTTTTCTAgCACTCCA R - TgTAggTTgTATTCgTgTATgC	60-55	141	1.5
D1Wox32	L - gCTACTgCCTTgCCCTCA R - TCACATTTACCTgTAgTTggAA	55-50	188	1.5
D1Mit11	L - ATAAgCCAgCCCCCATTC R - CCTACTgAAAgTgAAATgTCTgg	58-53	275	1.5
D1Mit1	L - ggggAAgTTTTAggAAgTCCC R - AgggAATgAAAATACAACACgC	55-50	146	1.5
D3Mit4	L - AAAAAACCAACCCCCTTCC R - gCAAAgAgATgCAACATCTgg	63-58	191	1.5
D3Wox14	L - gCATTgCCTgAgTAggATgT R - gTTTggCTgTAATTggCg	55-50	262	1.5
D3Mgh8	L - gCTgAACggATgCTgAgAg R - gggATTCCTAAgACAgTTgCC	55-50	156	1.5
D3Mgh16	L - AgTCAgggCTATgTATTgAgAACC R - CCTCTgACCCCTACATggg	55	120	1.5
GV88	L - ggAgCATgTgCTTTTAATACTT R - CAggggCTgTggCTgTAA	60-55	100	1.5
D4Mgh16	L - CAggAgCTgTCTgggACTTC R - gAACACTAgAgAAACTAggCAggC	60-55	259	1.5
D4Mgh7	L - gATCCAgCTCACATCTAATCCC R - CCAAATgCTCTTgCAgTCAA	60-55	145	1.5

D4Mit14	L - AggACAggTTTTTgggCTTT R - TCTgCCgCCACCTTAgAg	55-50	150	1.5
NG56	L - TgggTAggTCgTgTCTTCTC R - CCTggTTAgAggAgggAgTC	55-50	227	1.5
D5Wox15	L - ggTAgAggTgAgTggAATgAA R - CCTCCTCAgCTCTgCTAgTC	60-55	118	1.5
D5Mgh15	L - CACCTCgACCAACACCAAC R - TTAATCCCAACTgTgACATTCg	60-55	159	1.5
D5Mgh16	L - gCATACAgCTTTACAgTgCTgC R - AgACAAgggACATgCTCgAg	60-55	133	1.5
D6Wox21	L - TTgAgAAgCgTTAAAATATgTg R - TTggTTTACAgggTgAgAA	55-50	120	1.5
D6Mgh5	L - CAgTTAgCATAgAAAgCAAAggg R - ATAggAATAAAgAgTgCACgTTTg	55-50	104	1.5
D7Mit10	L - TgCCCCAAAAAggAAAAAC R - TCAgCTTCATACggAAgCAA	60-55	171	1.5
D7Mit7	L - ACAgCTggAATCCTCTggg R - gAgCTAgCCATgCAggAAAC	60-55	260	1.5
D8Wox22	L - gCAgTgTgAgAggAAAgTgTC R - gAAgTCCTCACCTgTgTTCAg	60-55	181	1.5
D8Mgh7	L - TgAAgAgTTTTACTgggTAgCTCC R - TggACCAggCAAgTTCTCTT	60-55	191	1.5
D8Wox13	L - CATCTgggTCTgTggTAAgg R - TCTgggAAggACTCTTggA	60-55	174	1.5
D8Mgh10	L - CTTTgATACTgTACCAACAgCACC R - AATgTCAggATggCAgAgAC	55	144	1.5
D9Mit1	L - gCTTTCAAACACCACAgggT R - ACAACTCCCATCTCTTgAgAgg	55-50	130	1.5
D9Wox13	L - CCTTTgCggggTgTTgTA R - ACCAACAATgCgACAgAgAAT	55-50	278	1.5
D9Mit4	L - gCATAATggAAgAAgACAACTACC R - TCCATgCATgTgTATCTgCA	55	200	1.5
D10Wox3	L - gAAgTCTTCACTTTTACTTgTgg R - gACCCTTTTgAgAgAACTTTTg	60	176	1.5
D11Mgh6	L - AACAgTCAAAAgAgATATCCAggg R - AAACAAATgATgTACATgCATACA	60-55	100	1.5
D12Wox2	L - TAACCTCCAAAggACCTCTC R - CTAgATAAggTgTATgTggCTC	60	194	1.0
D12Mgh3	L - TTCAACAACCACCTCACTTCC R - ggATTgAgggTggggTAAgT	60-55	124	1.5
D13Mgh1	L - AgAgAAAATATgTggACAgAAgCC R - CACTTCCCAAATgCTAgCgT	60-55	149	1.5
D13Wox4	L - CCTggACACTAATCCTATCTTg R - gggTAggTCTgAgggAAgg	55	180	1.0
REP328	L - AAgCCTgCTCCACTCCAC R - gCAgAggggAgAggTAATAA	60-55	257	1.5
D14Wox5	L - CAggAAgAgAAAgggAgTTgg R - gTCTgAAgTggTTgTgAgTTCC	63-58	154	1.5
D14Wox8	L - AAgAATAgCAgTgAATTggTg R - TTCATCATCCTTTCATAAAggC	60	151	1.0
D15Mit2	L - TACATggAAgCCAAATgCAA R - TACTgAgAAAATgggTCTgC	55-50	140	1.5
D15Mgh3	L - AgACCCAgggTAggCATTTT R - gATTACAATTCTgTCCAAgTCACg	55-50	146	1.5
D15Mgh6	L - AgCAgCggTATCTCCAgTgT R - gggTgACggAgCAgAgAAA	55-50	216	1.0

D16Wox12	L - TAgCAggATgTTgTAggTgC R - CCAgggTATTAAggTCggAC	60	230	1.0
D16Mit1	L - ggCTTgTgTggACACCTgTA R - AAAgAgCAgggAAgAgACAgg	55-50	164	1.5
R435, D16Wox10	L - AggCTTTgATTgCAggAAg R - AAAgAgCTgTCgTCCACAAC	60	118	1.5
D17Wox21	L - TAAggACCCCTgATACTCTgg R - AgATCTTTgTCAAATTCATggC	60-55	153	1.5
D17Wox13	L - AgTAgACAggAgTgggAAggA R - CTCTTTgggCAgCTTACATT	60	134	1.0
D17Wox10	L - ATCTgTgTgCgAgTgCgT R - CTggCgAAgTgACgTgAg	60	147	1.0
D18Wox12	L - CACATgTTTACTTTCTAAgCATTTg R - CCCCTCTTCTggACTTCATAg	60	148	1.5
D18Wox16	L - TCACAATAAAAAACTCCTCCAAC R - AgTCTgTgCCCTgTTCCCA	60-55	152	1.5
D19Wox2	L - ggTATgggAgTAACATgACCTC R- ggACACATACggTAAgCACATgC	63-58	129	1.5
D19Wox8	L - TgCCCgTCTCTgTTACTCAT R - CAAgAACCCTgAggCAATAA	60	111	1.0
D20Wox5	L - gAAAAATACTTCCACACACTAATg R - AAAgTCAAgCCCTggAgTg	60-55	259	1.0
D20Wox3	L - AggAAAtGGGtttCAgTTCC R - CAggATTCTgTggCAATCTg	55	125	1.0
DXWox3	L - gATCgTCCAgCATCgTgg R - gTTggTgCTACTCAAgATCgg	60	130	1.5

Raw Genotypic Data

Chromosome 2 New Markers (animals 1-50) Animals are displayed in same order as for phenotypes. H is a heterozygote, B a SS homozygote and A a WW homozygote. - indicates no genotype recorded for that animal.

D2Rat14	-BHAHHAHABB----------HHAHHBHAHAH-HHBH-HAH--HAAH
D2Rat18	BHAHHHHABB----------HHAHHBHAHAH-HHBB-HAH--HAAH
D2Rat167	-B--HHAA-HH----------HHHHHBBAHAB-HBBA-AAH--HBAH
D2Rat28	-BBAHHBHHBH----------HHHBHHBHBAB-HBHA-HAH--HABH
D2Mit18	-BBAHHBHHBH----------HHHBHHBHBAB-HBHA--AH--HBAH
D2Rat49	-BAABHAAABB----------AABBHHBHHHB-ABBA-BHH--HHBH
R405	-BAABHAHABB----------AABBHHBHHHB-ABBA-BHH--HHHH
D2Wox38	-HAABHAHABB----------AHBBHHBHBB-----A-BBB--AHHH
D2Rat58	-BAABBABAHB----------A-HHHHBHHHH-ABBA-BBB--BHHH

Chromosome 2 New Markers (animals 51-100)

D2Rat14	ABHBHHHBHHHHHHH-HHB-HH-HBHHAAABBAH-BBBHHAHAH--AHB-
D2Rat18	ABABHHABHHHHHHHAHAHHBH-HBHAHBHBBAH-HHHHHAHAH--AAB-
D2Rat167	AHAAHHHBHHHHHHHHHHHAAH-HBAB-HHHHABHBBHHHHHAA--H-B-
D2Rat28	HHABHH-BHHBHBHBHHHHBBH-HAAAHHHBBHHHBBHHHHA-A------
D2Mit18	HHAAAHHBHHBHBHAHHHHAAH-HBABHHHBBHHHBBHHHHAAA--AHB-
D2Rat49	HHAHH--HHHBHBBHHHAHBAH-HAABAABBHHHHHHHHBHHHH--AHB-
R405	HHAAHHHHHHBHBBHHHAHBAH-HHABAABBBHHHHHHHHHHHH-BAABH
D2Wox38	HHAAHHHHHH-HBBHHH--BA--HAABAAB--AH-H-HHAHHHH---AB-
D2Rat58	BHAHAHHHBHBHBBHBHHHBHB-BAABAABHBBHHHHHHBHHHH--AHB-

Chromosome 2 New Markers (animals 101-140)

D2Rat14	H-HBHHBABHAH----------
D2Rat18	H---HHBABHAH----------B--------H--------
D2Rat167	H-HBAHHAHHHA----------
D2Rat28	----------
D2Mit18	HAHBAHBHBAHA----------
D2Rat49	--B-AHBAHAAA----------
R405	HABBAHBAHAAA----------
D2Wox38	--BBAHB---AA----------
D2Rat58	H-BBHBAABAAA----------