Dissection of chromosome 18 blood pressure and salt-sensitivity quantitative trait loci in the spontaneously hypertensive rat

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DISSECTION OF RNO18 BLOOD PRESSURE AND SALT SENSITIVITY QUANTITATIVE TRAIT LOCI IN THE SPONTANEOUSLY HYPERTENSIVE RAT

Michelle D. Johnson[1], **Liqun He**[1], **Daniel Herman**[2], **Hiroko Wakimoto**[2], **Caroline A. Wallace**[1], **Vaclav Zidek**[3], **Petr Mlejnek**[3], **Alena Musilova**[3], **Miroslava Simakova**[3], **Jaroslav Vorlicek**[3], **Vladimir Kren**[3,4], **Ondrej Viklicky**[5], **Nathan R. Qi**[6], **Jiaming Wang**[7], **Christine E. Seidman**[2,8], **J.G. Seidman**[2], **Theodore W. Kurtz**[7], **Timothy J. Aitman**[1], and **Michal Pravenec**[3,4]

[1]Physiological Genomics and Medicine Group, MRC Clinical Sciences Centre, Imperial College, London W12 0NN, UK

[2]Department of Genetics, Harvard Medical School, Boston, MA 02115, USA

[3]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague 142 20, Czech Republic

[4]Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, Prague 12800, Czech Republic

[5]Institute for Clinical and Experimental Medicine, Prague 140 21, Czech Republic

[6]Department of Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109, USA

[7]Department of Laboratory Medicine, University of California, San Francisco, California 94107, USA

[8]Howard Hughes Medical Institute, 77 Avenue Louis Pasteur, Boston, MA 02115, USA

Abstract

Hypertension in humans and experimental models has a strong hereditary basis, but identification of causative genes remains challenging. Quantitative trait loci (QTLs) for hypertension and salt sensitivity have been reported on rat chromosome 18. We set out to genetically isolate and prioritise genes within the salt sensitivity and hypertension QTLs on the spontaneously hypertensive rat (SHR) chromosome 18, by developing and characterising a series of congenic strains derived from the SHR and normotensive Brown Norway (BN) rat strains. The SHR.BN-D18Rat113/D18Rat82 (SHR-18) congenic strain exhibits significantly lower blood pressure and is salt-resistant compared to SHR. Transplantation of kidneys from SHR-18 donors into SHR recipients is sufficient to attenuate increased blood pressure but not salt sensitivity. Derivation of

Correspondence to: Michal Pravenec, Institute of Physiology, Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Telephone: (+420)241062297 Fax: (+420)24108 2488. pravenec@biomed.cas.cz. Timothy J. Aitman, MRC Clinical Sciences Centre, Du Cane Road, London W12 0NN, United Kingdom, Telephone: (+440)2083834254 Fax: (+440)2083838577. t.aitman@csc.mrc.ac.uk.

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congenic sublines allowed separation of salt sensitivity from hypertension QTL regions. Renal expression studies with microarray and Solexa-based sequencing in parental and congenic strains identified four differentially expressed genes within the hypertension QTL region, one of which is an unannotated transcript encoding a previously undescribed, small non-coding RNA. Sequencing selected biological candidate genes within the minimal congenic interval revealed a non-synonymous variant in SHR Transcription factor 4. The minimal congenic interval is syntenic to a region of human chromosome 18 where significant linkage to hypertension was observed in family-based linkage studies. These congenic lines provide reagents for identifying causative genes that underlie the chromosome 18 SHR QTLs for hypertension and salt sensitivity. Candidate genes identified in these studies merit further investigation as potentially causative hypertension genes in SHR and human hypertension.

Keywords

hypertension; salt sensitivity; congenic; microarray expression profiling; candidate genes

INTRODUCTION

Essential hypertension is highly prevalent throughout the world and represents a major risk factor for coronary heart disease, kidney failure and stroke.[1] However, the molecular genetic basis of essential hypertension remains largely unknown.[2,3] The elucidation of genes responsible for human essential hypertension is especially challenging given the failure to identify statistically significant, replicated association by genome-wide association analysis.[4] Inbred animal models can be used to identify genes and pathways for complex cardiovascular traits for which the orthologous genes and pathways may contribute to the related human trait.[5–8] The spontaneously hypertensive rat (SHR) is a widely used animal model of essential hypertension and also develops salt sensitive hypertension. Salt sensitivity, defined as an increase in blood pressure with increased dietary salt, is found in 50–70% of human patients with essential hypertension.[9]

In this study, we set out to genetically isolate and prioritise the genes within the salt sensitivity and hypertension quantitative trait loci (QTLs) on SHR chromosome 18, by developing and characterising a series of congenic strains in which we transferred the corresponding region of chromosome 18 from the normotensive and salt resistant Brown Norway (BN) rat onto the genetic background of the salt sensitive SHR/Ola strain.

MATERIALS AND METHODS

SHR Congenic Strain and Subline Derivation

All animal studies were performed in agreement with the institutional animal care and use committee at the University of California, San Francisco and Animal Protection Law of the Czech Republic (311/1997); approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences, Prague. SHR/Ola rats were from the Prague colony and Brown Norway (BN/Crl) rats from Charles River Laboratories (Wilmington MA, USA). Please see http://hyper.ahajournals.org for details of the construction of these

congenic strains. The physical and genetic coordinates of the markers defining the SHR and BN limits in the four congenic lines are shown in Figure 1.

Blood Pressure Measurements

Arterial blood pressures were measured continuously by radiotelemetry in paired experiments between conscious, unrestrained SHR parental and age-matched congenic male rats. Pulsatile pressures were recorded as previously described.[10] To test for salt sensitivity, blood pressure measurements were taken for six days when rats were given 1% NaCl for drinking instead of tap water at 15 to 17 weeks of age for SHR-18, SHR-18B, and SHR-18C and from 19 to 21 weeks of age for SHR-18A. Comparison of radiotelemetry data between congenic and parental strain was carried out by two-way fixed ANOVA with one grouping factor for strains and one trial factor for blood pressure measurements at baseline and during 1% NaCl administration. F statistics and *P* values corresponding to the main effects for strain and trial factor effect for a strain are reported. Salt preference in SHR and SHR-18 strains was measured by a standard two bottle test to determine whether any differences in salt sensitivity were associated with differences in salt appetite. Please see http://hyper.ahajournals.org for details of blood pressure measurements and statistical analyses.

Kidney Transplantation

Kidney transplantation experiments were performed using methods similar to those previously described.[11] Transplantation procedures were performed in 9–11 week old male SHR progenitor strain with donor kidneys from either the SHR-18 congenic or the SHR progenitor strain. Transplant recipients were bilaterally nephrectomised SHR. Radiotelemetry transducers were implanted at 14–17 weeks of age and baseline blood pressures were taken at 18–20 weeks of age. To test for salt sensitivity, blood pressure measurements were taken for eight days when rats were given 1% NaCl for drinking instead of tap water at 19 to 21 weeks of age. At 23 weeks of age, transplant recipients were tested for renal function by determining glomerular filtration rates (GFR) and urine protein/creatinine ratios. Please see http://hyper.ahajournals.org for details of renal function tests and statistical analyses of blood pressure measurements taken before and during salt loading.

Microarray and Solexa Analyses

For generation of microarray data, RNA was extracted from 4 pairs of whole kidneys from SHR/Ola, BN/Crl, and SHR-18 males. For Solexa data, RNA was extracted from 3 pairs of whole kidneys from SHR/Ola, SHR-18 and SHR-18B males. Please see http://hyper.ahajournals.org for details of preparation of the RNA, statistical analyses for the microarray and Solexa analysis, and the mapping and validation of the Solexa analyses. The microarray data set has been submitted to ArrayExpress and can be accessed at http://ebi.ac.uk/ (Experimental Accession No. E-MIMR-1122).

5′ and 3′ Rapid Amplification of cDNA Ends (RACE) of rc_AA957526_at

The 5′ and 3′ sequence of rc_AA957526_at was determined by cloning and sequencing. Homologous sequences from human, mouse, dog and opossum genomic DNA were aligned to the rat sequence. To identify conserved small RNA, the multi-species alignment was run

in the RNAz web server[12] using the default parameters except for a basic window size of 200bp. Northern blots from BN and SHR kidney small RNA (<200bp) was probed with a single-stranded oligonucleotide probe. Please see http://hyper.ahajournals.org for details of the RACE procedure, *in silico* prediction, validation and Northern blotting. The 1.8kb sequence has been submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) (GenBank accession number FJ479701).

RT-PCR, DNA Sequencing and Restriction Fragment Length Polymorphism (RFLP) Analyses

SHR/Ola and BN/Crl genomic DNA and kidney random hexamer-primed cDNA served as templates for PCR and RT-PCR to amplify the protein coding regions of seven genes within the blood pressure QTL interval. Primers are listed in Table S1. The Transcription factor 4 (*Tcf4*) single nucleotide polymorphism (SNP) was tested in the genomic DNA of SHR, SHR-18, SHR-18B and 25 commonly used rat strains including the hypertensive strains SHR-Stroke Prone (SHRSP) and Dahl Salt-Sensitive (SS/Jr) using standard methods. Please see http://hyper.ahajournals.org for details of the amplification and RFLP protocols.

RESULTS

Mapping the Blood Pressure and Salt Sensitivity QTLs in Congenic Strains

Systolic and diastolic blood pressures in the congenic lines were compared (in paired experiments) to age-matched SHR controls at baseline and during days 4 and 5 of 1% NaCl administration (salt loading). At baseline, SHR-18, SHR-18A and SHR-18B showed lower systolic blood pressure compared to SHR controls whereas the SHR-18C strain showed similar systolic blood pressure levels to SHR (Figure 2). Salt loaded systolic blood pressure was significantly raised in all SHR and congenic strains with the exception of SHR-18 (Figure 2). Diastolic blood pressure did not show the same pattern as systolic blood pressure due to the large variation and narrower ranges associated with diastolic blood pressure (Figure S1). These data indicate the presence of blood pressure regulatory gene(s) within the SHR-18B congenic interval, excluding the region of overlap between SHR-18B and SHR-18C, as defined by the region between markers *D18Rat40* and *D18Rat82* (Figure 1). This region contains 88 protein coding genes in the Ensembl genome database (http://www.ensembl.org/Rattus_norvegicus/Info/Index). Similarly, the data indicate the presence of salt sensitivity susceptibility gene(s), located within the chromosomal interval defined by markers *D18Rat113* and *D18Rat99* (Figure 1). There was no significant difference in fluid consumption between the SHR-18 congenic strains and the SHR strain when corrected for body weight. Similarly, SHR (n=8) and SHR-18 (n=6) did not differ for their preference for salt (81.3% ± 3.7 versus 82.9% ± 4.7).

Kidney Transplantation

To test whether the blood pressure and salt sensitivity phenotypes are determined by genes expressed either within or outside the kidney, systolic and diastolic blood pressures were compared between bilaterally nephrectomised SHR recipients that were transplanted with either a kidney from SHR-18 congenic rats ($SHR_{SHR\text{-}18}$) (n= 8) or from SHR rats (SHR_{SHR}) (n=5). In the basal state prior to salt loading, the $SHR_{SHR\text{-}18}$ animals had lower systolic

blood pressures when compared to SHR_{SHR} controls (Figure 3). The nephrectomised and transplanted SHR_{SHR-18} and SHR_{SHR} rats showed highly significant rises in systolic blood pressure as a result of salt loading (Figure 3). Diastolic blood pressure did not show the same pattern as systolic blood pressure due to the large variation and narrower ranges associated with diastolic blood pressure (Figure S2). GFR was 1.2 ± 0.2 ml/minute for both groups. Protein urine/creatinine ratios (mg protein/mg creatinine) were 1.8 ± 0.2 for SHR_{SHR-18} animals and 1.7 ± 0.2 for SHR_{SHR} animals.

Gene Expression Analysis by Microarrays, Solexa Tag Analysis and quantitative RT-PCR (qRT-PCR)

To identify differentially expressed kidney transcripts within the congenic region of the SHR-18 congenic strain, gene expression analysis of the SHR, BN and SHR-18 kidney transcriptome was carried out with Affymetrix U34A-C microarrays. After filtering the microarray data for probe sets mapping to multiple locations in the genome, 88 transcripts were identified to be differentially expressed between SHR and BN of which 2 mapped to the SHR-18 and SHR-18B congenic interval that the blood pressure studies delineated as the minimal congenic interval for the SHR QTL hypertension gene(s) (p=0.05, FDR=0.05) (Table S2). Comparison of the SHR and SHR-18 strains identified 26 differentially expressed transcripts of which 2 mapped within the SHR-18 congenic interval and 1 within the SHR-18B congenic interval (p=0.05, FDR=0.05) (Table S3). Common to both the SHR/BN comparison and the SHR/SHR-18 comparison was a single unannotated transcript, rc_AA957526_at, which was 6.6 fold higher expressed in SHR compared to BN and 4.9 fold higher expressed in SHR compared to SHR-18 with *P* values of 3.5×10^{-9} and 8.2×10^{-10} respectively (Table S2, S3). Nineteen genes from the SHR-18B minimal blood pressure congenic interval were represented by microarray. To extend the microarray expression analysis and identify an unbiased set of differentially expressed transcripts within the blood pressure QTL interval, we carried out Solexa-based sequencing of the SHR, SHR-18 and SHR-18B kidney transcriptomes. Of 252 tags (representing 32 genes) that map within the SHR-18B congenic region, 69 (representing 15 genes) had a fold change of tag abundance ≥1.5 when SHR was compared to SHR-18 and SHR-18B. Five of these tags were found to be significantly differentially expressed ($p<0.05$) between SHR and SHR-18 and also between SHR and SHR-18B. The directional differential expression of three of these tags was confirmed by qRT-PCR (Figure S3). The list of tags that map within the blood pressure QTL interval and had a fold change of tag abundance ≥1.5 are given in Table S4.

Characterisation of rc_AA957526_at

Because the probe set representing the differential expressed transcript rc_AA957526_at mapped within both SHR-18 and SHR-18B differential segments, but was not a previously annotated gene, we carried out 5′ and 3′ RACE to determine its full-length sequence. The 5′ and 3′ RACE fragments formed an intronless 1.8kb sequence which did not have an open reading frame with homology to any known mammalian proteins. Semi-quantitative RT-PCR in SHR, SHR-18 and SHR-18B kidney cDNA confirmed the existence the 1.8kb differentially expressed transcript (Figure S4). When RT-PCR products were normalised to *Gapdh*, SHR-18 and SHR-18B had 1.2 and 1.6 fold less transcript compared to SHR, confirming higher expression in the SHR. The 1.8kb RACE sequence showed high

conservation of 73, 85, 74 and 67% in genomic DNA from human, mouse, dog and opossum respectively. Because all open reading frames lacked homology to mammalian proteins, we considered the possibility of this conserved 1.8kb fragment representing a non-protein coding RNA. Alignments of the rat, human, mouse, dog and opossum genomic DNA sequences were run through the RNAz web server to find thermodynamically stable and structurally conserved RNA elements. From a 200bp sequence within the 1.8kb fragment, RNAz predicted the presence of a small RNA encoded from the same strand as the RNA interrogated by the microarray (Figure S5). Northern blotting carried out in BN and SHR kidney small RNA showed the presence of a small transcript with approximate size of 100bp (Figure 4).

Sequence Analysis of Positional Candidate Genes

To provide an expression-independent set of candidate genes within the blood pressure QTL region which may lead to altered protein function, we sequenced seven genes within the minimal SHR-18B congenic region that had gene ontologies indicative of metabolic or regulatory processes that could be related to blood pressure control. The protein coding regions of cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (*Cidea*), melanocortin 2 receptor (*Mc2r*), melanocortin 4 receptor (*Mc4r*), melanocortin 5 receptor (*Mc5r*), protein tyrosine phosphatase, non-receptor type 2 (*Ptpn2*) and StAR-related lipid transfer (START) domain containing 6 (*Stard6*) were identical between BN and SHR. However, three SNPs were found in the SHR *Tcf4* coding sequence when compared to BN: G1182A, A1428G and G1984A. The G1948A sequence variant resulted in a non-synonymous change of alanine to threonine at codon 662. We tested 25 commonly used rat strains to determine if the variant was unique to either SHR or BN. The *Tcf4* non-synonymous sequence change was not found to be unique in BN or SHR and there was no consistent relationship between *Tcf4* genotype and hypertension across these strains.

DISCUSSION

Using congenic strains, our studies have defined regions on chromosome 18 that contain genes for increased blood pressure and salt sensitivity in the SHR strain. Our results showed that the SHR-18 congenic strain has pronounced salt resistance, a characteristic not shown in other SHR congenic strains[10,13–17]. The effects of chromosome 18 on salt sensitivity do not appear to be related to salt intake or fluid consumption as these were found to be similar in SHR-18 and the SHR progenitor strain. Previous linkage studies have suggested the presence of major QTLs on rat chromosome 18 with effects on blood pressure and/or salt sensitivity in three rat models of human essential hypertension: SHR,[18] SHRSP,[18,19] and the Dahl salt-sensitive rat (SS/Jr).[20] Blood pressure and salt sensitivity QTLs have been confirmed the SS-18^{BN} consomic strain.[21] Further congenic lines will need to be generated to determine if these hypertension and salt sensitivity QTLs in the Dahl rat are caused by the same genes as the QTLs that have been isolated in the SHR-18 strain.

Telemetric testing of blood pressure in the congenic strains showed that the SHR-18, SHR-18A, SHR-18B but not SHR-18C congenic intervals contains gene(s) for increased blood pressure in the SHR strain (Figure 1). Some or all of these SHR hypertension genes

are therefore contained within the minimal 11.6Mbp congenic interval of SHR-18B. We showed that bilaterally nephrectomised SHR rats transplanted with an SHR-18 kidney have significantly lower blood pressure than bilaterally nephrectomised SHR rats transplanted with an SHR kidney: the lower blood pressures observed in SHR animals with a transplanted SHR-18 kidney were similar to the lower blood pressures in the nontransplanted SHR-18 rats, thus providing evidence that the gene underlying the blood pressure QTL is expressed within the 18–20 week old SHR-18 kidney. In contrast, the transplanted SHR-18 kidney had no effect on salt sensitivity. The simple interpretation would be that the transplanted SHR-18 congenic kidney with intrarenally expressed chromosome 18 BN allele(s) is not sufficient for promoting salt-resistance in the SHR recipient. However, it should be noted that the transplantation procedure interrupts renal innervation and it is possible that under other experimental circumstances, for example, in the setting of intact renal nerves, that the differential segment of chromosome 18 might affect salt sensitivity by way of gene effects inside the kidney.

Microarray expression profiling in the kidney revealed a single transcript, represented by probe set rc_AA957526_at, that was differentially expressed between the SHR and BN progenitor strains, and also between the SHR and SHR-18 strains and that mapped within the blood pressure QTL region defined by the SHR-18B congenic subline. The rat 1.8kb extended sequence from this probe set did not have an open reading frame with homology to mammalian proteins, but showed strong conservation across species suggesting a possible functional significance of this transcript. *In silico* analyses using the RNAz web server[12] found one highly conserved small RNA. Northern blotting confirmed a small RNA transcribed from the same strand of RNA interrogated by the microarray. These data suggest that this locus encodes a small non-protein coding RNA that could mediate a novel mechanism for blood pressure regulation.

Solexa-based sequencing has the potential to provide a set of differentially expressed transcripts which is unbiased because, unlike microarrays, it is not reliant upon genome annotations or the presence of previously detected ESTs. These analyses of the kidney transcriptome identified five tags that mapped to the SHR-18B congenic interval that showed differential expression (tag abundance ratio ≥ 1.5, $p<0.05$) in SHR-18 and SHR-18B when compared to SHR (Table S4). Directional differential expression of the three tags in or close to Acetyl-CoA acyltransferase (*Acaa2*), Ras-related protein Rab-27B (*Rab27b*) and an annotated pseudogene (*LOC689364*) were confirmed by qRT-PCR (Figure S3) and may therefore be considered as attractive candidate genes for the chromosome 18 SHR blood pressure QTLs.

It is worth noting that the Solexa tags for these three genes were not represented on the Affymetrix microarray showing the value of the unbiased approach of Solexa sequencing compared to microarrays. However, the Solexa-based sequencing did not identify the putative non-protein coding RNA that was previously found to be differentially expressed between SHR and BN,[22] possibly due to the low number of tags which was found to be similar in all three strains.

It has been reported that the expression of *Acaa2* was significantly decreased in the kidneys of SS-18^{BN} consomic rats compared to SS controls using microarrays and qRT-PCR.[21] These results are consistent with the Solexa-based analysis when SHR-18 and SHR-18B rats showed significantly decreased *Acaa2* expression when compared to the SHR. Renal *Acaa2* expression is *cis*-regulated in the BXH/HXB recombinant inbred strains[22] and is non-significantly correlated to systolic blood pressure (r=0.29, P=0.13) (http://www.genenetwork.org/). Together these findings suggest that *Acaa2* is a promosing candidate for blood pressure regulation.

Because the gene(s) underlying the blood pressure QTL may not be dysregulated at the RNA level, but may vary in activity due to coding variants between BN and SHR, we sequenced selected genes within the SHR-18B congenic interval as an additional approach to prioritising and characterising candidate genes. We focused our attention on genes that may be indirectly involved in blood pressure regulation through their known and annotated functions relating to metabolic or regulatory processes. The only coding variant found in our selected set of genes was in *Tcf4*, where at amino acid 662, alanine was changed to a threonine in SHR. The significance of this coding variant in *Tcf4* is unclear because the affected codon, although conserved in mouse, dog and human, is not within an annotated functional domain according to InterPro. Point mutations, deletions and insertions in human *Tcf4*, some creating premature stop codons have been identified in humans diagnosed with the Pitt-Hopkins syndrome;[23–25] although hypertension is not a recognised feature of this syndrome. *Tcf4* has been shown to play an important role in vascular remodelling and in kidney development[26,27] and therefore could be putatively involved in blood pressure regulation. Whilst this sequence analysis is not a comprehensive study of all the protein-coding genes in the minimal congenic interval, it has prioritised one gene for further study over several other potential biological candidates.

Several genetic studies of human increased blood pressure have shown linkage or association to genes or regions that are syntenic to the SHR-18B congenic interval.[4,28–31] The human orthologues of *Acaa2*, *Rab27b*, *Tcf4* and the genomic region encoding the putative non-protein coding RNA map within 10Mbp of peak linkages for systolic blood pressure in two family-based studies[28,29] and for pulse and systolic blood pressure from the Family Blood Pressure Program.[30] These genes should therefore be considered attractive candidate genes for these three studies. However, they map more than 10Mbp from the peak of linkage markers for hypertension loci identified in a genome wide association study[4], a population based linkage study[31] and in the Framingham Heart Study.[32] Precise identification of the SHR hypertension QTL genes isolated in the congenic strains in this study may therefore be informative for elucidation of the linkages reported in these human studies.

PERSPECTIVES

These congenic studies have shown that the blood pressure and salt sensitivity QTLs previously reported on SHR chromosome 18 are encoded by separate genes on separate chromosomal segments. Kidney transplantation studies suggest that selective expression of allelic variants from chromosome 18 of the BN rat inside the SHR kidney is sufficient to

attenuate increased blood pressure but not salt-sensitivity. Renal gene expression analyses and sequencing of genes trapped within the blood pressure QTL region have identified five candidate genes which have not been previously implicated in hypertension. Further study of these genes will focus on detailed functional testing of these genes as determinants of the blood pressure QTLs in the SHR strain and of linkages to the corresponding regions of the human genome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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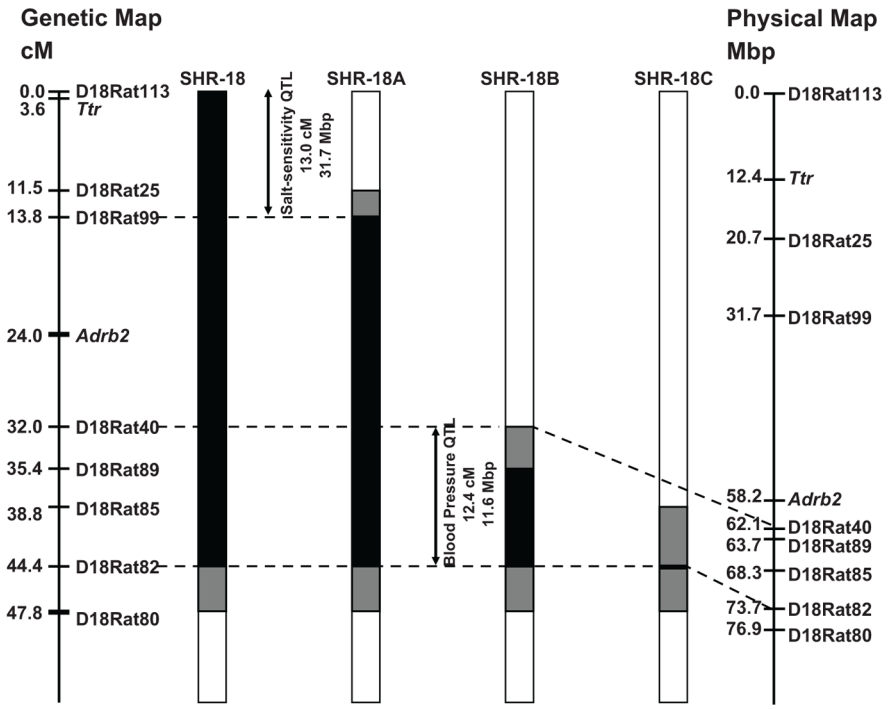

Figure 1.

Genetic and physical map showing the transferred segment of chromosome 18 in the SHR-18 congenic strain, and in the SHR-18A, SHR-18B and the SHR-18C congenic sublines. Map distances in centiMorgans (cM) and in Megabasepairs (Mbp) are based on the SHRSP x BN genetic map (http://rgd.mcw.edu/) and RGSC3.4, Ensembl release 45 respectively. Black bars represent the chromosomal region transferred from the BN strain, white bars from the SHR background and grey bars represent the recombination zones. The dashed lines represent the limits of the salt sensitivity and blood pressure regulatory regions.

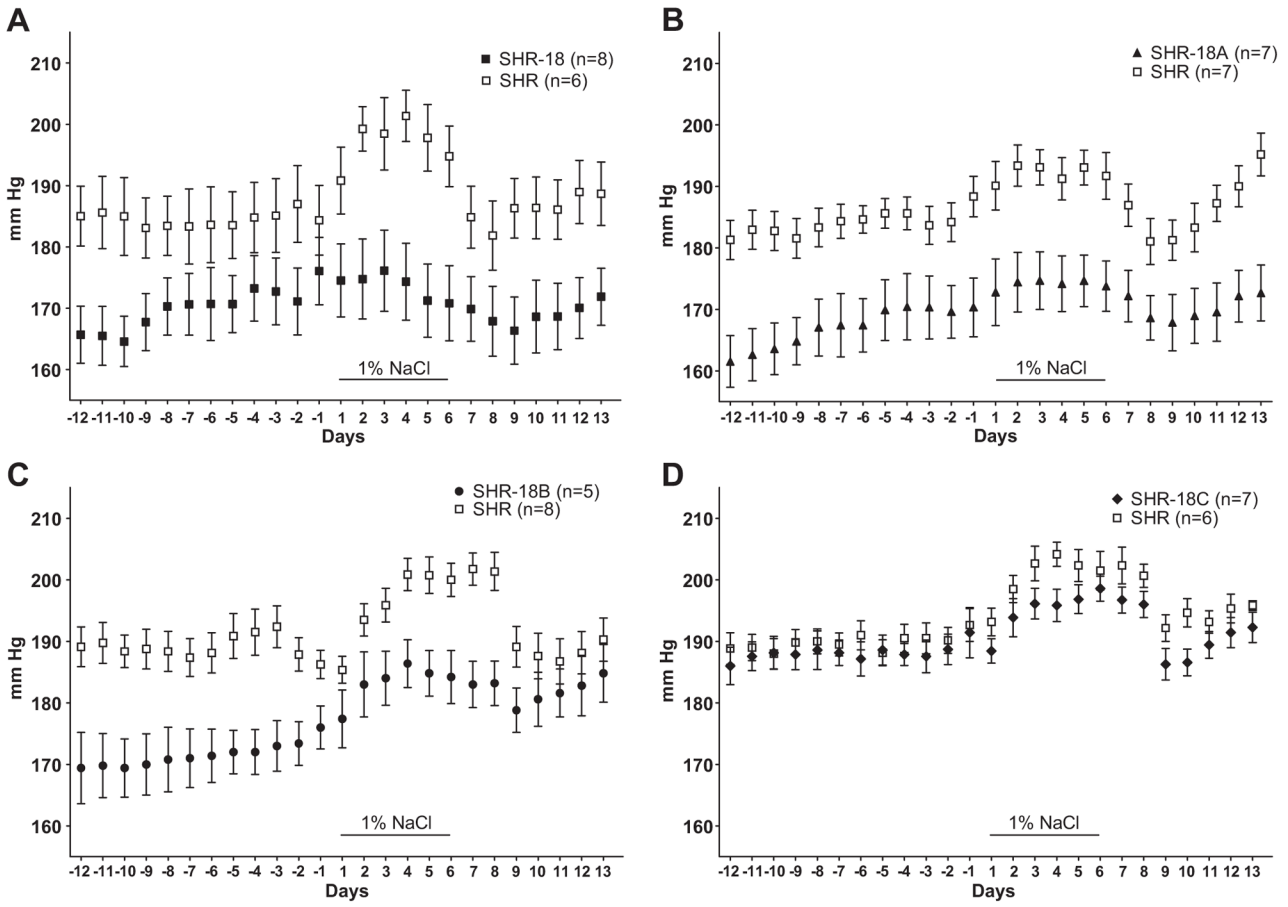

Figure 2.

Systolic blood pressure measured by radiotelemetry in (a) 15–17 week old SHR and SHR-18, (b) 19–21 week old SHR and SHR-18A, (c) 15–17 week old SHR and SHR-18B, (d) 15–17 week old SHR and SHR-18C strains. Baseline systolic blood pressure was significantly reduced in SHR-18 ($F_{1,12}$=4.84; P=0.048), SHR-18A ($F_{1,12}$=10.17; P=0.008) and SHR-18B ($F_{1,11}$=12.6; P=0.005). No significant reduction in systolic blood pressure was obtained in SHR-18C ($F_{1,11}$=0.27; P=0.61) strain vs SHR. All results according to main effect F values of two way repeated measures ANOVA are reported. Salt-loaded systolic blood pressure was significantly raised in all SHR and congenic strains SHR-18A ($F_{1,6}$ =27.7; P= 0.002), SHR-18B ($F_{1,4}$ =57.3; P= 0.0016) and SHR-18C ($F_{1,6}$ =10.3; P= 0.019) with the exception of SHR-18: SHR-18 ($F_{1,7}$ =0.88; P= 0.38). All F values computed for contrast comparison in two-way ANOVA. The baseline period is from −12 to −2 days and salt administration from day 1 as indicated. Values displayed are means ± SEM.

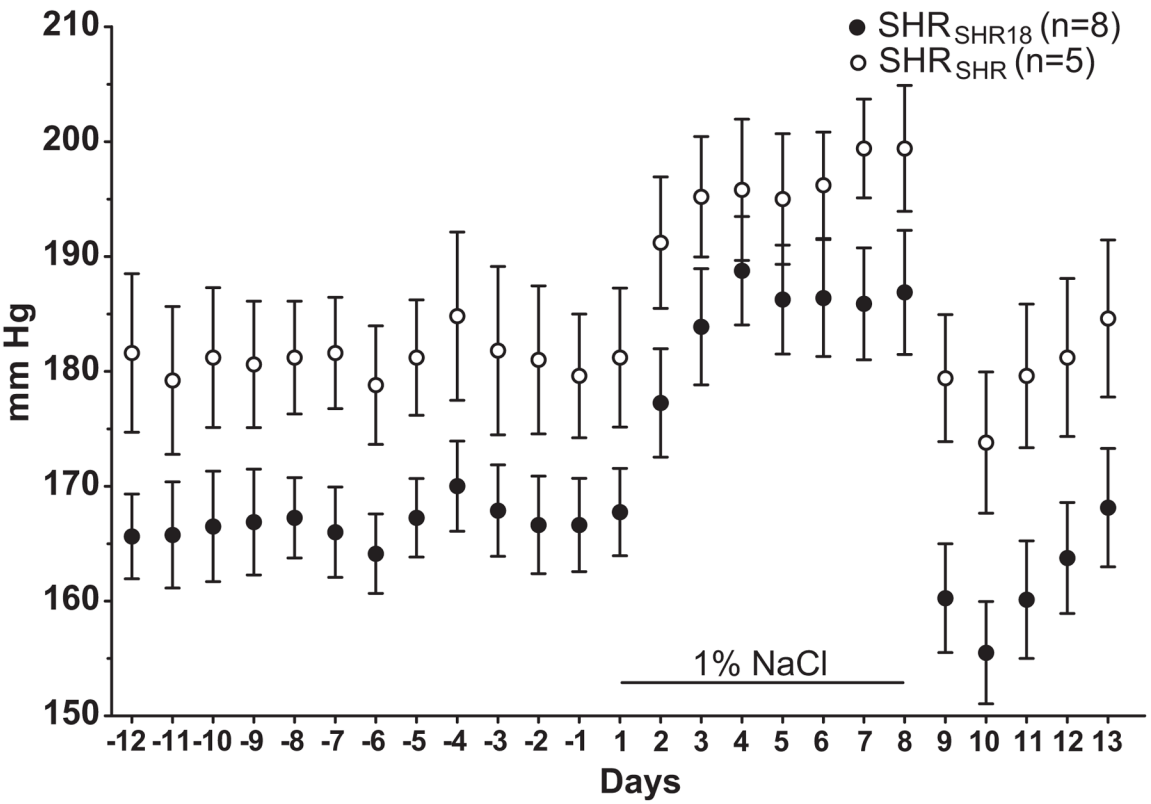

Figure 3.

Transplant recipients' systolic blood pressure during normal and high salt intake (1% NaCl as drinking water) measured by radiotelemetry at the age of 18–20 weeks. Reduction in baseline systolic blood pressure near the significance level was achieved in SHR_{SHR18} ($F_{1,11}$=4.50; P=0.057) vs SHR_{SHR} transplanted rats. All results according to main effect F values of two way repeated measures ANOVA. Salt-loaded systolic blood pressure was significantly raised in $SHR_{SHR\text{-}18}$ (F=101.6; P<0.0001). All F values computed for contrast comparison in two-way ANOVA. The baseline and salt administration period is indicated as in Figure 2.

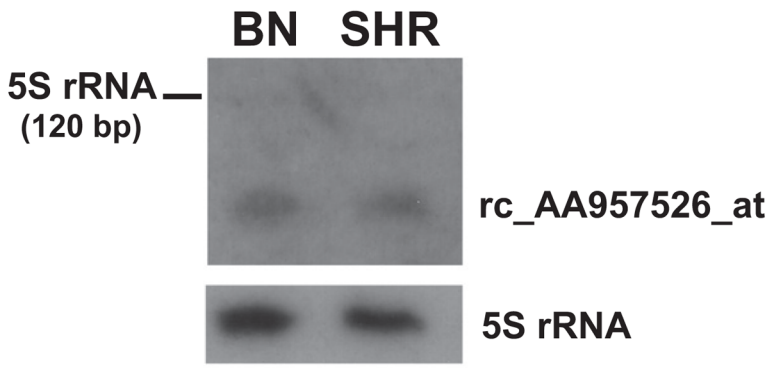

Figure 4.

Northern blot of SHR and BN kidney small RNA, probed with an antisense oligonucleotide derived from the extended RACE product for Affymetrix probe set rc_AA957526_at rat sequence with 5S rRNA as a loading control.