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QTL mapping for traits associated with stress neuroendocrine reactivity in rats

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Abstract

In the present study we searched for quantitative trait loci (QTLs) that affect neuroendocrine stress responses in a 20-min restraint stress paradigm using Brown–Norway (BN) and Wistar–Kyoto–Hyperactive (WKHA) rats. These strains differed in their hypothalamic–pituitary–adrenal axis (plasma ACTH and corticosterone levels, thymus, and adrenal weights) and in their renin–angiotensin–aldosterone system reactivity (plasma renin activity, aldosterone concentration). We performed a whole-genome scan on a F_2 progeny derived from a WKHA × BN intercross, which led to the identification of several QTLs linked to plasma renin activity (*Sr6*, *Sr8*, *Sr11*, *and Sr12* on chromosomes RNO2, 3, 19, and 8, respectively), plasma aldosterone concentration (*Sr7* and *Sr9* on RNO2 and 5, respectively), and thymus weight (*Sr10*, *Sr13*, *and Srl4* on RNO5, 10, and 16, respectively). The type 1b angiotensin II receptor gene (*Agtrlb*) maps within the confidence intervals of QTLs on RNO2 linked to plasma renin activity (*Sr6*, highly significant; LOD = 5.0) and to plasma aldosterone level (*Sr7*, suggestive; LOD = 2.0). *In vitro* studies of angiotensin II–induced release of aldosterone by adrenal glomerulosa cells revealed a lower receptor potency (log EC_{50} = −8.16 ± 0.11 M) and efficiency (E_{max} = 453.3 ± 25.9 pg/3 × 10^4 cells/24 h) in BN than in WKHA (log EC_{50} = −10.66 ± 0.18 M; E_{max} = 573.1 ± 15.3 pg/3 × 10^4 cells/24 h). Moreover, differences in *Agtr1b* mRNA abundance and sequence reinforce the putative role of the *Agtr1b* gene in the differential plasma renin stress reactivity between the two rat strains.

Introduction

The hypothalamic–pituitary–adrenal (HPA) axis (Selye 1973) and the sympathoadrenal system (Cannon 1935) are the primary neuroendocrine systems that react to acute stress, providing the energy necessary for behavioral adaptations to environmental challenges. Stress responses are highly controlled to restore homeostasis, and abnormal loss of this control has been implicated in the etiology and the maintenance of a wide range of physiologic or psychologic disorders in humans as well as in farm animals (Ehlert et al. 2001; Mormède et al. 2002). Other systems involved in the preservation of homeostasis can play a role during stress reactivity. For example, immobilization stress paradigms in rats are associated with increases in plasma renin activity and aldosterone levels (Sigg et al. 1978; Sowers et al. 1981; Paris et al. 1987; Golin et al. 1988; Sarrieau et al. 1998). Indeed, several interactions have been described between the renin–angiotensin–aldosterone system (RAAS) and HPA and sympathoadrenal stress responses.

Genetic variability has been reported for the HPA axis, sympathoadrenal system, or RAAS reactions to stress in rats (McCart et al. 1984; Armario et al. 1995; Courvoisier et al. 1996; Gomez et al. 1996; Sarrieau et al. 1998; Marissal–Arvy et al. 1999) and in humans (Inglis et al. 1999; Williams et al. 1993). Since neuroendocrine stress responses are consid-

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ered complex traits (Steckler 2001; Mormède et al. 2002), it remains quite difficult to distinguish between the molecular and the environmental effects. Genetic analyses in animal models allow one to dissect complex traits into simpler components (Lander and Schork 1994), and quantitative trait locus (QTL) mapping is a top–down strategy that has become very fruitful during the past decade (Altmuller et al. 2001; Glazier et al. 2002; Korstanje and Paigen 2002). In particular, QTL mapping has permitted detection of genomic regions that affect behavioral (Moisan et al. 1996, 2003; Ramos et al. 1999; Fernandez-Teruel et al. 2002; Ahmadiyeh et al. 2003) or endocrine (Dumas et al. 2000; Cui et al. 2003) stress responses in rats.

In previous studies, we reported functional differences in HPA axis activity and reactivity between the inbred rat strains BN and F344. In particular, the BN rat displays a different diurnal pattern of plasma corticosterone levels, a faster recovery after restraint stress, adrenals of larger size but less reactive to ACTH, a greater efficiency of glucocorticoid receptor, and an apparent insensitivity to adrenalectomy. For the latter trait we showed that this is a result of a Y73C substitution in the BN mineralocorticoid receptor. In addition, QTL associated with glucocorticoid receptor efficiency were detected in a F_2 cross between BN and F344 strains (Marissal–Arvy et al. 2004). In order to identify other genes associated with stress neuroendocrine reactivity, we looked for genomic loci involved in the HPA axis and RAAS reactivities to acute restraint stress in Brown–Norway (BN) rats and a new rat strain, Wistar–Kyoto–HyperActive (WKHA) that we previously characterized for stress reactivity (Castanon et al. 1993). Using a F_2 progeny derived from BN and WKHA parental strains, we identified several QTLs linked to neuroendocrine stress responses.

Materials and methods

Animals. A total of 239 rats of both sexes (38 BN, 51 WKHA, 4 Wistar, 24 F_1, and 122 F_2) were used in this study. BN/OrlIco and Wistar rats were provided by IFFA Credo (L'Arbresle, France) and WKHA rats were from inbred WKHA/Bx rats bred in the laboratory since 1994 and initially provided by E. Hendley (University of Vermont, Burlington, VT) (Hendley and Ohlsson 1991). BN and WKHA rats were crossed in order to generate the F_1 progeny. Animals from the F_1 population were then mated to generate the F_2 progeny. When bred in the lab, rats were weaned and separated by sex at 28 days of age. All animals were housed four per cage with food and water *ad libitum* in an animal quarter maintained at 22 ± 1°C with a 12:12 h light/dark cycle (lights on at 07:00 h). All the restraint stress experiments were conducted when rats were 15 weeks old, between 09:00 h and 12:00 h. Studies of the AT_{1B} receptor were performed on 12-week-old male rats in the morning. Animal studies were conducted in accordance with the institutional guidelines.

Restraint stress procedure. Basal endocrine levels in the parental BN and WKHA strains were studied in eight rats from each strain and sex. They were housed for two weeks as described above before blood was collected.

For poststress endocrine levels, animals (14 BN, 27 WKHA, 24 F_1, and 122 F_2) were weighed and then restrained for 20 min in plastic bag restrainers with breathing holes (Harvard Apparatus, Baling, Courtaboeuf, France), as previously reported (Sarrieau et al. 1998). Rats were then killed by decapitation. Trunk blood was collected into chilled tubes coated with a 10% EDTA solution and centrifuged (4500*g*, 4°C, 15 min) and plasma was stored at −20°C for subsequent measurement of ACTH, corticosterone, aldosterone, and plasma renin activity.

For all animals, adrenal (ADR) and thymus (THY) glands were removed and weighed in order to estimate structural differences of HPA regulation between strains. A piece of lung was removed from BN, WKHA, and F_2 rats and stored at −80°C for subsequent DNA extraction.

Analytical methods. Plasma ACTH was measured by an immunoradiometric assay using a commercial kit (Nichols Institute Diagnostic, San Juan Capistrano, CA). Plasma corticosterone levels (CORT) were determined by radiocompetitive protein binding following extraction with absolute ethanol, using ^{3}H-corticosterone as the radioligand and transcortin from rhesus monkey plasma as the binding protein as previously described (Sarrieau et al. 1998). Aldosterone concentration in plasma (ALDO) was determined by radioimmunoassay, the specificity characteristics of which have been previously reported (Leboulenger et al. 1982). Plasma renin activity (PRA) was assayed with a commercial radioimmunoassay kit measuring the amount of angiotensin I generated *in vitro* (REN-CT2, CIS Bio International, Saclay, France).

Genotyping. DNA extraction and purification were performed as previously described (Sambrook et al. 1989). Initially, all animals were genotyped with 100 polymorphic microsatellite markers dis-

tributed throughout the whole genome at 20-cM intervals on average as predicted by the RGD database (http://rgd.mcw.edu/GENOMESCANNER/). They were chosen so that alleles differ by at least 8 bp between BN and WKHA strains. Following a first genome scan, the total number of markers was increased to 128 to better cover some large map intervals remaining from the first genotyping and to improve the precision of the localization of significant QTLs. Oligonucleotides for rat microsatellite markers were synthesized by Eurogentec (Seraing, Belgium). Genotypes were determined by polymerase chain reaction (PCR) as previously described (Moisan et al. 1996). Alleles were visualized on ethidium bromide–stained 3% agarose gels (Kalys, France).

Sequencing of the AT_{1B} receptor DNA. DNA was extracted from lung using the Wizard extraction kit (Promega, France). Nine overlapping PCR fragments were obtained from genomic DNA of each strain using oligonucleotides derived from the GenBank rat *Agtr1b* sequence (gi: 546904): AT1bPro1 (Fwd: 5′-ttgacatgaattttccacttacg-3′; Rev: 5′-agaagcggcctggaagtaat-3′), AT1bPro2 (Fwd: 5′-ggggtgaggtgcaagagtta-3′; Rev: 5′-gctagctcccccttttaga-3′, AT1bPro3 (Fwd: 5′-ccacagctgcactgtcaaat-3′; Rev: 5′-atccttccccacaatcttcc-3′; AT1b5′UTR (Fwd: 5′-tcccccaaagactaaagttgaa-3′; Rev: 5′-ggggcagtcatcttggatt-3′), AT1b1 (Fwd: 5′-tgttttctccaggtgcattt-3′; Rev: 5′-gcggaggcgagacttcatt-3′), AT1b2 (Fwd: 5′-tgccagcgtcagtttcaat-3′ Rev: 5′-gataatgcccagctgaatga-3′), AT1b3 (Fwd: 5′-cacgccaagaaatgatgaca-3′; Rev: 5′-aaggattgctggaggttgaa-3′), AT1b3′UTR1 (Fwd: 5′-caaagcctgcaagtgaagtg-3′ Rev: 5′-gtgtcaggaacaatggtgtca-3′) ATlb3′UTR2 (Fwd: 5′-caaaaggagatgggaggtca-3′; Rev: 5′-tgctacaacttcaatcaaacaaca-3′). Sequencing was done by Genomex (Grenoble, France).

Quantitative real-time PCR. Reverse transcription was performed using 2 μg of RNA from adrenal glands of WKHA and BN animals, according to the Superscript II protocol (Invitrogen). The quantitative real-time PCR assay was based on primers that specifically amplify *Agtr1b* (Fwd: 5′-gcttgaaagaagcccagagc-3′; Rev: 5′-aagggtcatgtctcccttgg-3′) and *S16* (Fwd: 5′-aggagcgatttgctggtgtgg-3′; Rev: 5′-gctacca gggcctttgagatg-3′) genes and spanned one intronic region to detect contamination by genomic DNA. *S16* was used as a housekeeping gene. Quantitative real-time PCR was conducted with an initial denaturation step (95°C, 15 min) and 40 cycles of amplification (94°C for 30 sec, 58°C for 45 sec, and 72°C for 30 sec), using the Quantitect SYBR Green PCR Kit (Qiagen) and a final 0.5 μM concentration of primers. Each sample was run in duplicate. Amplification products were visualized on ethidium bromide–stained agarose gels (1%) to check the fragment length and the absence of nonspecific products (not shown). A melting curve analysis was also performed to check the absence of primer dimers (not shown). Results are given as the ratio between the *Agtr1b* and the *S16* relative quantities.

In vitro studies of AT_{1B} receptor. Adrenal glands were removed from male rats (8 BN, 8 WKHA, and 4 Wistar rats as controls). Preparation of glomerulosa cell cultures was performed as previously described (Lenglet et al. 2002). Briefly, the adrenal capsules containing the glomerulosa cells were separated from the inner zones and washed in DMEM medium (Sigma, St. Louis, MO, USA) supplemented with 1% of the antimycotic/antibiotic solution. The tissues were gently stirred for 30 min at 37°C in culture medium containing collagenase (1 mg/ml) and deoxyribonuclease I (25 μg/ml) (Sigma) in a 95% O_2/5% CO_2 atmosphere. The tissues were dissociated by gentle aspiration with a sterile 10-ml pipette, and dispersed cells were filtered on a nylon sieve (100-μm mesh opening). The cells were harvested by centrifugation (100*g* for 10 min), and the pellet was suspended in DMEM medium supplemented with 5 mg/ml insulin, 10 mg/ml transferrin, 10 mM L-ascorbic acid, and 5% fetal bovine serum. The residual tissues were subjected to a second period of digestion/dispersion as described above. Glomerulosa cells were plated in 15-mm 24-well culture dishes (500 μl medium/well) at a density of 3×10^4 cells/well and grown at 37°C in a humidified incubator with an atmosphere of 95% O_2/5% CO_2. After 24 h of culture, cells were stimulated by 10 nM ACTH, 10 mM KCl, or 100 pM to 1 μM angiotensin II (Ang II) for BN and WKHA, and only by 100 pM to 1 μM Ang II for Wistar. Aldosterone concentration was determined by radioimmunoassay without prior extraction, in 100–200-μl aliquots for each cell culture medium, as previously described (Lenglet et al. 2002).

Statistical analysis. All values are given as means ± SEM. Phenotypic data for parental strains and F_1 animals were compared by two-way ANOVA, with strain (or cross) and sex as between-subjects factors. All variables were characterized by a normal distribution. Significant ANOVA were followed by Tukey's multiple comparison tests.

Data for *in vitro* studies were compared by two-way ANOVA, with strain and stimulation as between-subjects factors. All variables were characterized by a normal distribution. Significant

ANOVA were followed by Tukey's multiple comparison tests. The concentration–response curves were fitted using the Prism program (GraphPad Software, Inc., San Diego, CA) and maximum response (E_{max}) and EC_{50} were derived from this analysis. For the F_2 progeny, genotypic data were first analyzed with Map Manager QTX software, developed and adapted by Dr.K.F. Manly (Manly et al. 2001), in order to construct a complete linkage map (in centimorgan, cM) containing all the microsatellite markers used herein. Phenotypic variables were characterized by a normal distribution except for plasma renin activity which has been log-normalized, and adjustment for sex (*Z*-score) was made for variables that show differences with gender but no sex and strain interaction by two-way ANOVA. QTL analysis was conducted on the whole population (122 rats) and also for each sex separately (59 male and 63 female rats) using Map Manager QTX. First, we did a whole-genome scan in each population with no assumption about the mode of heritability to calculate LOD scores and genome-wide LOD thresholds. When a QTL was at least suggestive, phenotypic data were grouped according to the genotype of the closest marker and analyzed by one-way ANOVA to determine the mode of inheritance (additive, BN dominant, or BN recessive). LOD scores and genome-wide LOD thresholds were then recalculated on the basis of the appropriate model of inheritance. QTL linkage was evaluated by single-interval mapping using the Haley–Knott regression method (Haley and Knott 1992) implemented by the software. Genome-wide LOD thresholds for each trait were calculated by permutation tests (1000 permutations with $p = 0.01$) (Churchill and Doerge 1994) and were labeled as ''suggestive'' (37th percentile), ''significant'' (95th percentile), or ''highly significant'' (99.9th percentile) according to Lander and Kruglyak (1995). Confidence intervals (CI) were estimated by bootstrap analysis (Visscher et al. 1996; Walling et al. 1998) instead of the classic 1-LOD support interval (Lander and Botstein 1989), since it has been shown to be more reliable over all QTL strengths (Darvasi and Soller 1997).

Results

Basal endocrine levels. BN and WKHA strains differ in a number of basal endocrine levels (Fig. 1): BN females show higher levels of ACTH (by 42%), corticosterone (by 55%), and renin activity (by 44%) compared with WKHA females, but renin activity is 34% lower in BN males compared with WKHA males and aldosterone is 25% higher in BN females but 92% lower in BN males compared with WKHA rats of the same sex. Within-strain sex differences were found for all parameters, females having higher levels than males.

Neuroendocrine stress responses. As expected all hormonal levels as well as plasma renin activity were strongly increased after the 20-min restraint stress period compared with basal levels (Fig. 2). Compared with BN, poststress ACTH (35%) and corticosterone (49%) levels were higher in WKHA females and in males for corticosterone (38%). Conversely, poststress aldosterone levels were higher in BN females (49%) and no significant differences were detected between males. Poststress plasma renin activity was higher in WKHA males but not significantly different in females.

Taking in to consideration the values obtained in nonstress animals (Fig. 1), it appears that the WKHA rats have a higher ACTH and corticosterone response to stress but BN rats display a higher response for aldosterone levels, especially males. Responses of plasma renin levels to stress appear higher in WKHA rats, in females in particular.

Comparisons of the F_1 generation with the parental strains suggested that BN alleles have a dominant effect on poststress corticosterone and ACTH levels (at least in females). Conversely, WKHA alleles are dominant for aldosterone levels and for plasma renin activity, but only in males. As for plasma renin activity in females, the inheritance appears to be codominant (Fig. 2).

In both experiments WKHA rats have a lower thymus relative weight compared with that of BN, which may reflect the higher corticosterone response to stress of this strain. Adrenal gland relative weight is either slightly higher in BN rats or not different between strains, depending on the experiment (Figs. 1 and 2).

QTL analysis of the whole population. One hundred twenty-eight microsatellites markers were used to genotype the F_2 population. Markers were mapped as predicted by the RGD database (http://rgd.mcw.edu), with a mean genetic distance of 17.7 ± 0.9 cM. We used the nomenclature from the RGD as a guide to naming stress response–related QTLs (to date they range from *Sr1* to *Sr5*). The model-based genome scan for the whole population led to the identification of two significant QTLs linked to plasma renin activity and named *Sr6* and *Sr12* (Table 1). *Sr6* is a highly significant BN dominant locus (LOD = 5.0) on RNO2, whereas *Sr12* (LOD = 3.7, RNO8) is BN recessive. A suggestive

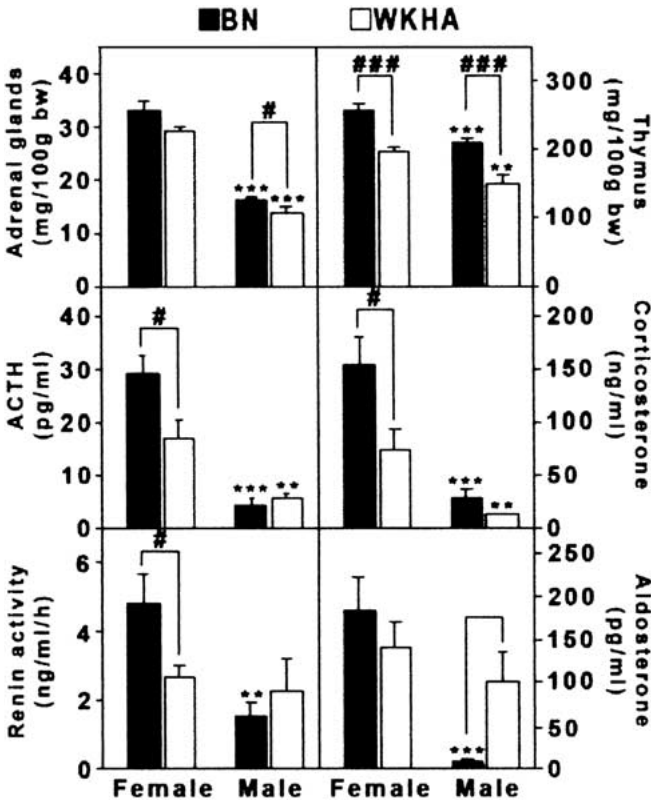

Fig. 1. Basal neuroendocrine levels for BN and WKHA rats. Results are given as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for differences between sexes within strains. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for differences between strains, b.w.-body weight.

QTL was linked in a BN dominant fashion to aldosterone levels (*Sr7*, LOD = 2.0) on RNO2, and its CI (37–60 cM) strongly overlapped with the CI of *Sr6* (48–62 cM) (Table 1, Fig. 3A). The gene encoding the type 1b angiotensin II receptor (*Agtr1b*) maps in the CI of both *Sr6* and *Sr7* with D2Mit17 as the closest marker (4 Mb from *Agtr1b*).

QTL analysis of the male and female populations. As we observed strong within-strains sex differences for several neuroendocrine phenotypes, we performed a QTL analysis on male and female progenies separately to detect putative autosomal sex-specific loci. *Sr6*, *Sr7*, and *Sr12* were detected in both males and females but with a weaker LOD score than the one calculated for the whole population. Thus, these QTLs were not considered sex-specific. The model-based genome scan for the female population led to the identification of only one female-specific QTL, linked to thymus weight on RNO5 (*Sr10*, LOD = 2.9; Table 1) with a BN recessive mode of inheritance (Table 1). In the male population we identified five autosomal male-specific loci. We found that a locus strongly linked to aldosterone levels (*Sr9*, LOD = 4.3) in a BN dominant fashion mapped in the same region as the female-specific *Sr10* QTL (Table 1, Fig. 3B). In addition, two QTLs were found linked to plasma renin activity (*Sr8* and *Sr11*). *Sr8* is a robust BN recessive locus (LOD = 4.0) on RNO3 that had been initially detected in the whole-population genome scan before we found that the contribution of the female progeny to this QTL was minor or null (Fig. 2B). *Sr11* is also a BN recessive QTL (LOD = 3.6) on RNO19. Finally, two male-specific QTLs were found linked to thymus weight (*Sr13* and *Sr14*). *Sr13*, located on RNO10, is a BN recessive locus (LOD = 3.3), whereas *Sr14* is an additive QTL (LOD = 2.8) on RNO16 (Table 1). Details concerning confidence intervals of the QTL as well as models of inheritance can be found in Table 3.

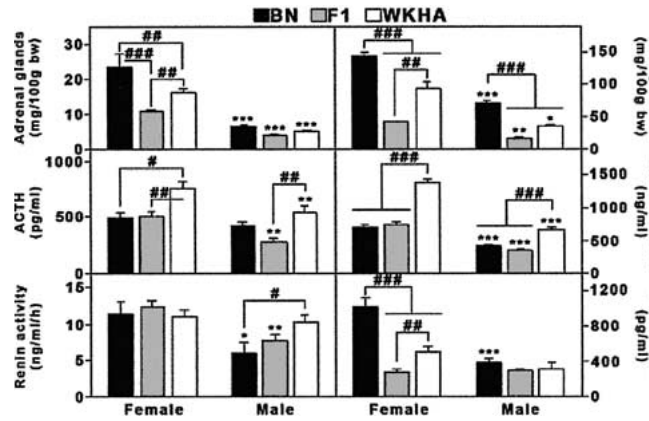

Fig. 2. Neuroendocrine parameters measured after a 20-min restraint stress. Bar graphs for parental strains BN and WKHA (HA) and the F_1 progeny show mean values ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. females. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for differences between parental strains.

***Analysis of AT_{1B} receptor gene as a positional candidate for QTLs* Sr6 *and* Sr7.** The 5′-flanking region (1374 bp) and the two exons of the AT_{1B} receptor gene were sequenced from BN and WKHA parental genomic DNA. We did not detect any differences in the coding region of *Agtr1b* between the two rat strains. In contrast, we found two SNPs in the 5′-flanking region (positions -840 and -431), and five SNPs in the 3′-UTR (positions 1375, 1376, 1481, 1609, and 2062) (Table 2). Although none of these SNPs maps to a known consensus site for transcriptional regulation, we looked at putative differences in *Agtr1b* mRNA quantities between BN and WKHA strains in adrenal glands. As shown in Fig. 4, we found 3.5 times more *Agtr1b* mRNA in WKHA than in BN male rats.

Table 1. Significant QTLs identified in the genome scan

QTL	*Pop*[a]	*Trait*	*Peak marker*	*Chr*	*LOD*	*WKHA/WKHA*[b]	*WKHA/BN*[b]	*BN/BN*[b]
Sr6	m + f	Renin[c]	D2Rat180	2	5.0	$17.7 \pm 1.2^{***\dagger\dagger\dagger}$	11.0 ± 0.7	11.6 ± 0.9
Sr7	m + f	Aldo[d]	D2Rat277	2	2.0	$1675 \pm 148^{*\dagger}$	1222 ± 80	1235 ± 93
Sr8	m + f	Renin	D3Mit3	3	3.2	$11.2 \pm 1.1^{**}$	$12.2 \pm 0.8^{***}$	$16.0 \pm 1.0^{\dagger\dagger\dagger}$
Sr8	m	Renin	mD2Mit78	3	4.0	$8.9 \pm 0.8^{**}$	$9.1 \pm 0.6^{***}$	$16.2 \pm 1.4^{\dagger\dagger\dagger}$
Sr9	m	Aldo	D5Rat6	5	4.3	$1459 \pm 118^{**\dagger\dagger}$	877 ± 77	623 ± 153
Sr10	f	Thymus[e]	D5Rat6	5	2.9	$62.0 \pm 4.8^{**}$	$63.3 \pm 2.1^{***}$	$82.9 \pm 4.7^{\dagger\dagger\dagger}$
Sr11	m	Renin	D19Rat71	19	3.6	$13.8 \pm 1.4^{***}$	$13.1 \pm 0.8^{***}$	$7.3 \pm 0.9^{\dagger\dagger\dagger}$
Sr12	m + f	Renin	ACPH	8	3.7	$13.4 \pm 0.9^{*}$	$13.2 \pm 1.0^{*}$	$9.8 \pm 0.9^{\dagger}$
Sr13	m	Thymus	D10Rat71	10	3.3	$38.1 \pm 1.8^{***}$	$37.3 \pm 2.0^{**}$	$26.2 \pm 2.6^{\dagger\dagger}$
Sr14	m	Thymus	D16Rat66	16	2.8	$28.1 \pm 2.5^{**\dagger}$	$34.9 \pm 1.7^{*}$	$42.2 \pm 2.5^{\dagger}$

[a]Population used for the linkage analysis (m + f for the whole population, m or f for the male or the female progeny, respectively).
[b]Mean phenotypic value ± SEM for rats homozygous for the WKHA allele (WKHA/WKHA) heterozygous (WKHA/BN) and homozygous (BN/BN) for the BN allele.
[c]Plasma renin activity (ng/ml/h).
[d]Plasma aldosterone (pg/ml)
[e]Thymus weight over body weight (mg/100 g body weight).
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. BN/BN.
$^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. WKHA/BN.

Because the AT_{1B} receptor mediates the aldosterone secretory response to angiotensin II during stress (Armando et al. 2001), we studied the aldosterone secretory response to different factors in male BN or WKHA adrenal glomerulosa cells in culture (Fig. 5A). Male Wistar rats were used as control. Basal levels of aldosterone secretion were not different in cultures from BN and WKHA rats. High concentrations of ACTH (10 nM) or KCl (10 mM) were used as controls for secretion capacity of glomerulosa cells, and both factors strongly stimulated aldosterone secretion with the same amplitude in both strains. On the other hand, a large difference ($p < 6.7 \times 10^{-3}$) was observed between BN and WKHA rats for the total secretion of aldosterone when stimulated by a high concentration (1 μM) of Ang II.

In dose–response experiments, aldosterone secretion was measured for a 5-log range of Ang II concentration (Fig. 5B). In WKHA, the response was the same as in Wistar (same EC_{50}, no difference for E_{max}). In BN the response was reduced with a 2.5-log lower EC_{50} than in Wistar and in WKHA, and a significantly lower E_{max} ($p < 3.0 \times 10^{-3}$ when compared to Wistar, $p < 0.05$ when compared to WKHA). Therefore, these results suggest that AT_{1B} receptor is less sensitive to Ang II stimulation and less efficient to activate the secretion of aldosterone in BN than in WKHA glomelurosa cells *in vitro*.

Discussion

Neuroendocrine stress reactions are the outcome of complex interactions between a large gene network and environmental factors. Therefore, QTL mapping is a relevant way to explore genetic variability because one can consider the whole genome as a candidate for the presence of polymorphic loci, explaining the variability of complex quantitative traits. A few studies have been carried out on behavioral (Moisan et al. 1996, 2003; Ramos et al. 1999; Fernandez–Teruel et al. 2002; Ahmadiyeh et al. 2003) or endocrine (Dumas et al. 2000; Cui et al. 2003) stress responses in rats.

Three QTLs associated with thymus relative weight (*Sr10*, *Sr13*, *Sr14*) were detected. Thymus contains only glucocorticoid receptors on which corticosterone acts to induce cellular apoptosis in this gland. WKHA rats display a higher corticosterone response to stress and accordingly have a smaller thymus weight. Interestingly, males and females do not share the same locus. No genomic locus linked to HPA axis-specific parameters, such as ACTH, corticosterone levels, or adrenal weight, was identified despite strong differences between parental strains. One possible explanation is that the genetic determinism for these traits is multigenic with a weak effect of individual genes on the phenotypes, at least in these models. Indeed, only a suggestive QTL has been reported for late corticosterone levels in a progeny of 188 F_2 (F344 × LEW) male rats (Potenza et al. 2004). Another explanation may be that the end of the 20-min restraint period was not ideal because it has been reported that the strongest differences of corticosterone responses to the same stress paradigm were observed during the phase of recovery after stress (Sarrieau and Mormede 1998).

However, several QTL related to the renin–angiotensin–aldosterone system were identified in this study. First, *Sr6*, associated with renin activity,

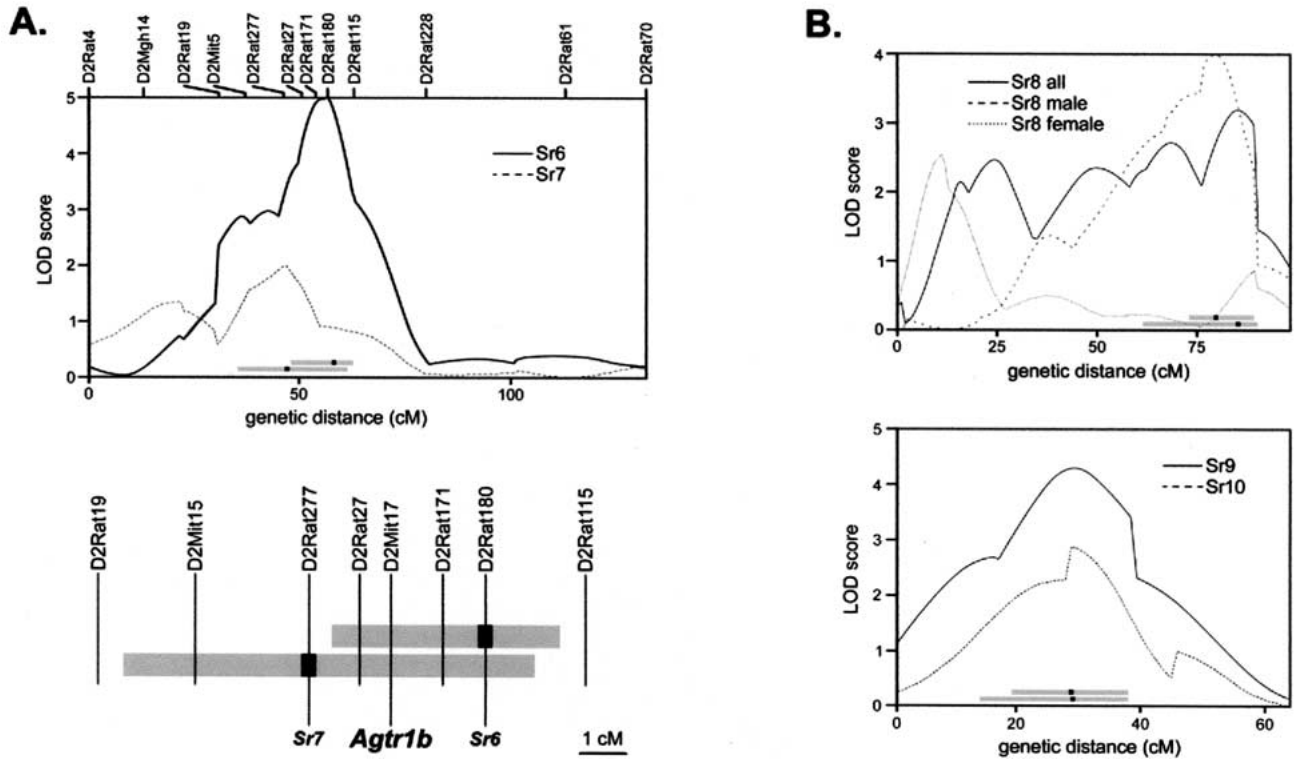

Fig. 3. Mapping of *Agtr1b*. (**A**) *Agtr1b* is localized in the overlap of the confidence intervals of *Sr6* and *Sr7* on RNO2. *Top*: The plain line represents the LOD-score curve for *Sr6* across RNO2 (highly significant LOD threshold = 4.4); the dashed line represents the LOD-score curve for *Sr7* (suggestive LOD threshold = 1.6). Gray bars represent confidence intervals calculated by bootstrap analysis, with the black square at the peak position of each QTL. Marker map is shown at the top of the graph. *Bottom*: Enlargement of the overlapping region of *Sr6* and *Sr7*. *D2Mit17*, the nearest marker from *Agtrlb*, is mapped between *D2Rat27* and *D2Rat171* in the overlap of confidence intervals of *Sr6* and *Sr7*. (**B**) Plain, dashed, and dotted lines represent the LOD score curves along the chromosome for each QTL as indicated in the graph legends. Gray bars represent confidence intervals calculated by bootstrap analysis, with the black square at the peak position of each significant QTL. *Top*: *Sr8* on RNO3 is male-specific. Significant LOD threshold = 2.9 for the whole population; significant LOD threshold = 2.9 for males; suggestive LOD threshold =1.5 for females. *Bottom*: *Sr9* and *Sr10* perfectly overlap on RNO5. Significant LOD threshold = 2.9 for *Sr9*; significant LOD threshold = 2.8 for *Sr10*.

Table 2. SNPs identified by direct sequencing in BN- and WKHA-like *Agtr1b*

SNP	*WKHA*	*BN*
−840	G	A
−431	A	C
1375	C	T
1376	A	G
1481	A	C
1609	A	G
2062	T	G

The position for each SNP is given from the first nucleotide of the first exon. The intron is not taken in to consideration.

and *Sr7*, associated with aldosterone levels, strongly overlapped on the long arm of RNO2, close to the type 1b Ang II receptor (AT_{1B}) gene (*Agtrlb*). AT_{1B} is expressed in the zona glomerulosa of rat adrenal gland (Gasc et al. 1994; Johren et al. 2003), which suggests a role in the stress response in rat (Armando et al. 2001; Leong et al. 2002; Johren et al. 2003). Indeed, at least three independent studies support the hypothesis that stimulation of aldosterone synthesis by Ang II during stress is mediated through AT_{1B} receptor activation (Balla et al. 1991; Gigante et al. 1997; Armando et al. 2001). Moreover, it is well known that renin synthesis and activation are regulated by Ang II and aldosterone, and some studies show that the AT_{1B} receptor is present in the rat kidney (Kakar et al. 1992; Gasc et al. 1994; Lenkei et al. 1998; Armando et al. 2001), where renin is synthesized. Thus, given its chromosomal localization and its biological function, *Agtr1b* was a good positional candidate to investigate.

By direct sequencing we detected several polymorphisms in *Agtr1b* between the two strains. All the SNPs were localized in the 5′-flanking region or in the 3′-UTR of the gene, meaning that the protein structure was not affected. However, this result

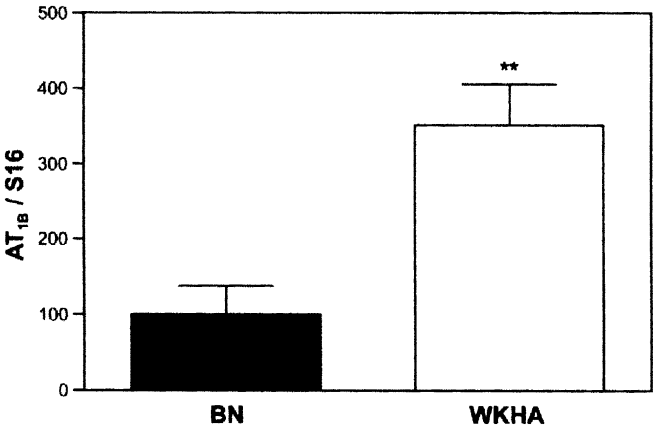

Fig. 4. *Agtr1b* mRNA expression in adrenal glands of BN and WKHA male rats (n = 8 for each strain). Results are given as the ratio between the *Agtr1b* and the housekeeping gene S16 relative quantities of mRNA.

suggested a difference in the regulation of the transcription of the gene or the half-life of the mRNA. This was confirmed by estimating *Agtrlb* mRNA abundance in each strain that appears lower in BN rats. Moreover, using *in vitro* studies of adrenal zona glomerulosa cells, we found that the AT_{1B} receptor displayed functional differences in BN compared with WKHA and Wistar rats. Indeed, Ang II was both less potent and less efficient in BN than in WKHA, whereas stimulation of aldosterone secretion by other specific factors (i.e., ACTH and KCl) was the same in both strains, showing that the general functioning of adrenal glomerulosa cells did not differ between the strains. The differences in mRNA abundance and sensitivity of adrenal AT_{1B} receptor to Ang II in BN compared with those in WKHA rats strongly support the hypothesis that *Agtr1b* is potentially the causal gene for *Sr6* and *Sr7*. Indeed, the WKHA allele is the high allele for both QTLs. Poststress plasma renin levels are also higher in the WKHA parental strain, which fits with *Sr6* QTL data. However, WKHA poststress aldosterone levels are lower than in BN parental strains. This may be explained by the fact that aldosterone levels are regulated by many factors, *Sr7* reflecting only the regulation via the AT_{1B} receptor. In an independent study, a QTL associated with left ventricular mass has been detected in a region overlapping with *Sr6* and *Sr7* in a SHR × DRY F_2 cross (Innes et al. 1998). This QTL was recently replicated in a SHR × F344 F_2 cross (Di Nicolantonio et al. 2003) and fine-mapped around the *Agtr1b* gene, which is also a good candidate for this trait. Very interestingly, the WKHA strain derives from a SHR × WKY F_2 cross and has retained the trait of left ventricular hypertrophy from SHR rats (Hendley and Ohlsson 1991). Although further investigations are required to prove it, it may well be that the WKHA *Agtr1b* allele is responsible for both poststress renin activity and cardiac hypertrophy.

Second, *Sr11* male-specific QTL associated with renin activity, has been mapped on the 19q12 band of RNO19, close to the mineralocorticoid receptor (MR) rat gene (*Nr3c2*). We have recently reported a mutation in the MR of the BN rat (Marissal–Arvy et al. 2004) which is strongly linked to the insensitivity to adrenalectomy previously described in this strain (Marissal–Arvy et al. 2000). Interestingly, the genome-wide linkage analysis for sensitivity to adrenalectomy performed in the same study showed that the *Nr3c2* locus is involved in a stronger manner in the male progeny. This may be related to the male specificity of *Sr11* reported herein. Moreover, another recent study demonstrated an increase of the renin gene expression by aldosterone in juxtaglomerular cells, and this genomic effect was mediated through the MR (Klar et al. 2004). Therefore, we consider *Nr3c2* a good candidate gene for *Sr11*.

Table 3. Significant QTLs identified in the genome scan

QTL	*Chr*	*Pop*	*Trait*	*Pos (cM)*	*CI (cM)*	*LOD*	*Model*	*High allele*
Sr6	2	m + f	Renin	56	48–62	5.0	Dominant	WKHA
Sr7	2	m + f	Aldo	47	37–60	2.0	Dominant	WKHA
Sr8	3	m + f	Renin	85	63–89	3.2	Recessive	BN
Sr8	3	m	Renin	69	60–79	4.0	Recessive	BN
Sr9	5	m	Aldo	26	19–38	4.3	Dominant	WKHA
Sr10	5	f	Thymus	28	14–38	2.9	Recessive	BN
Sr11	19	m	Renin	24	14–27	3.6	Recessive	WKHA
Sr12	8	m + f	Renin	15	0–31	3.7	Recessive	WKHA
Sr13	10	m	Thymus	19	18–37	3.3	Recessive	WKHA
Sr14	16	m	Thymus	31	19–49	2.8	Additive	BN

Pop: population used for the linkage analysis (m + f for the whole population, m or f for the male or the female progeny respectively); Pos: genetic position of the QTL peak (in cM); Model: model of inheritance used to perform the linkage analysis (the BN allele is considered for dominance/recessivity); High allele: allele of the peak nearest marker associated with the highest phenotypic value; Renin: plasma renin activity; Aldo: plasma aldosterone; Thymus: thymus weight over body weight.

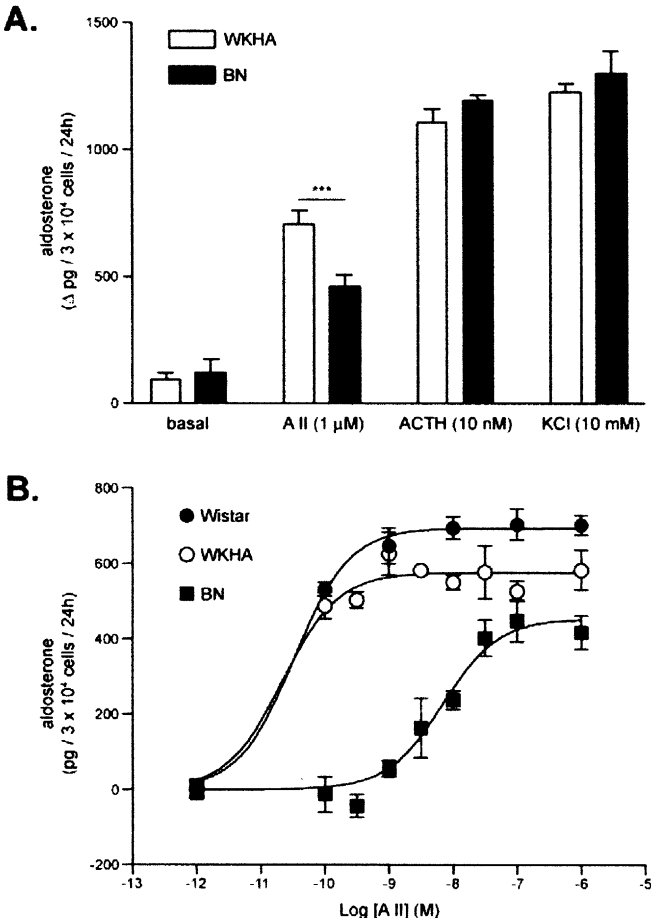

Fig. 5. AT_{1B} is less potent and less efficient in BN than in Wistar and WKHA rats. All the experiments were performed on adrenal glomerulosa cell cultures from Wistar, BN, and WKHA rats. (**A**) Total secretion of aldosterone stimulated by different factors. Results are given as mean + SEM. $***p < 0.001$. (**B**) Concentration–response curves for the induced secretion of aldosterone after different stimulations by Ang II. Values for log EC_{50} are −10.48 ± 0.07 M, −10.66 ± 0.18 M, and −8.16 ± 0.11 M and values for E_{max} are 694.1 ± 10.75 pg/3 × 10^4 cells/24h, 573.1 ± 15.3 pg/3 × 10^4 cells/24 h, and 453.3 ± 25.9 pg/3 × 10^4 cells/24 h for Wistar, WKHA, and BN, respectively.

Third, *Sr9* and *Sr10* perfectly overlapped on the 5q22 band of RNO5 close to two aquaporin isoform genes (*Aqp3* and *Aqp7*). Even though these two QTLs were not linked to the same trait, there is strong evidence for the implication of aquaporins in both aldosterone regulation and thymocyte apoptosis. The expression of AQP3 and AQP7 has been clearly demonstrated in kidney [AQP3 in collecting duct and AQP7 in proximal tubule (Matsuzaki et al. 2002)] and in immature dendritic cells (de Baey and Lanzavecchia 2000). Moreover, it has been reported that the expression of *Aqp3* is enhanced by water deprivation and correlates with an increase of aldosterone secretion (Murillo–Carretero et al. 1999). Finally, aquaporins have been shown to play a crucial role in the rate of thymocyte apoptosis, especially during the primary event of apoptotic volume decrease (Jablonski et al. 2004). Therefore, we consider *Aqp3* (and maybe *Aqp7*) a good candidate gene for *Sr9* and *Sr10*.

Interestingly, the BN strain used herein (BN/OrlIco) has a constitutive activation of MR mechanisms regulating the hydroelectrolytic balance at the level of the kidney and/or the brain (Marissal–Arvy et al. 1999, 2000, 2004), and the additive effects of the MR locus and a QTL on RNO4 strongly determine most of the MR-related phenotypes in BN rats (Marissal–Arvy et al. 2004). Moreover, the BN strain shows elevated levels of angiotensin I-converting enzyme (ACE) compared with various other rat strains (Challah et al. 1998) linked to allelic variants of the ACE gene. Here, we show that AT_{1B} receptors are less potent and efficient in BN rats, resulting in a decreased adrenal sensitivity to Ang II. Altogether, these atypical features of the main regulators of water and salt homeostasis in BN rats pave the way for interesting pathophysiological studies in hypertension research.

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