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Gain of Function Mutation in the Mineralocorticoid Receptor of the Brown Norway Rat*

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The aim of this research was to identify the molecular bases of differences in sensitivity to corticosteroid hormones between Brown Norway and Fischer 344 rats. We previously showed an apparent insensitivity to adrenalectomy in Brown Norway rats. Based on our first hypothesis of a different activity/reactivity of the mineralocorticoid signaling pathway between the two rat strains, we sequenced Brown Norway and Fischer 344 mineralocorticoid receptor cDNA and identified a tyrosine to cysteine substitution (Y73C) in the N-terminal part of the Brown Norway mineralocorticoid receptor. As a first step, this substitution gave us a means to distinguish the Brown Norway allele from the Fischer 344 at the mineralocorticoid receptor locus in an F2 population. We showed a strong genetic linkage between the mineralocorticoid receptor genotype and sensitivity to adrenalectomy. A subsequent genome-wide linkage analysis confirmed the involvement of the mineralocorticoid receptor locus and implicated other loci, including one on chromosome 4, which collectively explain a large part of the strain differences in corticosteroid receptor responses. *In vitro* studies further revealed that the Y73C substitution induces greater transactivation of the mineralocorticoid receptor by aldosterone, and surprisingly by progesterone as well, which could substitute for aldosterone after adrenalectomy in Brown Norway rats. We challenged this hypothesis *in vivo* and showed that plasma progesterone is higher in Brown Norway male rats and partially compensates for aldosterone after adrenalectomy. This work illustrates the interest of a pluristrategic approach to explore the mineralocorticoid receptor signaling pathway and its implication in the regulation of hydroelectrolytic homeostasis and blood pressure.

Adrenal steroids act through two receptor subtypes: the mineralocorticoid receptor (MR),[1] which exhibits a high affinity for aldosterone, deoxycorticosterone (DOC), and endogenous glucocorticoids (K_d = 0.5–1.0 nM), and the glucocorticoid receptor (GR), which binds cortisol and corticosterone with a lower affinity (K_d = 2.5–5.0 nM) than synthetic agonists like dexamethasone or RU28362 (K_d = 0.5–1.0 nM). Like other nuclear receptors, MR and GR bind to *cis*-acting DNA elements in the regulatory regions of target genes (1). The MR is located predominately in sodium-transporting epithelia and in the limbic system. It is involved in the maintenance of blood pressure (2) and brain function (3). The GR has a widespread distribution and is involved in almost all organic functions, including carbohydrate and lipid metabolism, modulation of immune responses, and behavior (4–6). Both receptor types are also involved in the control of hypothalamic-pituitary-adrenal axis activity and reactivity to stress (7). Numerous diseases, such as hypertension, autoimmunity, obesity, mood, and behavioral disorders, are associated with disturbed corticosteroid secretion or action (8–10). Vulnerability to such dysfunctions shows high interindividual variation, and the involvement of genetic factors in this variability has been demonstrated by family and twin studies in humans and by the comparison of inbred strains and selection experiments in animals (11).

In the present work, we focused on two inbred rat strains, Brown Norway (BN) and Fischer 344 (F344), shown to display different corticotropic axis activity and reactivity (12, 13). To unravel the network of interactions between the components of the hypothalamic-pituitary-adrenal axis, BN and F344 rats were treated with adrenalectomy (ADX) to remove the feedback regulation exerted by corticosteroids (14, 15). As classically described, ADX induced weight loss and markedly increased saline intake in F344 rats. It also led to a sustained increase in urinary Na^+/K^+ ratio in these rats.[2] Conversely, in the BN rat, ADX induced no weight loss on the first week and even increased the growth rate on the third week (14). ADX did not alter saline and food intake and induced only very transient effects on the Na^+/K^+ excretions, suggesting a ligand-independent MR activation in BN rat. A treatment with DOC had no effect in BN rats, whereas it restored saline intake of the F344 ADX rats to control levels (15). Alternatively, a treatment with the GR-specific agonist RU28362 induced greater weight loss, thymus involution, and decrease in food intake and plasma transcortin concentration in BN than in F344 rats, suggesting a greater efficiency of GR activity in BN rats (15). This apparent insensitivity to ADX and the greater GR efficiency of the BN rat were observed in F1 hybrid F344xBN rats, indicating dominance of the BN allele(s) on these traits (16).

We thus aimed to identify the molecular bases of such dif-

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[1] The abbreviations used are: MR, mineralocorticoid receptor; DOC, deoxycorticosterone; GR, glucocorticoid receptor; BN, Brown Norway; F344, Fischer 344; ADX, adrenalectomy; SD, Sprague-Dawley; QTL, quantitative trait loci; CTX, castration; ANOVA, analysis of variance; LOD, logarithm of the odds; VE, variance; STAT, signal transducers and activators of transcription.

[2] N. Marissal-Arvy and P. Mormède, submitted for publication.

ferences in corticosteroid receptor function between BN and F344 rats. In the present work, sequencing, associated to a linkage study of a BNxF344 F2 population and followed by an *in vitro* study, allowed us to prove the implication of the MR in these differences.

EXPERIMENTAL PROCEDURES

Animals

Experiments were made in accordance with the principles and guidelines of the French legislation on animal welfare: Journal Official number 87-848. The BN and F344 rats were purchased from Charles River/IFFA Credo (L'Arbresle, France). F1 hybrids were obtained by crossbreeding BN with F344 rats, and then F1 were bred inter se to obtain the F2 population, of which 132 males and 95 females were studied. All of the rats were housed in a temperature-controlled room (23 ± 1 °C) with a light/dark cycle of 12/12 h (lights on at 0700 h). Food and saline were provided *ad libitum*.

MR cDNA Sequencing

Because our first hypothesis was a different activity/reactivity of the mineralocorticoid signaling pathway between the BN and F344 rat strains, we sequenced MR cDNA in both strains. The coding sequence of BN and F344 MR was determined by reverse transcription-PCR method, using total RNA extracted from 100 mg of fresh kidney with guanidinium thiocyanate, followed by centrifugation onto a cushion of cesium chloride, as described by Glisin *et al.* (17).

RNA (5 μg) was denatured at 65 °C for 5 min and was added to a 20-μl volume of 1× reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$) containing 10 pmol of antisense primer, 1 mM dNTP, 10 mM dithiothreitol, and 20 units of RNasin (Promega). The reaction was conducted for 1 h at 42 °C with 200 units of Moloney murine leukemia virus enzyme (Promega) added in two doses of 100 units separated by 30 min. RNA was reverse transcribed from 12 different MR cDNA specific primers (Invitrogen), based on the sequence of the Sprague-Dawley (SD) MR cDNA (18) (GenBank™ accession number M36074): 5′-CAGGGTATCTGCACTGTCGCTCTAT-3′ (position −193 from the first ATG), 3′-TGGCAAAATCCCAGACCGA-5′ (255), 5′-CAAAGGCTACCACAGTCTCCCTGAA-3′ (8), 3′-TCACCAGCTGCTCCATGTTTTGA-5′ (423), 5′-TCGGTCTGGGATTTTGCCAT-3′ (236), 3′-AGGACATGGAGTTGATGCCCA-5′ (642), 5′-TTGCGTGCCATCGTGAAGA-3′ (528), 3′-TTGTTGAGATTTGCCGGGCT-5′ (913), 5′-AGCCCCACACATGCGAGCAA-3′ (747), 3′-AGCTACCATCAAAGCCGGGCA-5′ (1452), 5′-GGAAACAGCAAAATCAGCCCCA-3′ (1239), 3′-AGGTCACCGTGTGGTTTCCATGA-5′ (1699), 5′-GTGCCCGGCTTTGATGGTAG-3′ (1431), 3′-GTTGCCCTTCCACGGCTCTT-5′ (1905), 5′-GGTGTGAATTCGGGTGGACA-3′ (1545), 3′-GCAGGACAGTTCTTTCGCCG-5′ (1972), 5′-CTTCTTCAAAAGAGCCGTGGAAG-3′ (1877), 3′-TGGATCATCTGTTTCGCTGCCA-5′ (2326), 5′-ATTACGCATGCACTCACACCATC-3′ (2178), 3′-TGGCTCTTGAGGCCATCTTTTG-5′ (2656), 5′-GCCAACTCCTCTATTTTGCTCCAGA-3′ (2460), 3′-CGACCAACTGTCAACTCAGCCATCA-5′ (3060), 5′-ATGCGCCAGATCAGCCTTCAAT-3′ (2544), and 3′-GCTCCAGACCCTTGACGTGATTT-5′ (3101). The amplifications were carried out in a 50-μl reaction volume by combining 5 μl of reverse transcription product with 10 pmol of each primer, 200 μM dNTP, and 1 unit of TaqDNA polymerase (Promega) in 1× PCR buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% Triton X-100, and 1.5 mM $MgCl_2$). PCR products were purified with a QIAquick PCR purification kit (Qiagen). Sequencing was carried out and checked on two rats of each strain and on both strands by Genome Express (Grenoble, France).

Phenotyping of the F2 BNxF344 Population

F2 BNxF344 rats (n = 227) were submitted at 6–7 weeks of age to the phenotypic measurements previously described as the most discriminant between BN and F344 inbred strains (15): weight loss, food and saline intake in response to ADX or to treatments with MR or GR-specific ligands (DOC or RU28362, respectively) after ADX, and adrenal weight. Bilateral ADX was performed after a control period of 6 days. During the first 10 days, the rats were provided with 0.9% saline. Then they were given 0.9% saline containing 5 μg/ml of DOC as drinking fluid during 10 days. After a 6-day period of wash-out with saline only, the rats were submitted to a treatment with RU28362 in saline at 5 μg/ml during 10 days, followed by another 6-day wash-out period with saline only. Then all of the rats were killed by decapitation. Trunk blood was collected into chilled tubes coated with a 10% EDTA solution and centrifuged (4,500 × g, 15 min, 4 °C), plasma was stored at −80 °C. Plasma corticosterone concentrations were determined as previously described (13) and were below assay detection limits.

Genotyping of the F2 BNxF344 Population

Restriction Polymorphism—The genomic DNA from F2 rats was extracted as classically described (19). Sequencing revealed a digestion site (restriction fragment-length polymorphism) that allowed us to distinguish the BN MR allele from the F344 MR one in the F2 population. It was localized at the middle of the DNA fragment obtained by amplification with the sense primer 3′-CAAAGGCTACCACAGTCTCCCTGAA-5′ and the antisense primer 3′-TCACCAGCTGCTCCATGTTTTGA-5′ (416 bp for the F344 and 209 + 207 bp for the BN allele). PCRs were made as described above, in a 50-μl reaction volume. BsaMI (20 units) was directly added to the PCR mix that was incubated for 1 h at 65 °C, and digestion products were visualized on ethidium bromide-stained 1% agarose gel.

Genome Scan—To localize the quantitative trait loci (QTL) implicated in MR- and GR-related traits, a genome scan of the F2 population was made with 100 microsatellite markers (Genosys or Eurogentec) selected for their polymorphism between BN and F344 strains (*www.rgd.mcw.edu*) and by covering evenly the whole genome. BsaMI RLFP was added to microsatellite markers of chromosome 19. PCRs were performed as previously, in a 20-μl reaction volume by combining 50 ng of genomic DNA with 5 pmol of each primer, 200 μM dNTP, and 0.4 unit of TaqDNA polymerase (Promega) in 1× PCR buffer. The alleles were visualized on ethidium bromide-stained 3% agarose gel.

Functional Implications of the Y73C Substitution in Vitro

To assess the functional outcome of the Y73C substitution revealed by sequencing, we investigated transactivation properties of BN and F344 MR by transient transfection assays.

MR Expression Vectors—The expression vector pcDNA3-rMR was constructed from the pGEM3-rMR plasmid given generously by Dr. P. D. Patel (University of Michigan Medical School) and containing the coding sequence of the SD rMR (18). A 3.5-kb KpnI-Eco47III fragment was excised and subcloned into the KpnI-EcoRV site of an expression vector pcDNA3 (Invitrogen). The mutations revealed by the sequencing were obtained by site-directed oligonucleotide mutagenesis by Cybergene (Genopole, Evry, France) and were verified on both strands by direct sequencing.

Cell Culture and Transfections—Rabbit RCSV3 cells (provided by Dr. P. Ronco, Hôpital Tenon, Paris, France) were grown in a defined medium composed of Dulbecco's modified Eagle's medium/Ham's F-12 supplemented as previously described (20). They were co-transfected by the calcium phosphate method (Profection kit; Promega) with the plasmid pcDNA3-rMR of BN or F344 sequence, murine mammary tumor virus-luciferase reporter construct (pFC31Luc, a gift from Dr. H Richard-Foy, CNRS, Toulouse, France), and a plasmid encoding for β-galactosidase (pSVβgal; Clontech Laboratories, Inc.) as an internal control for transfection efficiencies. The day after transfection, the cells were rinsed with phosphate-buffered saline, and steroids (Sigma) were added for 24 h. Finally, the cells were washed twice with cold phosphate-buffered saline and lysed, and the transfection products were analyzed as previously described (20). Results were standardized for transfection efficiency and expressed as the ratio of luciferase activity over β-galactosidase activity in fold induction compared with MR activation in absence of treatment. BN and F344 MR transactivations were compared with increasing doses of aldosterone (10^{-12}–10^{-7} M) or progesterone (10^{-10}–10^{-5} M).

Role of Progesterone in the Insensitivity of BN Rats to ADX in Vivo

To test the hypothesis of a protection against ADX by progesterone in BN rats, the effects of ADX, castration (CTX), and the combined surgery on body weight gain and on food and saline intakes were compared between 6-week-old BN and F344 males (n = 8–10 rats/group). Control rats (n = 8/strain) were treated with sham ADX and sham CTX. Blood samples were collected by tail nicks from half the rats of each group 1, 2, 3, and 7 days after surgery and by decapitation 14 days after surgery. Plasma concentrations of progesterone were measured with a RIA kit (CisBio, Schering, France).

Data Analysis

For the QTL search, the physiological data were expressed as percentages of variation compared with the last day of control values for the effects of ADX on body weight and on food and saline intakes (on the 10th day after surgery) and compared with the last day of ADX values

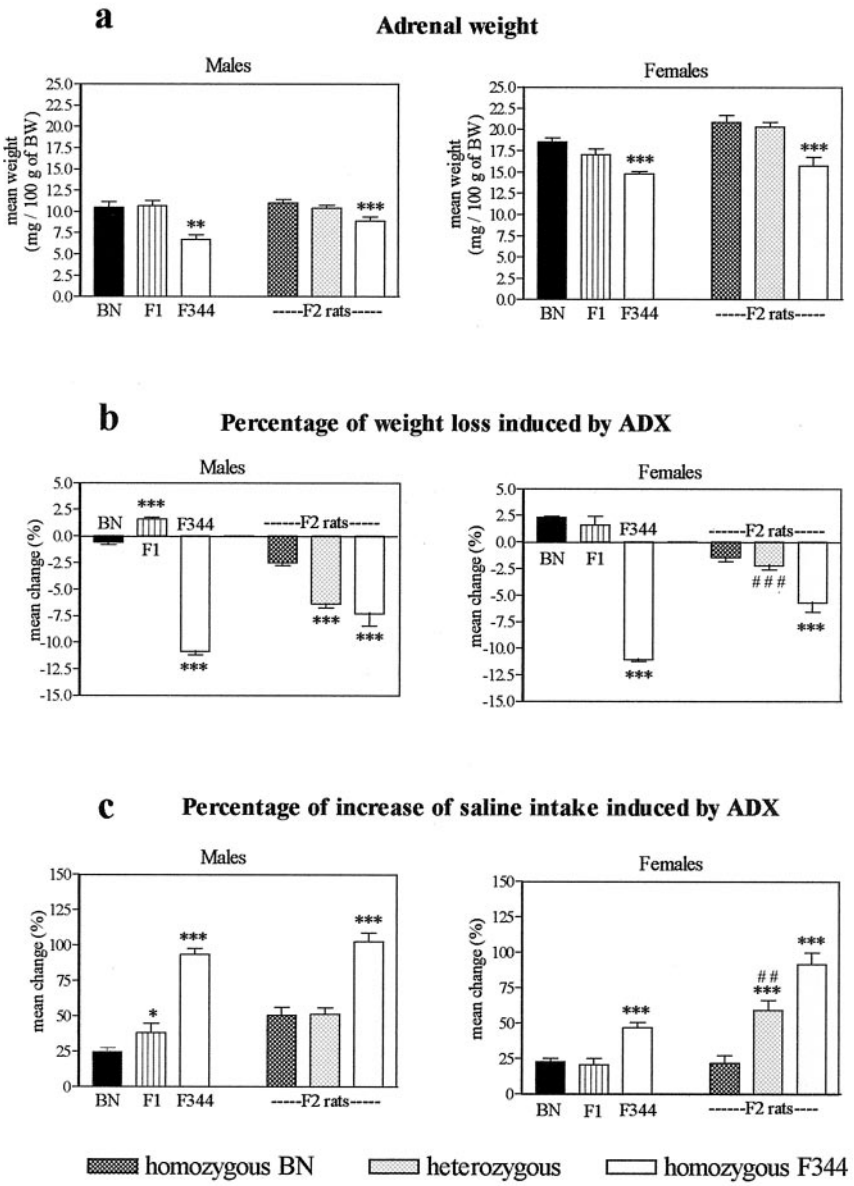

FIG. 1. **Phenotypes of the F2 rats grouped according to their MR genotype in rats homozygous for the BN allele (36 males and 33 females), rats homozygous for the F344 allele (33 males and 20 females), and heterozygous (61 males and 41 females).** *a*, adrenal weight. *b*, weight loss. *c*, increase in saline intake, 10 days after ADX. The data for inbred rats were extracted from our previous work (13, 15). Differences from the homozygous rats for the BN MR allele were: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Differences from the homozygous rats for the F344 MR allele were: ##, $p < 0.01$; ###, $p < 0.001$.

for the effects of DOC and RU28362 on these parameters (on the 10th day of treatment). For each marker, the data were submitted to a two-way ANOVA with sex and allele as two between subject factors. Linkage analysis was made separately on males and females when ANOVA showed a significant sex-allele interaction ($p < 0.05$). Linkage analysis was performed with MapManager (21). Linkage was considered as suggestive when the logarithm of the odds (LOD) score was higher than 2.8 and significant when the LOD score was higher than 4.3 (22). Genes localized in the QTL regions were found on web sites (ratmap.gen.su.se/ and rgd.mcw.edu).

For the other studies, the data were submitted to a two-way ANOVA with strain and treatment as two between subject factors. Post-hoc Newman-Keuls tests were performed when the ANOVA was significant ($p < 0.05$). Kinetic constants of the transfection study were calculated by means of Prism software (GraphPad, San Diego, CA).

RESULTS

Sequencing Reveals a Y73C Substitution in the N-terminal Part of the BN MR

The sequencing showed three polymorphisms between BN, F344, and SD MR (18): the first in codon 73 from TAC in SD and F344 rats to TGC in BN rats, leading to a tyrosine to cysteine substitution, the second in codon 221 from GGT in SD rats to GGC in BN and F344 rats (no amino acid substitution), and the third in codon 487 from GAC in SD rats to GGC in BN and F344 rats (asparagine to glycine substitution). The single nucleotide polymorphism in codon 73 disclosed a digestion site for the BsaMI enzyme in the BN MR gene that provided a means to test a potential linkage between the MR locus and sensitivity to ADX in F2 BNxF344 rats.

F2 Rats Bearing the BN MR Locus Are Partly Protected against the Effects of ADX

The F2 rats were submitted to the phenotypic measurements the most discriminant between BN and F344 strains (15). At first, the BsaMI restriction site was used to distinguish the BN and F344 MR alleles in the F2 population.

F2 females had heavier adrenals than males (19.53 ± 0.51 mg/100 g *versus* 10.19 ± 0.24 mg/100 g of body weight; $p < 0.0001$). As seen in pure BN and F344 rats (13), F2 rats homozygous for the BN MR allele showed heavier adrenals than rats homozygous for the F344 allele (Fig. 1*a*). As with F1 BNxF344 hybrids (16) and in agreement with a dominance effect of the BN locus, heterozygous F2 rats showed the same adrenal weights as rats homozygous for the BN MR allele.

Fig. 1*b* shows that ADX induced a greater weight loss in male than in female F2 rats (−5.55 ± 0.39% *versus* −2.68 ± 0.34%; $p < 0.0001$). In both sexes, rats homozygous for the

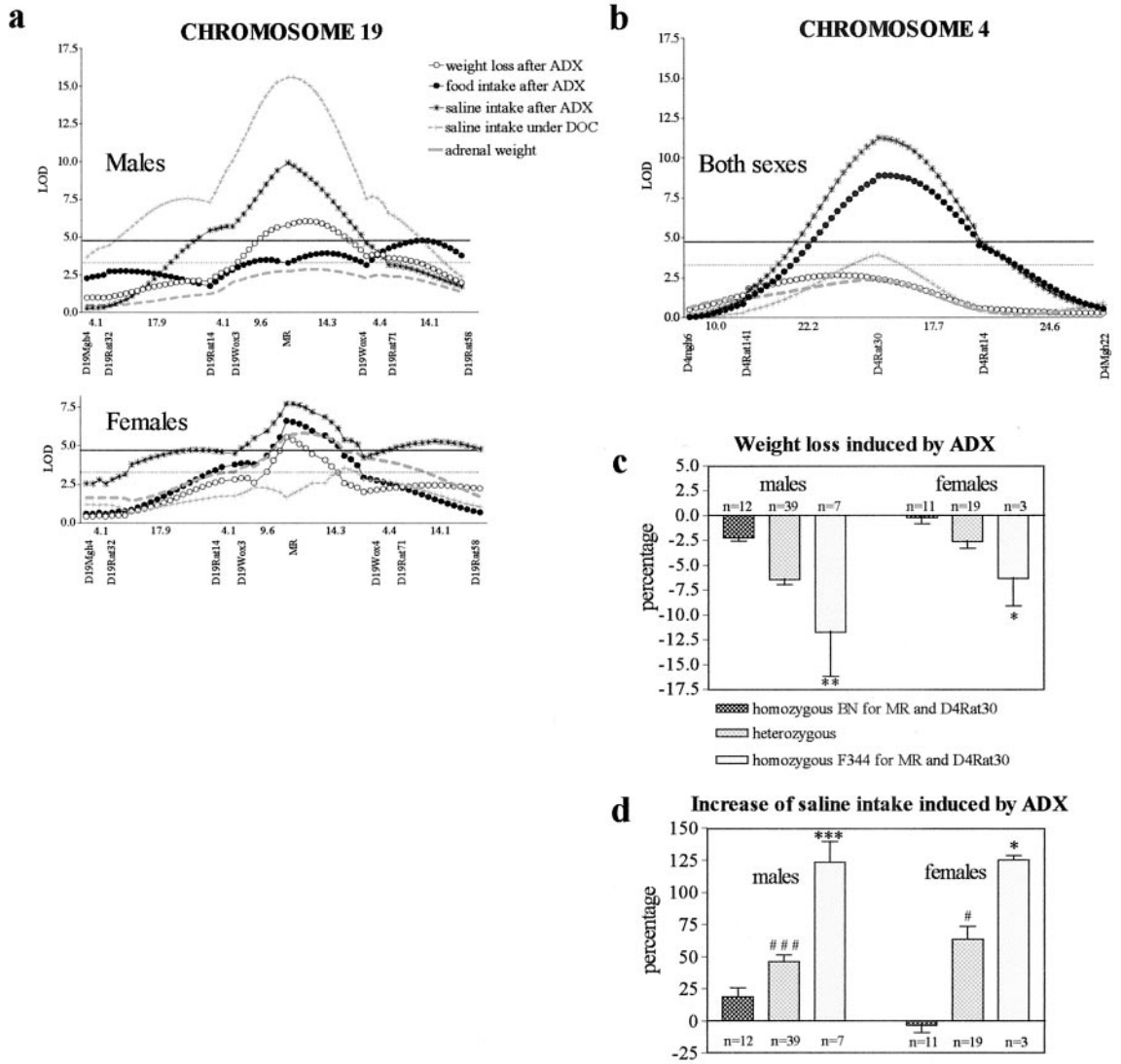

FIG. 2. **QTL on chromosomes 4 and 19 influence rat sensitivity to ADX.** *a*, MR-related QTL on chromosome 19. *b*, MR-related QTL on chromosome 4. The *dotted lines* represent the suggestive threshold, and the solid lines represent the significant threshold. Distances between markers (*cM*) are noted below the *abscissa*. *c*, percentage of weight loss. *d*, percentage of increase in fluid intake, induced by ADX in inbred rats, F1 hybrids, and F2 rats grouped according their genotype in rats homozygous for the BN MR and D4Rat30 alleles, rats homozygous for the F344 MR and D4Rat30 alleles, and rats heterozygous for both markers. Differences from the homozygous rats for the BN MR allele were: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Differences from the homozygous rats for the F344 MR allele were: #, $p < 0.05$; ###, $p < 0.001$.

F344 MR allele lost more weight than rats homozygous for the BN allele ($-6.70 \pm 0.80\%$ *versus* $-2.00 \pm 0.25\%$; $p < 0.0001$), which resulted partly from the decrease in food intake provoked by ADX in rats bearing the F344 MR alleles ($-33.23 \pm 3.31\%$ *versus* $+2.99 \pm 4.23\%$ in homozygous BN; $p < 0.0001$; data not shown). ADX increased saline intake of all rats but to a greater extent in males than in females ($p < 0.0001$), and in homozygous F344 than in homozygous BN ($+98.09 \pm 5.07\%$ *versus* $+36.65 \pm 4.29\%$; $p < 0.0001$), as previously shown in inbred strains (Fig. 1*c*). Conversely, DOC treatment induced a greater decrease in saline intake in rats homozygous for the F344 MR allele than in rats homozygous for the BN allele ($-27.90 \pm 2.60\%$ *versus* $-3.22 \pm 2.13\%$; $p < 0.0001$; data not shown). For most traits, the phenotype of F2 rats heterozygous for the MR allele was intermediate between homozygous or alike BN.

These data reveal a strong genetic linkage between the MR genotype and sensitivity to ADX. Nevertheless, the phenotypic differences found between F2 rats homozygous BN and F344 for the MR allele were globally smaller than the differences measured between BN and F344 inbred strains, suggesting the implication of additional loci in sensitivity to ADX. Therefore, a genome scan followed by a multipoint analysis of the F2 BN×F344 population was achieved to map markers and localize loci implicated in corticosteroid receptor-related traits.

QTL on Chromosomes 4 and 19 Influence Rat Sensitivity to ADX

Evaluation of MR Function—The multipoint analysis confirmed the linkage of the MR locus on chromosome 19 (Fig. 2*a*) with adrenal weight in females (LOD = 5.83, explaining 24.4% of the variance (VE)) that did not reach significance in males (LOD = 2.87, VE = 10.7%). A sex-dependent QTL in this region was also confirmed for the weight loss induced by ADX (LOD = 6.04 in males and 5.54 in females), the increase in saline intake induced by ADX (LOD = 9.91, VE = 29.8% in males; LOD = 7.74, VE = 31.6% in females), and the DOC treatment effect on saline intake (LOD = 15.56, VE = 45.6% in males; LOD = 3.56 between MR and D19Wox4, VE = 16.1% in females).

A sex-independent QTL was found on chromosome 4 for the decrease in food intake (D4Rat30, LOD = 8.87; Fig. 2*b*) induced

TABLE I
QTL for GR-related traits
Shown are the BN/BN mean values ± S.E. for rats homozygous for the BN allele of the closest marker, F344/F344 for rats homozygous for the F344 allele, and BN/F344 for heterozygous rats.

GR-related traits	Chrom	Marker	LOD	VE	$p <$	BN/BN	BN/F344	F344/F344
				%		%	%	%
Body weight	1	D1Rat256	3.46	7.0	0.001	−8.78 ± 0.64 (n = 59)	−7.64 ± 0.32 (n = 100)	−6.23 ± 0.41 (n = 64)
Food intake	1	D1Rat256	7.17	14.4	0.0001	−20.94 ± 3.90 (n = 59)	−18.06 ± 3.36 (n = 100)	−15.28 ± 4.80 (n = 64)
Fluid intake	4	D4Rat30	8.34	19.7	0.0001	10.57 ± 4.00 (n = 61)	−10.05 ± 5.86 (n = 120)	−23.87 ± 2.89 (n = 37)
	19	Males	4.54	15.6	0.0001	−8.08 ± 4.16 (n = 36)	−2.62 ± 3.74 (n = 61)	−28.90 ± 3.56 (n = 33)
			+ D4Rat30 = 7.04	+ D4Rat30 = 22.4	0.0001			
			5.50	24.0	0.0001	16.27 ± 5.97 (n = 33)	−8.57 ± 3.19 (n = 41)	−16.64 ± 3.34 (n = 20)
		MR females	+ D4Rat30 = 12.00	+ D4Rat30 = 47.8	0.0001			
			4.60	22.0	0.001	6.39 ± 6.25 (n = 35)	−15.30 ± 3.96 (n = 63)	−17.18 ± 2.67 (n = 26)
	14	D14Rat13	+ MR = 8.37	28.0	0.0001			
		In males only	+ D4Rat30 = 6.23	20.3	0.001			
			All = 10.24	31.0	0.0001			

FIG. 3. **The Y73C substitution increases MR transactivation in response to aldosterone (*a*) and progesterone (*b*).** Differences between BN and F344 MR were: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

by ADX. The D4Rat30 allele also contributed to 20.8% of the VE (LOD = 11.26) of saline intake after ADX and to the effect of DOC treatment on saline intake (LOD = 3.94, VE = 8.9%).

As compared with Fig. 1*c*, Fig. 2*c* shows that the additive effects of both MR and D4Rat30 loci explained 24.4% of the variance of weight loss in males and 34.6% in females and also accounted for a large part of the BN *versus* F344 difference in ADX effect on saline intake (47.5% of the VE in males, 56.0% in females, global LOD score = 28.61; Fig. 2*d*). Taken together, both alleles determined 51.3% of the VE for DOC effect in males (LOD = 15.44) and 20.5% in females (LOD = 4.43).

In summary, the additive effects of the MR and D4Rat30 loci accounted for most of the MR-related differences measured between the BN and F344 inbred strains, although the respective influence of these loci varied largely with the sex and trait under consideration.

Evaluation of GR Function: Effects of Treatment with RU28362—QTL implicated in GR-related traits are shown in Table I. A transgressive linkage was found for the weight loss and decrease in food intake induced by RU28362 on chromosome 1 (D1Rat256).

The MR and D4Rat30 loci were linked to the decrease in fluid intake induced by RU28362. Another suggestive QTL was also shown on chromosome 14 but only in males (D14Rat13). Taken together, these three markers explained 31.0% of the variance for this trait (LOD = 10.24) in F2 male rats.

The Y73C Substitution Increases MR Transactivation in Response to Aldosterone and Progesterone

We compared transactivation properties of BN and F344 MR by transient transfection assays. No difference was found in the constitutive transcriptional activity of the MR between the two sequences. Fig. 3*a* shows that aldosterone-induced transactivation was twice higher for the BN MR than for the F344 MR ($p < 0.001$), with a EC_{50} in the same order of magnitude (5×10^{-11} M; see *inset* of Fig. 3*a*). Progesterone is classically de-

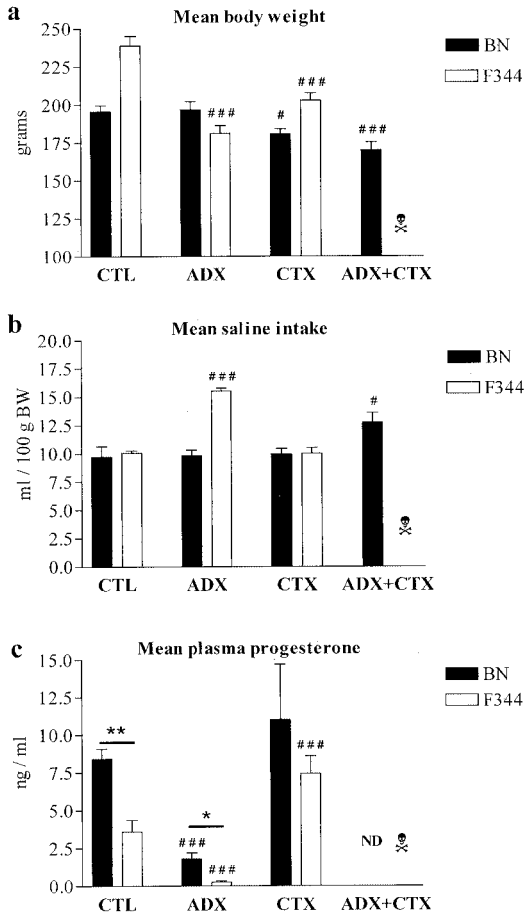

FIG. 4. **Effects of CTX, ADX, and combined surgery (ADX+CTX) in BN and F344 rat strains (on the 7th day after surgery) on body weight (*a*), saline intake (*b*), and plasma concentration (*c*) of progesterone.** All of the F344 rats that were both ADX and CTX died in the 2–3 days following surgery. Progesteronemia of ADX+CTX rats was below assay detection limits. Differences between BN and F344 MR were: *, $p < 0.05$; **, $p < 0.01$. The treatment effects *versus* the control group were: #, $p < 0.05$; ###, $p < 0.001$.

scribed for its antagonistic properties on MR action in presence of aldosterone. Indeed, the inhibition of transactivation induced by progesterone (10^{-6} M) in the presence of a high concentration of aldosterone (10^{-8} M) was proportionally the same for the BN MR as for the F344 MR ($-76.11 \pm 3.79\%$ in BN *versus* $-86.05 \pm 3.85\%$ in F344; data not shown). However, this steroid has been shown to behave as a partial agonist of the MR in absence of aldosterone in man and in rat (23). Fig. 3*b* shows that the partial agonistic activity of progesterone was three times greater for the BN MR than for the F344 MR ($p < 0.001$). This finding suggests that the Y73C substitution could protect BN rats against the detrimental effects of ADX by a partial substitution for aldosterone by progesterone after ADX.

Castrated BN Become Sensitive to ADX

Finally, we challenged our hypothesis of aldosterone substitution by progesterone as an MR agonist after ADX in BN rats. To that end, we compared CTX, ADX and ADX+CTX effects on body weight, food and saline intakes, and plasma concentration of progesterone between BN and F344 males (Fig. 4).

As previously shown (14), contrary to BN, the F344 rats lost weight after ADX ($p < 0.001$), an effect involving the expected decrease in their food intake ($p < 0.001$). Similarly, ADX increased the saline intake of F344 rats only. The plasma concentration of progesterone was greater in BN than in F344 males in the control situation ($p < 0.01$) and after ADX ($p < 0.05$). ADX induced a sizeable fall in plasma progesterone in both strains ($p < 0.001$). Plasma progesterone was undetectable during the first 3 days after ADX and slightly increased at 7 and 14 days after ADX (data not shown).

CTX decreased weight in equal proportions in both strains, probably because of the loss of the anabolic effect of testosterone (24), because food intake remained stable in both strains after this surgery. As expected, CTX did not modify saline intake. On the other hand, CTX increased plasma progesterone in F344 rats only.

All F344 rats that were both ADX and CTX died within 2–3 days following surgery, as compared with 2 of 14 BN rats. Compared with the BN rats with CTX only, the BN rats with both ADX and CTX lost weight ($p < 0.001$) and increased their saline intake ($p < 0.05$). These data suggest that progesterone from testicular origin may substitute for aldosterone after ADX in BN male rats.

DISCUSSION

Our previous studies underscored functional differences in hypothalamic-pituitary-adrenal axis activity/reactivity between BN and F344 rat strains (12, 13). We showed that, compared with F344 rats, the BN rats displayed a different diurnal pattern of plasma corticosterone levels, a faster recovery after restraint stress, and adrenals of larger size but less reactive to ACTH. Unlike the F344 that lost weight, decreased their food intake, increased their saline intake and their urinary Na^+/K^+ ratio after ADX, the BN rats were insensitive to ADX, suggesting a ligand-independent activation of their MR (14, 15). Moreover, a treatment with a GR agonist (RU28362) induced greater weight lost, anorexia, and thymolysis in BN than in F344 ADX rats. Therefore, the aim of the present study was to find the molecular bases of the functional differences in corticosteroid receptors between BN and F344 rats.

As a first step, the sequence of the MR cDNA was compared between BN and F344 strains. We found a single nucleotide polymorphism in the second exon of the BN MR compared with the F344 and SD, resulting in a tyrosine to cysteine substitution at residue 73 of the N-terminal part of the receptor. This substitution revealed a restriction site for the BsaMI enzyme, which provided the means to genotype an F2 BNxF344 population for the MR allele. The F2 rats were submitted to the phenotypic measurements the most discriminant between BN and F344 inbred strains: adrenal size, effects of ADX on body weight, food and saline intakes, and their response to DOC or RU28362 treatments (15). ADX induced weight loss and increase in saline intake in all F2 rats but to a greater extent in the rats homozygous for the F344 MR allele than in those homozygous for the BN MR allele, confirming the biological implication of the MR locus in the differential sensitivity of parental strains to ADX. Nevertheless, the phenotypic differences found between F2 rats homozygous BN and F344 for the MR allele were lower than the differences measured between inbreds (15), suggesting the implication of additional loci in their differential sensitivity to ADX.

A genome scan and a multipoint analysis of the F2 population confirmed a strong linkage between the MR locus, adrenal weight, and sensitivity to ADX and localized other loci involved in the MR and GR-related traits, including a locus on chromosome 4 (D4Rat30 marker). When considered together, these two markers accounted for most of the differences relative to MR function measured between BN and F344 inbred strains. The rat MR locus contains MR gene (*Mlr*) but also other candidate genes involved in MR function, such as the catalytic α subunit of protein kinase A (*Prkaca*, that regulates MR transactivation function) (25), and the 11 β-hydroxysteroid dehydrogenase type 2 (*Hsd11b2*, that inactivates glucocorticoids to

provide the specificity of aldosterone for MR in target tissues) (26). The D4Rat30 region contains two positional and functional candidate genes: the 2β isoform of the Na^+/K^+-ATPase (*ATP2b2*) and the α subunit of the amiloride-sensible sodium channel (*Scnn1a*), both targets of the MR regulation of electrolytic homeotasis (27–29). MR action on the renal isoform 1α of the Na^+/K^+-ATPase is now well documented for its antinatriuretic effects (30). The MR is also known to promote the synthesis of the 2β subunit of the Na^+/K^+-ATPase in the colon to improve Na^+ reabsorption (27). This isoform was also implicated in the increase in salt appetite induced by DOC in brain structures (28). The epithelial sodium channel is composed of three homologous subunits (α, β, and γ) and is responsible for salt reabsorption in the kidney (31). In the rat cortical collecting duct, aldosterone increases epithelial sodium channel α-subunit mRNA without affecting the mRNA encoding the two other subunits (29). Mutations of these subunits can cause human diseases by increasing channel function (the β and γ subunits in Liddle's syndrome) (32) or by decreasing channel function in recessive type I pseudohypoaldosteronism (the α subunit) (33). Iwai *et al.* (31) screened for sequence variations in *Scnn1a* and associated a A2139G polymorphism in this gene to blood pressure in human. Therefore, beyond the functional differences of the MR that we document in this paper, several other candidate genes will have to be studied to understand completely the molecular mechanisms responsible for the differential MR function between BN and F344 rat strains.

The weight loss and the decrease in food intake induced by RU28362 treatment were influenced by one or several genes localized in the region of the D1Rat256 marker. One could expect that a MR harboring a gain of function could potentiate GR-related effects, notably by heterodimerization (34) but, apart from the fluid intake under RU28362, these parameters were not influenced by MR-related markers.

To investigate the functional consequences of the Y73C substitution in the MR N-terminal part, we compared transactivation properties of BN and F344 MR by transient transfection *in vitro*. Aldosterone induced a transactivation two times greater for the BN MR than for the F344 one. Even though the inhibition of transactivation induced by progesterone in presence of aldosterone was proportionally the same in both strains, the partial agonistic activity of progesterone was three times greater for the BN MR than for F344. The Y73C substitution removes a potential phosphorylation site, but to our knowledge, no phosphorylation of tyrosine has been described on the MR to date. This substitution takes place in the 1–167 fragment of the MR, harboring the AF-1a function, and shown to interact with specific co-factors such as RNA helicase A and the co-integrator CBP/p300 (35) and protein inhibitor of activated STAT (PIAS) family members (36). It also induces a potential site for the formation of a disulfide bond, which could consolidate the interaction between the N-terminal and the C-terminal regions of the MR recently proposed by Rogerson and Fuller (37). This process could increase the half-life of the steroid-receptor complex (38), allowing progesterone to strengthen its partial agonist status.

In our *in vivo* experiments, we show that plasma progesterone concentration is decreased by ADX but remains seven times higher in BN than in F344 rats. These higher levels of progesterone after ADX together with a stronger agonistic activity of progesterone on the Y73C variant of MR may explain at least partially the low sensitivity of BN rats to ADX. We confirmed this hypothesis by comparing the effects of CTX, ADX, and ADX+CTX in BN and F344 rats. Contrary to the BN rats with CTX only, the BN rats that were both ADX and CTX lost weight and increased their saline intake. The partial agonist effect of progesterone could also be implicated in the lesser effects of ADX on body weight or saline intake in females than in males of both rat strains and of the F2 population. On the other hand, the survival of BN rats with both ADX and CTX, contrary to F344 rats that died within the first 3 days after surgery, and their moderate increase in saline intake could result from the implication of other genes located in the second MR-related QTL on chromosome 4.

This work illustrates the interest of a pluristrategic approach to explore genetic variations in the MR function and its implication on hydroelectrolytic homeostasis. Comparison of rat strains diverging for physiological outputs of the MR signaling pathway led to a functional hypothesis that was explored by gene sequencing and QTL mapping, completed by *in vitro* investigations and confirmed *in vivo*. Because the gain of function of the MR signaling pathway in the BN rat has no obvious effect on blood pressure (39), it can be hypothesized that other mechanisms compensate for the functional consequences of this mutation. For instance, as suggested by Iwai *et al.* (31), a lower level of epithelial sodium channel α-subunit expression might lead to lower sodium reabsorption in the kidney and might provide protection against the gain of function mutation in the MR of BN rats. These data show the way to exciting physiopathological perspectives for the study of MR signal transduction and system genomics in hypertension research.

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