**Cyp11b1 Null Mouse, a Model of Congenital Adrenal Hyperplasia**

Linda J. Mullins‡, Audrey Peter‡, Nicola Wrobel‡, Judith R. McNeilly§, Alan S. McNeilly§, Emad A. S. Al-Dujaili‡, David G. Brownstein†, John J. Mullins‡, and Christopher J. Kenyon‡

From the Centres for Cardiovascular Science and Reproductive Biology, University of Edinburgh, Edinburgh EH16 4TJ, Scotland, United Kingdom

Patients with congenital adrenal hyperplasia arising from mutations of 11β-hydroxylase, the final enzyme in the glucocorticoid biosynthetic pathway, exhibit glucocorticoid deficiency, adrenal hyperplasia driven by unsuppressed hypothalamo-pituitary-adrenal activity, and excess mineralocorticoid activity caused by the accumulation of deoxycorticosterone. A mouse model, in which exons 3–7 of Cyp11b1 (the gene encoding 11β-hydroxylase) were replaced with cDNA encoding enhanced cyan fluorescent protein, was generated to investigate the underlying disease mechanisms. Enhanced cyan fluorescent protein was expressed appropriately in the zona fasciculata of the adrenal gland, and targeted knock-out was confirmed by urinary steroid hormone profiles and, immunocytochemically, by the absence of 11β-hydroxylase. The null mice exhibited glucocorticoid deficiency, mineralocorticoid excess, adrenal hyperplasia, mild hypertension, and hypokalemia. They also displayed glucose intolerance. Because rodents do not synthesize adrenal androgens, changes in reproductive function such as genital virilization of females were not anticipated. However, adult homozygote females were infertile, their ovaries showing an absence of corpora lutea and a central proliferation of disorganized stromogenic tissue. Null females responded normally to superovulation, suggesting that raised systemic progesterone levels also contribute to infertility problems. The model reveals previously unrecognized phenotypic subtleties of congenital adrenal hyperplasia.

The final steps leading to the production of glucocorticoids and mineralocorticoids are undertaken by 11β-hydroxylase and aldosterone synthase, encoded by two closely linked genes, Cyp11b1 and Cyp11b2, respectively, that share 95% sequence homology. In rodents, the common substrate, deoxycorticosterone, is converted into the mineralocorticoid, aldosterone, in the adrenal zona glomerulosa by aldosterone synthase and into corticosterone, the main glucocorticoid, by 11β-hydroxylase in the zona fasciculata. Cortisol rather than corticosterone is the zona fasciculata. Unlike rodents, human adrenals produce significant amounts of the androgen dehydroepiandrosterone (DHEA).²

Patients with congenital adrenal hyperplasia (CAH) have a markedly reduced capacity to produce glucocorticoids. In ~90% of cases CAH is caused by deficiency of 21-hydroxylase, the penultimate enzyme in the pathways of both aldosterone and cortisol synthesis (1). In 8% of CAH cases, point mutations in or deletion of Cyp11b1 drastically reduce or completely destroy 11β-hydroxylase activity (2–4).

Adrenal hyperplasia is a consequence of increased ACTH secretion in the absence of normal negative feedback control by glucocorticoids of the hypothalamo-pituitary-adrenal axis. In 21-hydroxylase-deficient patients, ACTH drives the production of progesterone and 17-hydroxyprogesterone and channels earlier 17-hydroxylated intermediates in the glucocorticoid pathway toward the synthesis of adrenal androgens. CAH due to 11β-hydroxylase deficiency causes increased synthesis of the 11-deoxycorticosteroids deoxycortisol and deoxycorticosterone (a weak mineralocorticoid) as well as increased adrenal androgen production. In all CAH patients, it is the raised adrenal androgen production in early development leading to premature puberty in males and ambiguous external female genitalia in females that is of greatest concern (5–7).

Rodents, which do not synthesize adrenal androgens, allow us to study the equally pernicious effects of glucocorticoid deficiency, progesterone excess, and disturbances of mineralocorticoid hormone synthesis. Studies of mice carrying a severely mutated 21-hydroxylase gene have been useful but are limited by problems of viability because of the lack of both mineralocorticoid and glucocorticoid hormones (8). In this study we have targeted Cyp11b1 in the expectation that, by preserving mineralocorticoid synthesis, mice will survive.

The Cyp11b1 gene was replaced with a fluorescent reporter gene, and the resultant transgenic line was assessed for reduced 11β-hydroxylase activity and pathophysologies associated with altered steroid hormone activity. We report anomalies in steroid hormone profiles, glucose tolerance, blood pressure, salt handling, and reproductive performance, and we suggest that the Cyp11b1 knock-out mouse represents a good model of CAH.

---

¹ This work was supported by the Medical Research Council (United Kingdom). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

² The abbreviations used are: DHEA, dehydroepiandrosterone; ECFP, enhanced cyan fluorescent protein; CAH, congenital adrenal hyperplasia; WT, wild type; ACTH, adrenocorticotropic hormone; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; GnRH, gonadotrophin releasing hormone; ES, embryonic stem; ANOVA, analysis of variance.

---

*This work was supported by the Medical Research Council (United Kingdom). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*
Cyp11b1 Null Mice Model CAH

TABLE 1
Primer and probe sequences for PCR and RT-PCR

| Primer and probes | Homology arm primers |
|-------------------|-----------------------|
|                   | ex1for                 |
|                   | ex2rev                 |
|                   | ex8for                 |
|                   | ex9rev                 |

| PCR screen primers | RT-PCR primers and probe |
|--------------------|--------------------------|
| 11BWT for          | 11Bex3for                |
| BAC1 for           | 11B34FAM probe           |
| BAC2 for           | 6-FAM-TACCAGAACTACGGG-MGB |

EXPERIMENTAL PROCEDURES

Generation of 11β-Hydroxylase Null Mice—A BAC clone (ASBAC, a gift from Prof. K. Parker), which has a 125-kb insert and spans the entire Cyp11b2-Cyp11b1 locus, with approximately 60 kb upstream and 45 kb downstream of the genes was used to produce the construct for subsequent generation of Cyp11b1 null mice. Specific targeting of the Cyp11b1 gene by replacing exons 3–7 with an IRES-ECFP cDNA and neomycin/kanomycin resistance construct was achieved through BAC recombineering (9, 10). The target construct contained an 11β-hydroxylase-specific, PCR-generated, 780-bp 5′ homology arm spanning exons 1–2 of Cyp11b1, and a 644-bp 3′ homology arm spanning exons 8 and 9 respectively. (PCR primers are given in Table 1.) The ex2rev primer was designed to place a STOP codon in all three reading frames. An IRES-ECFP cassette was positioned between the homology arms, placing its expression under the 11β-hydroxylase promoter. The ECFP reporter was directed to cell membranes by the addition of a 20-amino acid farnesylation signal (from H-ras) at the C-terminus, and a 10-amino acid c-Myc epitope (9E10) was placed at the N terminus. For selection of the targeting construct, a floxed Kan-neo cassette, driven by the PGK-em7 promoters (gift from Prof. N. Copeland), was placed downstream of the reporter cassette. All fragments generated by PCR were verified by sequencing. The construct was electroporated into strain DY380; colonies were screened using chloramphenicol plus kanomycin, and correct homology arm spanning exons 1–2 of Cyp11b1, and a 644-bp 3′ homology arm spanning exons 8 and 9 respectively. (PCR primers are given in Table 1.) The ex2rev primer was designed to place a STOP codon in all three reading frames. An IRES-ECFP cassette was positioned between the homology arms, placing its expression under the 11β-hydroxylase promoter. The ECFP reporter was directed to cell membranes by the addition of a 20-amino acid farnesylation signal (from H-ras) at the C-terminus, and a 10-amino acid c-Myc epitope (9E10) was placed at the N terminus. For selection of the targeting construct, a floxed Kan-neo cassette, driven by the PGK-em7 promoters (gift from Prof. N. Copeland), was placed downstream of the reporter cassette. All fragments generated by PCR were verified by sequencing. The construct was electroporated into strain DY380; colonies were screened using chloramphenicol plus kanomycin, and correct targeting was confirmed by PCR and Southern blot hybridization (using exon-specific probes).

ES cells (E14TG2a, derived from mouse strain 129/Sv (11)) were transfected with the Cyp11BKO construct by electroporation using a dual pulse (Easyjet Plus Optipulse; pulse 1, 550 V, 25 microfarads, 481 ohms; interpulse delay, 0.00 s; pulse 2, 100 V, 1500 microfarads, 481 ohms). Clones incorporating the BAC DNA were isolated under neomycin selection. Homologous targeting was assessed by Southern blot hybridization (using a cDNA probe), and by using a panel of four PCRs to determine the presence of both the targeted and endogenous Cyp11b1 gene and the loss of flanking BAC vector sequences.

(For primers see Table 1.) Fluorescence in situ hybridization analysis and karyotyping was performed in cell spreads to confirm that BAC DNA had integrated homologously.

Breeding—Successful knock-out of the Cyp11b1 gene was achieved by homologous recombination in both bacteria (BAC recombineering) and 129/Sv-derived ES cells. Following injection of Cyp11b1 null ES cells into C57BL/6 blastocysts, chimeras capable of germ line transmission were backcrossed to C57BL/6. All experiments described here were carried out on animals with a 75% C57BL/6, 25% 129/Sv genetic background. Because homozygous females were infertile, all homozygous progeny were generated by crossing male homozygotes with female heterozygotes.

Phenotyping—All experimental animal studies were undertaken under UK Home Office license, following review by local ethics committee. Mice were maintained in a 12-h light-dark cycle (on at 07.00 h) under controlled conditions of humidity (50 ± 10%) and temperature (21 ± 2°C) and fed standard mouse chow (SDS Ltd., Whitham, Essex, UK) and water ad libitum unless otherwise stated.

Systolic blood pressure was measured by a tail cuff technique (12) on at least three separate occasions. Data shown are the means ± S.E. of the average of the recordings for each animal. Prior to recording these values, mice were fully accustomed to the warming and restraint procedures that this method requires.

Metabolic Cage Studies—Animals (minimum n = 5) were habituated to food containing 0.3% NaCl (SDS Ltd.) that was formulated as a gel to prevent food wastage (13). Mice were housed singly in metabolic cages (model 3600M021, Techniplas UK Ltd.). After acclimatization to the cages, body weight, food and water intake, and urine and fecal production were monitored daily over 4 consecutive days. Urine was collected and frozen for later analysis of steroid hormones and electrolytes.

Steroid Profile Determination—Urine samples were analyzed for corticosterone, aldosterone, deoxycorticosterone, DHEA, and testosterone by in-house ELISAs using previously characterized specific antibodies (14–18). The protocol for the ELISAs has been described previously (14). Urinary K+ and Na+ were determined by flame atomic absorption spectrophotometry and flame emission spectrophotometry, respectively, using a SpectRAA-300 spectrophotometer.

Blood was collected by cardiac puncture into heparin-coated syringes and analyzed for plasma renin concentration, angiotensinogen, corticosteroids, triglycerides, and insulin as described previously (19). Plasma ACTH was measured by radioimmunoassay (MP Biomedicals, New York). Plasma electrolytes were analyzed using ion-selective electrodes (Electrolyte Analyzer, Roche Applied Science).

Quantitative Real Time PCR Analysis—Total RNA was extracted from various tissues using TRIzol reagent (Invitrogen). The RNA was treated with DNA-free (Ambion), quantified by spectrophotometer, and its integrity confirmed on an agarose gel. 3 μg of RNA was reverse-transcribed, using the Superscript II first strand cDNA synthesis kit (Invitrogen), and the resultant cDNA was amplified by Taqman RT-PCR on the ABI Prism 7000 sequence detection system. Primers and probes
were designed to be specific for endogenous Cyp11b1, spanning exons 3 and 4 (see Table 1). Reporter-specific primers and probes were used for RT-PCR of targeted Cyp11b1. Reactions were carried out in triplicate and normalized to 18S for adrenal samples.

Liver function was assessed by pre-optimized RT-PCR assays specific for phosphoenolpyruvate carboxykinase (Pepck), tyrosine aminotransferase (Tat), and angiotensinogen (Agt) and pancreatic function by serum glucocorticoid-inducible kinase 1 (Sgk1) assay and insulin (Ins2) assays. Values are reported as the ratio of target/TATA box-binding protein (Tbp) or wbscr1 (Williams Beuren syndrome chromosome region 1) mRNA and expressed in arbitrary units.

Histology and Immunohistochemistry—Tissues were fixed in 4% normal buffered formalin, weighed, dehydrated, and embedded in paraffin. 6-Micrometer-thick sections were mounted on slides and stained with hematoxylin and eosin. Adrenal sections were stained using anti-11β-hydroxylase (a kind gift of Celso Gomez-Sanchez) or anti-green fluorescent protein (mouse IgG2a monoclonal antibody, JL-8, BD Biosciences) as primary antibody and peroxidase-conjugated rabbit anti-mouse IgG secondary antibody. Whole heads were fixed in 4% normal buffered formalin, before careful dissection of the brain. Pituitary sections were immunostained using anti-ACTH primary antibody.

Tissues of the male and female reproductive tracts were fixed for 5 h in Bouin’s solution and then transferred to 70% ethanol. Immunostaining of ovary sections was carried out as described previously (20). To assess cell proliferation in ovaries of null mice, bromodeoxyuridine (Sigma; 0.1 mg/0.1 ml saline) was injected intraperitoneally 2 h before sacrifice.

Fluorescence Microscopy—Tissues were removed and immediately snap-frozen in OCT embedding medium on dry ice. Frozen 10-μm sections were viewed directly using a confocal fluorescence microscope (Zeiss LSM 510 META).

Glucose and Insulin Tolerance Tests—Glucose tolerance in male and female mice was assessed after a 5-h fast, in serial blood samples taken at 0, 30, 60, and 120 min following administration of glucose (2 g/kg body weight, intraperitoneally). Blood glucose was measured using a One-Touch Ultrasmart blood glucose meter (Lifespan Scotland Ltd.). Glucose tolerance tests were repeated in male mice before and after KCl had been administered in drinking water (1%) for 7 days. Insulin tolerance was determined in fasted animals by administering insulin intraperitoneally (1 unit/kg body weight Humulin S; Lilly, Basingstoke, Hampshire, UK) and measuring blood glucose at time intervals as described for the glucose tolerance test. Plasma insulin was measured using a commercial kit (mouse insulin ELISA kit, Crystal Chem Inc., Downers Grove, IL). Sgk and Ins2 mRNA levels were measured by real time RT-PCR in pancreatic tissues collected from male mice (see above).

Statistics—Appropriate analyses were performed using Student’s t tests and one-way or two-way ANOVA, as described in the text and figure legends, to determine statistical significance. Error bars in figures represent mean ± S.E.

RESULTS

Generation of Knock-out Model—The targeting construct (Fig. 1a) was designed such that 11β-hydroxylase was insertionally inactivated by the replacement of exons 3–7 of Cyp11b1 with a gene encoding a fluorescent reporter (ECFP). Reporter expression was placed under the control of the endogenous Cyp11b1 promoter by virtue of the three-frame STOP-IREs motif, and sequence encoding a 20-amino acid farnesyl-tail was included to anchor the resultant reporter to cell membranes. The floxed Kan-neo cassette was included for antibiotic selection in both bacterial cells (BAC recombineering) and mammalian cells (ES cell homologous recombination). BAC recombineering was used to specifically target the Cyp11b1 gene in a BAC containing both Cyp11b1 and the highly homologous Cyp11b2 gene, and demonstrated the fidelity of the homologous recombination system in Escherichia coli. The targeted BAC was subsequently introduced into E14TG2a ES cells (derived from the 129/Sv mouse) by electroporation. Correct targeting events were identified using a panel of four PCR screens for the presence of both endogenous and targeted Cyp11b1 and the absence of flanking BAC sequences. The possibility of additional random integration events was discounted by fluorescence in situ hybridization analysis. One chimera from each homologously targeted ES line was found to be capable of germ line transmission, resulting in the generation of two independent knock-out lines (designated LM1B and LM2B). Only experiments on line LM1B are described.

Targeting—Fig. 1 summarizes functional data establishing that Cyp11b1 was successfully knocked out. Urinary steroid analysis showed that the main product of 11β-hydroxylase activity was reduced by 9.5- and 18-fold in homozygous males and females, respectively, compared with wild type mice, whereas the substrate, deoxycorticosterone, was dramatically increased by 27- and 44-fold (Fig. 1b). Steroid levels in urine collected from heterozygotes was not significantly different from those in wild type mice, although the ratio of deoxycorticosterone:corticosterone was significantly increased in males. Residual corticosterone in urine of null mice probably reflects the 11β-hydroxylase activity of aldosterone synthase. No Cyp11b1 mRNA was detected by real time RT-PCR in adrenals of homozygous null mice, which instead expressed high levels of reporter mRNA. Immunocytochemistry confirmed that the substitute ECFP transgene and not 11β-hydroxylase was expressed appropriately in the zona fasciculata of homoygote null mice and vice versa for wild type mice (Fig. 1c). As expected, a strong fluorescence signal of the farnesylated ECFP marker was detected in cell membranes of adrenals of null mice (Fig. 1d). The signal was weaker in heterozygotes and absent in wild type mice.

Glucocorticoid Deficiency—Adrenal hypertrophy, the hallmark of CAH, was evident in homozygous null mice (Fig. 2a and Table 2). Histological examination indicated that the zona fasciculata is wider because of cell hypertrophy (Fig. 2b), although proliferation tests would be required to exclude simultaneous hyperplasia. A comparison of males and females indicated that female adrenals are generally larger but that adrenal hypertrophy of female null mice is less than males.
These sex and genotypic differences in adrenal glands correspond to relative differences in urinary steroid secretion. We have confirmed that male Cyp11b1 null mice do not respond appropriately to restraint/handling stress with an increase in plasma corticosterone levels (Fig. 2c). The cause of CAH in patients is activation of the hypothalmo-pituitary-adrenal axis because of the lack of negative feedback control. The pituitary glands of null mice appeared hypertrophied, and preliminary immunochemical evidence indicated increased numbers of corticotropes3 reflecting stimulation of the HPA axis. Plasma ACTH values were higher (p < 0.001) in both male and female Cyp11b1 null mice compared with wild type controls (male, WT 468 ± 105, n = 9; cf. null 1639 ± 186 pg/ml, n = 14; female, 583 ± 68, n = 6; cf. 1119 ± 86 pg/ml, n = 6).

Table 2 shows difference in weights of various tissues in proportion to body weight in null and wild type mice. Adipose tissue weights were generally reduced in null mice of both sexes consistent with the idea that glucocorticoids promote fat deposition. Interestingly, thymus weights, which are usually inversely correlated with glucocorticoid activity, were decreased rather than increased in males and were unaffected in females. The observed increase in brain mass is not an artifact caused by the reduced adiposity of null mice because differences were significant even when uncorrected for variations in body weight. Brain growth and development occur in late gestation and early postnatal life, with glucocorticoids signaling the end of cell proliferation. Increased brain mass in adult life could therefore reflect perinatal glucocorticoid deficiency. The kidney, another tissue whose growth is regulated by glucocorticoids, was increased in males but not females. The increased heart mass may reflect changes in blood pressure (see below). No change in angiotensinogen, phosphoenolpyruvate kinase, or tyrosine aminotransferase mRNA levels were observed in null animals by RT-PCR.

Glucocorticoids are known to antagonize insulin actions and to inhibit insulin secretion. We therefore expected Cyp11b1 null mice to show increased clearance of plasma glucose following an intraperitoneal injection of glucose and a quicker response to insulin in fasted mice. However, responses to glucose tolerance tests were indistinguishable between null and wild type female mice, whereas male null mice were glucose-intolerant (Fig. 3, left, upper panel). Because the response to insulin injection was similar for both wild type and null animals (Fig. 3, left, lower panel), this indicated an impairment of insulin synthesis or secretion in Cyp11b1 null males. The expression of insulin by pancreatae of male mice was not affected by genotype (Ins2 mRNA, WT 1.26 ± 0.76; null 0.82 ± 0.64; not significant), but plasma insulin levels were reduced in male null mice (3.46 ± 1.7; cf. 1.74 ± 0.3 ng/ml; p < 0.05) with a similar trend in females (3.24 ± 1.51, cf. 1.09 ± 0.42 ng/ml; p = 0.14). These data suggest an inhibitory effect on insulin secretion. As recent studies indicate that serum glucocorticoid kinase (Sgk) mediates an inhibitory control of insulin secretion, via up-regulation of the K+ channel activity (21, 22), we compared Sgk mRNA in pancreatic tissues from null and WT mice. Our expectation was...

3 J. R. McNeilly, A. S. McNeil, D. Brownstein, J. J. Mullins, L. J. Mullins, and C. J. Kenyon, unpublished data.
that glucocorticoid deficiency would be suppressive, whereas excess mineralocorticoid activity (see below) might be stimulatory. However, no differences were observed (Sgk mRNA, WT 1.31 ± 0.42; null 1.12 ± 0.35).

We then considered whether glucose intolerance was because of electrolyte imbalance in null mice caused by excess mineralocorticoid. A repeat glucose tolerance test on a second cohort of null mice confirmed the original observation. The provision of KCl in the drinking fluid for 7 days did improve glucose clearance but was equally effective in null and WT mice (Fig. 3, right).

**Excess Mineralocorticoid Activity**—Deoxycorticosterone, a weak mineralocorticoid, was increased more than 30-fold in null males and females (Fig. 1b). This caused hypokalemia (Table 3) and suppression of the renin angiotensin system. Plasma renin concentration was lower in null male mice compared with controls (629 ± 170, cf. 141 ± 90 nmol of angiotensin 1·h⁻¹·l⁻¹, p < 0.05) and plasma angiotensinogen was not significantly affected (290 ± 54, cf. 437 ± 42 nmol·ml⁻¹, p < 0.1). Hypokalemia and renin suppression led to a decrease in normal levels of aldosterone (Table 5). Excess mineralocorticoid increased blood pressure, an effect that was more marked in females than males (Table 3). Urinary sodium and potassium excretions were not affected indicating that mice had already escaped from the sodium retaining actions of deoxycorticosterone (Table 4). Fluid turnover was greater in null mice, but this could be related to glucocorticoid deficiency rather than mineralocorticoid excess.

**Increased Androgen Activity**—Mice express negligible amounts of adrenal 17-hydroxylase, so it is unclear why urinary testosterone was raised in both male and female null mice; DHEA was not significantly affected (Table 5). There is no indication of virilization in females and only a small, nonsignificant increase in testicular mass.

**Progesterone Excess**—Male and female null mice showed increased urinary progesterone levels (Table 5), which appear to be of no phenotypic consequence in the fertile and viable males but may be responsible for the infertility observed in homozygous null females. Urinary estradiol levels were not affected (data not shown). Reproductive function of female null mice is compromised in two respects.

Homozygous females proved to be infertile, although smears suggested that some of the younger null females were cycling normally, and older mice treated with pregnant mare serum and human chorionic gonadotrophin did ovulate. Ovaries of 5-month-old virgin homozygous females were indistinguishable in external appearance when compared with those of age-matched wild type animals (Fig. 4a), but their internal morphology was clearly abnormal. Homozygote ovaries contained pre-ovulatory follicles but no clearly defined corpora lutea. The remainder of the ovary was filled with lobular amorphous cells, which were proliferating cell nuclear antigen-negative and did
Cyp11b1 Null Mice Model CAH

**TABLE 2**
Tissue weights of WT and null mice
Values shown are means ± S.E. Genotype effects were tested by one-way ANOVA for males and females and by two-way ANOVA for overall comparisons. bwt, body weight.

| Sex/genotype | Adrenal | Heart | Liver | Brain | Kidney | Mesenteric adipose | Gonadal adipose | Subcutaneous adipose | Brown adipose |
|--------------|---------|-------|-------|-------|--------|-------------------|----------------|---------------------|--------------|
|              | mg/g bwt| mg/g bwt| mg/g bwt| mg/g bwt| mg/g bwt| mg/g bwt| mg/g bwt| mg/g bwt| mg/g bwt |
| Male/null    | 0.095 ± 0.01 | 7.15 ± 0.21 | 56.0 ± 1.1 | 15.1 ± 0.5 | 7.51 ± 0.21 | 7.08 ± 0.77 | 15.8 ± 1.9 | 11.3 ± 0.8 | 4.21 ± 0.33 |
| Male/WT      | 0.033 ± 0.004 | 5.90 ± 0.08 | 45.6 ± 2.1 | 12.9 ± 0.7 | 6.10 ± 0.9 | 24.7 ± 2.7 | 15.51 ± 2.30 | 6.70 ± 0.48 |
| p value male | 0.001     | 0.001     | 0.003    | 0.03    | 0.001    | 0.02    | 0.03     | 0.02     | 0.005 |
| Female/null  | 0.289 ± 0.067 | 7.06 ± 0.52 | 49.4 ± 2.7 | nm       | 6.69 ± 0.46 | 6.56 ± 1.21 | 32.8 ± 1.9 | 6.56 ± 1.21 | 2.58 ± 0.31 |
| Female/WT    | 0.132 ± 0.01 | 5.90 ± 0.27 | 40.5 ± 2.7 | nm       | 6.68 ± 0.33 | 14.46 ± 1.71 | 32.2 ± 0.4 | 14.46 ± 1.71 | 3.39 ± 0.27 |
| p value female | 0.08    | 0.11    | 0.05    | nm      | 1.0      | 0.003    | 0.8      | 0.03     | 0.1 |
| p value all  | 0.004     | 0.02     | 0.02    | nm      | 0.3      | 0.001    | 0.02     | 0.02     | 0.003 |

**FIGURE 3. Glucose and insulin tolerance tests.** Left, upper panels show responses of male and female animals to glucose injection (intraperitoneally; downward arrows). Lower panels show response of male and female animals to insulin injection (intraperitoneally; upward arrows). (Open circles, WT; filled circles, null animals). Values shown are means ± S.E. ANOVA with repeated measures show that glucose tolerance in null mice is impaired in male (p < 0.001) but not female mice. * indicates significant difference (p < 0.01) of null compared with WT mice at particular time points. Right, glucose tolerance tests carried out before and after high K⁺ diet in male mice. Values shown are means ± S.E. When all data were analyzed using a general linear model of ANOVA, both genotype and dietary potassium significantly affected glucose tolerance (p < 0.001) without interacting. When analyzed separately, WT (p < 0.001) and null mice (p < 0.01) showed improved glucose tolerance with high potassium diet. Similarly, glucose tolerance was impaired in null compared with WT mice when fed either a normal (p < 0.01) or high potassium (p < 0.01) diet. Inj, intraperitoneal injection.

**TABLE 3**
Blood pressure and plasma electrolytes
Genotype effects were tested by one-way ANOVA for males and females and by two-way ANOVA for overall comparisons.

| Sex/genotype | Systolic blood pressure | Plasma [Na⁺] | Plasma [K⁺] | Hematocrit |
|--------------|-------------------------|--------------|-------------|------------|
|              | mm Hg       | mEq/L         | mEq/L     | %          |
| Male/null    | 128.7 ± 4.5 | 146.6 ± 3.9  | 6.42 ± 0.29 | 47.3 ± 1.2 |
| Male/WT      | 115.8 ± 4.7 | 150.0 ± 1.2  | 7.36 ± 0.36 | 47.4 ± 0.9 |
| p value male | 0.05        | 0.4           | 0.09       | 0.9        |
| Female/null  | 136.9 ± 3.2 | 146.7 ± 2.0  | 4.73 ± 0.18 | 40.3 ± 0.4 |
| Female/WT    | 113.0 ± 2.2 | 145.0 ± 2.1  | 6.38 ± 0.29 | 40.5 ± 1.4 |
| p value female | 0.001    | 0.6           | 0.001      | 0.9        |
| p value all  | 0.001       | 1.0           | 0.003      | 0.8        |

not stain positively for bromodeoxyuridine, indicating that they were not proliferating. Immunohistochemistry, with antibodies against 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase, suggested that the cells were nonsecreting luteinized granulosa cells.

Older homozygote females (>8 months old) showed signs of adenomyosis. Uteri were grossly enlarged with multiple cysts (Fig. 4, c and d). Histopathology revealed endometrial hyperplasia in 6-month-old animals (Fig. 4b).

**DISCUSSION**
A mouse model of CAH has been created by targeted replacement of Cyp11b1 with a gene for the fluorescent protein ECFP. The urinary steroid profile for Cyp11b1 knock-out mice is similar to that of patients carrying null mutations of 11β-hydroxylase with evidence of glucocorticoid depletion and mineralocorticoid and progesterone excess. Because rodents do not normally synthesize adrenal androgens, increases in DHEA and testosterone are modest in comparison with CAH patients.
null mice show clear signs of glucocorticoid deficiency, which reflect the 10–20-fold reduction in urinary corticosterone that we have observed. Increased adrenal mass, a hallmark of CAH, is because of a lack of negative feedback control by corticosterone of the HPA axis. This leads to hypertrophy of corticotropes in the anterior pituitary, which in turn produce ACTH, the main trophic factor for the adrenal cortex and for the Cyp11b1 promoter. In null mice this promoter drives the adrenal expression of ECFP in the zona fasciculata. Much higher ECFP signal is seen in the homozygote compared with heterozygote adrenals as might be expected from the near normal urinary corticosterone values for heterozygotes. Interestingly, adrenal histopathology suggested that increased ACTH drive causes hypertrophy rather than hyperplasia of zona fasciculata cells, although further tests are needed for confirmation. In the absence of adrenal Cyp11b1 expression, null mice are unresponsive to stress stimulation as demonstrated by their plasma corticosterone concentration.

Evidence of glucocorticoid deficiency among target tissues is seen in fat beds. Just as increased endocrine and intracrine glucocorticoid activity in humans and mice cause central obesity (23, 26, 27), reduced glucocorticoid levels in null mice are associated in all male and most female adipose tissues with a reduction in fat mass. The leanness of Cyp11b1 null mice contrasts with the obesity of another model of glucocorticoid deficiency, the Pomc null mouse (28). The difference is explained by opposing effects of components of the HPA axis on energy balance. Obesity in POMC null mice is attributed to an absence of the appetite-suppressive effects of brain melanocortin receptors, whereas leanness in Cyp11b1 null mice probably reflects the absence of a direct glucocorticoid effect in adipose tissues with no measurable change in appetite. It is significant that, when glucocorticoid activity is restored in Pomc null mice, obesity is exacerbated (29, 30).

Although weights of adipose tissue were reduced in null mice, those of kidney, heart, brain, and liver were increased. Possibly these differences are linked to functional phenotypes (e.g. heart and blood pressure, kidney and polydipsia, liver and fatty acid metabolism). One unifying explanation for these observations is that glucocorticoid deficiency delays organ maturation allowing the early growth phase in perinatal life to be extended. Certainly the converse effects of glucocorticoid excess support this view (31). For example, perinatal treatment with dexamethasone reduces nephron number in adult life and promotes developmental changes in the kidney, which are responsible for urinary concentration (32, 33).

Excess glucocorticoids are known to be diabetogenic because they antagonize the actions, synthesis, and secretion of insulin (21, 34, 35). We anticipated that Cyp11b1 null mice would have the opposite phenotype. Unexpectedly, neither male nor female null mice showed improvements in glucose tolerance tests, and in fact, males appeared to be glucose-intolerant. This impairment could not be explained by reduced insulin actions nor were there genotypic differences in the expression of the insulin gene in pancreatic tissue. As the synthesis and actions of insulin were unaffected in null mice but plasma insulin levels were lower, we then considered whether other steroidogenic actions might account for the effect on glucose-mediated insulin secretion. A key mediator in the control of insulin secretion is Sgk1, which has a direct genomic effect on the expression of K+ chan-

| Sex/genotype | Food intake | Fluid intake | Urine volume | Urinary sodium | Urinary potassium | Urinary Na/K |
|--------------|-------------|--------------|--------------|----------------|------------------|-------------|
| Male/null    | 0.13 ± 0.01 | 0.32 ± 0.03  | 0.15 ± 0.02  | 11.9 ± 1.08    | 9.4 ± 0.9        | 1.26 ± 0.06 |
| Male/WT      | 0.1 ± 0.01  | 0.18 ± 0.01  | 0.10 ± 0.01  | 8.3 ± 0.6      | 7.7 ± 0.9        | 0.99 ± 0.04 |
| p value male | 0.07        | 0.03         | 0.11         | 0.15           | 0.7              | 0.03        |
| Female/null  | 0.17 ± 0.01 | 0.44 ± 0.04  | 0.22 ± 0.03  | 15.5 ± 1.1     | 15.8 ± 1.3       | 1.02 ± 0.06 |
| Female/WT    | 0.18 ± 0.02 | 0.31 ± 0.03  | 0.12 ± 0.03  | 14.2 ± 0.6     | 18.1 ± 3.7       | 0.86 ± 0.07 |
| p value female | 0.7       | 0.05         | 0.05         | 0.5            | 0.6              | 0.13        |
| p value all   | 0.9         | 0.01         | 0.01         | 0.13           | 0.7              | 0.01        |

*Table 4: Fluid balance and urinary electrolytes* Averages of daily individual values were compared by one-way ANOVA for males and females and by two-way ANOVA for overall comparisons. bwt, body weight.

| Sex/genotype | Aldosterone | Progesterone | DHEA | Testosterone |
|--------------|-------------|--------------|------|-------------|
| Male/null    | 0.016 ± 0.003 | 2.77 ± 0.75 | 16.7 ± 4.2 | 5.21 ± 0.28 |
| Male/WT      | 0.042 ± 0.01  | 0.77 ± 0.08  | 10.7 ± 2.0  | 2.74 ± 0.4  |
| p value male | 0.02         | 0.01         | 0.3      | 0.01        |
| Female/null  | 0.57 ± 0.08   | 1.27 ± 0.02  | 132.3 ± 28.9 | 1.78 ± 0.38 |
| Female/WT    | 2.51 ± 0.51   | 0.68 ± 0.15  | 73.6 ± 28.0 | 0.96 ± 0.3  |
| p value female | 0.001     | 0.01         | 0.3      | 0.03        |
| p value all   | 0.02         | 0.01         | 0.3      | 0.03        |

*Table 5: Urinary steroid profiles* Averages of average daily individual values were compared by one-way ANOVA for males and females and by two-way ANOVA for overall comparisons. bwt, body weight.
null mice show similar signs, including hypokalemia, renin suppression, and raised blood pressure. Urinary electrolytes were unaffected indicating that mice, like humans, escape from the acute sodium retaining actions of mineralocorticoids after a few days of exposure. It is interesting that hypertension was achieved without recourse to dietary sodium loading or reductions in renal mass, which we find to be obligatory in an adult model of aldosterone-dependent hypertension (44). This highlights the fact, as shown here and in other rodent models of hypermineralocorticoidism (45, 46), that chronic lifelong exposure to mineralocorticoid excess has cumulative adverse effects on blood pressure control. The outcome also appears to be more severe in female than males. This is perhaps to be expected because rodent female adrenal size and plasma corticosteroid values are generally larger than males. The causative steroid is almost certainly deoxycorticosterone, the immediate substrate for 11β-hydroxylase. Deoxycorticosterone is a relatively weak mineralocorticoid, which achieves pathophysiological significance only because its secretion is increased by 30–100-fold in null mice. The excess deoxycorticosterone leads to suppression of the renin angiotensin system, which in turn reduces the secretion of the normal mineralocorticoid aldosterone.

Clinically, CAH is characterized by increased adrenal androgen production with marked virilizing effects particularly in females. Cyp17, the gene responsible for adrenal DHEA production, is virtually absent in mouse adrenal glands, so very little androgen is produced. It has been suggested that adrenal precursors to corticosterone synthesis can stimulate testosterone synthesis by gonadal tissue (47). An alternative possibility stems from recent studies of Cyp11b1 expression in murine ovaries and testes (48). Because gonadal 11β-hydroxylase is thought to convert testosterone to 11-hydroxytestosterone, this would favor the accumulation of testosterone. Either of these processes could contribute to the modest increases in urinary testosterone in null mice. However, there are no obvious pathophysiological consequences of androgen excess, only a trend toward increased testicular mass. It seems unlikely that androgen excess contributes to abnormalities in glucose metabolism as has been noted in female CAH patients (49). First, glucose metabolism in female null mice was unaffected, and second, glucose intolerance has been noted in androgen receptor knock-out mice (50). Glucose intolerance is marked in aromatase knock-out mice, which have high testosterone levels, but this is associated with insulin resistance and obesity that are not seen in Cyp11b1 null mice (51).
The female reproductive system is profoundly affected at two levels in null mice, either of which could cause infertility. In older female null mice, endometrial hyperplasia and adenomyosis are common. It is interesting that prolonged treatment with progesterone has been used as an experimental method of inducing adenomyosis (52), that adrenal progesterone synthesis has been linked to uterine estrogen receptor expression (53), and that progesterone secretion in null mice of both sexes is increased. Because adrenal progesterone secretion is thought to contribute significantly to circulating levels (54, 55), we suggest that increases of urinary progesterone in null mice represent an accumulation of 11β-hydroxylase precursors. Similarly, in female CAH patients, urinary 17α-hydroxyprogesterone is raised and can be used as a titratable marker for restoring normal progesterone activity when treating infertility problems with glucocorticoid replacement (56).

The appearance of the ovary is radically different in null mice. There are no corpora lutea. Instead there is an accumulation of ill-defined stromal tissue, which appears to be steroidogenic as it stains positively for 3β-hydroxysteroid dehydrogenase and weakly for 17α-hydroxylase. We suggest these cells are luteinized granulosa cells that subsequently fail to luteolize because of sustained high levels of adrenal progesterone production. Normally these cells would be bound within the basement membrane of the corpus luteum. Failure to develop a basement membrane could be due to glucocorticoid deficiency. This hypothesis is supported by previous human studies which show that glucocorticoids are involved in the transition of follicles to corpora lutea following ovulation. After follicle rupture, a local increase in cortisol is required for efficient membrane repair and corpus luteum formation (57). Although it has been concluded that the increase in cortisol is caused by a shift in the local expression of 11β-hydroxysteroid dehydrogenase enzymes from type 2 (which inactivates glucocorticoid hormones) to type 1 (which regenerates glucocorticoid), it should be noted that a primary deficiency in glucocorticoid synthesis has been linked to uterine estrogen receptor expression (53), rendering adenomyosis (52), that adrenal progesterone synthesis has been demonstrated adverse effects on blood pressure control due to the mineralocorticoid activity of deoxycorticosterone. Glucose intolerance is a novel finding of Cyp11b1 null mice that, as far as we are aware, has not been reported for CAH patients. Although contraindicated for conditions of glucocorticoid deficiency, similar findings have been described for patients with hyper-aldosteronism. This would suggest patients with CAH due to 11β- but not 21-hydroxylase deficiency might have impaired insulin sensitivity because of mineralocorticoid excess. We have also found that female null mice are infertile not because of androgen excess but probably because of excess adrenal progesterone synthesis that affects both ovarian function and morphology of the uterus. Androgen-independent effects of CAH on female reproductive function have not been noted previously and might contribute to problems of menstruation and conception.

Acknowledgments—We are grateful for the assistance of Mike Millar, Sheila MacPherson, Margaret Ross, Ian Swanston, and Nik Morton.

REFERENCES
1. Speiser, P. W., and White, P. C. (2003) N. Engl. J. Med. 349, 776–788
2. Krone, N., Riepe, F. G., Gotze, D., Korsch, E., Rister, M., Commentz, I., Partsch, C. J., Grotzinger, J., Peter, M., and Sippell, W. G. (2005) J. Clin. Endocrinol. Metab. 90, 3724–3730
3. Paperna, T., Gershoni-Baruch, R., Badarneh, K., Kasinetz, L., and Hochberg, Z. (2005) J. Clin. Endocrinol. Metab. 90, 5463–5465
4. Barr, M., Mackenzie, S. M., Wilkinson, D. M., Holloway, C. D., Friel, E. C., Miller, S., MacDonald, T., Fraser, R., Connell, J. M., and Davies, E. (2006) Clin. Endocrinol. 65, 816–825
5. Cerame, B. I., Newfield, R. S., Pascoe, L., Curnow, K. M., Nimkarn, S., Roe, T. F., New, M. I., and Wilson, R. C. (1999) J. Clin. Endocrinol. Metab. 84, 3129–3134
6. Motaghed, R., Betensky, B. P., Slowinska, B., Cerame, B., Cabrer, M., New, M. I., and Wilson, R. C. (2005) J. Pediatr. Endocrinol. Metab. 18, 133–142
7. Simm, P. I., and Zacharin, M. R. (2007) Horm. Res. 68, 294–297
8. Riepe, F. G., Tatzel, S., Sippell, W. G., Pleiss, J., and Krone, N. (2005) Endocrinology 146, 2563–2574
9. Copeland, N. G., Jenkins, N. A., and Court, D. L. (2001) Nat. Rev. Genet. 2, 769–779
10. Liu, P., Jenkins, N. A., and Copeland, N. G. (2003) Genome Res. 13, 476–484
11. Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) Nature 326, 292–295
12. Evans, A. L., Brown, W., Kenyon, C. I., Maxted, K. J., and Smith, D. C. (1994) Med. Biol. Eng. Comput. 32, 101–102
13. Ahn, D., Ge, Y., Stricklett, P. K., Gill, P., Taylor, D., Hughes, A. K., Yanagisawa, M., Miller, L., Nelson, R. D., and Kohan, D. E. (2004) J. Clin. Investig. 114, 504–511
14. Al-Dujaili, E. A. (2006) Clin. Chim. Acta 364, 172–179
15. Al-Dujaili, E. A., and Edwards, C. R. (1981) Clin. Chim. Acta 114, 277–287
16. Al-Dujaili, E. A., Hubbard, A. L., van Heyningen, V., and Edwards, C. R. (1984) J. Steroid Biochem. 20, 849–852
17. Al-Dujaili, E. A., Williams, B. C., and Edwards, C. R. (1981) Steroids 37, 157–176
18. Wulff, C., Wilson, H., Rudge, J. S., Wiegang, S. J., Lunn, S. F., and Fraser,
