CYP11B1-NULL MOUSE - A MODEL OF CONGENITAL ADRENAL HYPERPLASIA

Linda J Mullins¹, Audrey Peter¹, Nicola Wrobel¹, Judith R McNeilly², Alan S McNeilly², Emad AS Al-Dujaili¹, David G Brownstein¹, John J Mullins¹ and Christopher J Kenyon¹

From Centres for Cardiovascular Science¹ and Reproductive Biology², University of Edinburgh, Edinburgh, EH16 4TJ, United Kingdom

Address correspondence to CJ Kenyon, Centre for Cardiovascular Science, Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK; Fax: 0044 131 242 6779; E-mail: chris.kenyon@ed.ac.uk

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 cyano fluorescent protein (ECFP), was generated to investigate the underlying disease mechanisms. ECFP was expressed appropriately in the zona fasciculata of the adrenal gland and targeted knockout was confirmed by urinary steroid 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.

Since rodents do not synthesise adrenal androgens, changes in reproductive function such as genital virilisation of females were not anticipated. However, adult homozygote females were infertile, their ovaries showing an absence of corpora lutea and a central proliferation of disorganised steroidealogenic tissue. Null females responded normally to superovulation, suggesting that raised systemic progesterone levels also contribute to infertility problems. The model reveals previously unrecognised 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 (DOC), 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 main glucocorticoid in humans because Cyp17 is expressed in the zona fasciculata. Unlike rodents, human adrenals produce significant amounts of the androgen dehydroepiandrosterone (DHEA).

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 towards 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 which is of greatest concern (5-7). Rodents, which do not synthesise 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 due to the lack of both mineralocorticoid and glucocorticoid hormones (8). In the present 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 pathophysiologies associated with altered steroid hormone activity. We report anomalies in steroid hormone profiles, glucose tolerance, blood pressure, salt handling and reproductive performance, and suggest that the Cyp11b1-knockout mouse represents a good model of CAH.

**EXPERIMENTAL PROCEDURES**

*Generation of 11β-hydroxylase null mice- A BAC clone, (ASBAC, a gift from Prof. K. Parker), which has a 125kb insert and spans the entire Cyp11b2 – Cyp11b1 locus, with approximately 60kb 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 to 7 with an IRES-ECFP cDNA and neomycin/kanomycin selection cassette while leaving the Cyp11b2 gene untouched, was achieved through BAC recombineering (9,10). The targeting construct contained an 11β-hydroxylase-specific, PCR-generated, 780bp 5’ homology arm spanning exons 1-2 of Cyp11b1, and a 644bp 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 3 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-terminal, and a 10 amino acid c-myc epitope (9E10) was placed at the N-terminal. 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 selected using ....*
over four consecutive days. Urine was collected and frozen for later analysis of steroid hormones and electrolytes.

**Steroid profile determination**- Urine samples were analysed for corticosterone, aldosterone, deoxycorticosterone, DHEA and testosterone by in house ELISAs using previously characterised 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 analysed for plasma renin concentration (PRC), angiotensinogen, corticosteroids, triglycerides and insulin as previously described (19). Plasma ACTH was measured by radioimmunoassay (MP Biomedicals, NY). Plasma electrolytes were analysed using ion-selective electrodes (Roche Electrolyte Analyser).

**Quantitative real-time PCR analysis**- Total RNA was extracted from various tissues using Trizol reagent (Invitrogen, UK). The RNA was treated with DNA-free (Ambio, USA), quantified by spectrophotometer, and its integrity confirmed on an agarose gel. 3µg RNA was reverse transcribed, using the Superscript II first strand cDNA synthesis kit (Invitrogen, UK), 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 normalised to 18S for adrenal samples.

Liver function was assessed by pre-optimised RT-PCR assays specific for phosphoenolpyruvate carboxykinase (*Pepck*), tyrosine amino transferase (*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 (*Thp*) or *wbcr1* (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 hydroxyxylase (a kind gift of Celso Gomez-Sanchez) or anti-GFP (Beckton Dickenson mouse IgG2a monoclonal; JL-8) 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 5h in Bouin’s solution and then transferred to 70% ethanol. Immunostaining of ovary sections was carried out as previously described (20). To assess cell proliferation in ovaries of null mice, BrdU (Sigma, Poole, UK; 0.1mg/0.1ml saline) was injected ip 2h before sacrifice.

**Fluorescence microscopy**- Tissues were removed and immediately snap-frozen in OCT embedding medium on dry ice. Frozen 10µm 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 5h fast, in serial blood samples taken at 0, 30, 60 and 120 min following administration of glucose (2g/kg body weight, ip). 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 ip insulin (1U/kg body weight Humulin S; Lilly, Basingstoke, Hampshire, U.K.) 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, Crysall Chem Inc, Illinois, USA). *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 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 ± SEM.

**RESULTS**

**Generation of knockout model**- The targeting construct (Figure.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 3-frame STOP-IREs motif, and sequence encoding a 20 amino acid farnesylation 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 E.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 4 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 FISH analysis. One chimaera from each homologously targeted ES line was found to be capable of germline transmission, resulting in the generation of two independent knockout lines (designated LM1B and LM2B. Only experiments on line LM1B are described).

**Targeting**- Figure 1 summarises 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, whilst the substrate, deoxycorticosterone, was dramatically increased by 27 and 44 fold (Figure. 1b). Steroid levels in urine collected from heterozygotes was not significantly different from those in wild type mice though 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 RTPCR 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 homozygote null mice and vice versa for wild type mice (Figure 1c). As expected, a strong fluorescence signal of the farnesylated ECFP marker was detected in cell membranes of adrenals of null mice (Figure. 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 (Figure. 2a and Table 2). Histological examination indicated that the zona fasciculata (ZF) is wider because of cell hypertrophy (Figure. 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 difference 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 (Figure. 2c). The cause of CAH in patients is activation of the HPA axis due to lack of negative feedback control. The pituitary glands of null mice appeared hypertrophied and preliminary immunochemical evidence indicated increased numbers of corticotropes (unpublished data) 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 artefact caused by the reduced adiposity of null mice since differences were significant even when uncorrected for variations in body weight. Brain growth and development occurs in late gestation and early postnatal life, with glucocorticoids signalling 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,
phosphoenol-pyruvate kinase or tyrosine amino transferase mRNA levels were observed in null animals by RT-PCR. Glucocorticoids are known to antagonise insulin actions and to inhibit insulin secretion. We therefore expected Cyp11b1 null mice to show increased clearance of plasma glucose following an ip injection of glucose and a quicker response to insulin in fasted mice. However, GTT responses were indistinguishable between null and wild type female mice, while male null mice were glucose intolerant (Figure. 3a – upper panel). Since the response to insulin injection was similar for both wild-type and null animals (Figure 3a – 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; NS) 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 (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 due to electrolyte imbalance in null mice caused by 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 considered whether glucose intolerance was due to electrolyte imbalance in null mice caused by excess mineralocorticoid activity. A repeat GTT on a second cohort of null mice confirmed the original observation. The provision of KCl in the drinking fluid for seven days did improve glucose clearance but was equally effective in null and wt mice (Figure 3b).

Excess Mineralocorticoid Activity- Deoxycorticosterone, a weak mineralocorticoid, was increased more than 30 fold in null males and females (Figure. 1b). This caused hypokalemia (Table 3) and suppression of the renin angiotensin system. PRC was lower in null male mice compared with controls (629±170 cf 141±90 nmol Ang1.ml⁻¹.h⁻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 which was more marked in females than males (Table 3). Urinary sodium and potassium excretion 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 virilisation in females and only a small, non-significant increase in testicular mass.

**Progesterone excess-** Male and female null mice showed increased urinary progesterone levels (Table 5), which appears 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, though smears suggested that some of the younger null females were cycling normally and older mice treated with PMS and hCG 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 (Figure 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 PCNA negative and did not stain positively for BrdU, indicating that they were not proliferating. Immunohistochemistry, with antibodies against 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase suggested that the cells were non-secreting luteinised granulosa cells.

Older homozygote females (>8 months old) showed signs of adenomyosis. Uteri were grossly enlarged with multiple cysts (Figures 4c & d). Histopathology revealed endometrial hyperplasia in 6 month old animals (Figure 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 knockout mice is similar to that of patients carrying null mutations of 11β-hydroxylase with evidence of glucocorticoid depletion and mineralocorticoid and progesterone excess. Since rodents do not normally synthesise adrenal androgens increases in DHEA and testosterone are modest in comparison to CAH patients. This means the phenotypic consequences of Cyp11b1 knockout can be analysed without complications of the virilisation that characterises CAH patients.

The severity of CAH symptoms range from salt wasting conditions, which threaten neonatal life, to milder forms which become apparent in later life. Individuals with CYP11B1 null mutations survive because aldosterone and corticosterone synthesis, although reduced, are not wholly abolished. Residual corticosterone synthesis can be attributed to aldosterone synthase, which catalyses the conversion of DOC to aldosterone via three enzymatic functions involving hydroxylation at positions 11 and 18 and oxidation at position 18. Some corticosterone is released as an intermediate on the way to the final product, aldosterone (23). The most severe symptoms of CAH are caused by 21 hydroxylase, which compromises both aldosterone and cortisol/corticosterone synthesis and markedly affects viability of both humans and mice (24,25). Cyp11b1 null mice show clear signs of glucocorticoid deficiency, which reflect the ten- twenty fold reduction in urinary corticosterone that we have observed. Increased adrenal mass, a hallmark of CAH, is due to 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 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 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 Pome 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 (eg 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 antagonise 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+ channels and Na+/K+ ATPase sub-units that trigger
the exocytotic release of insulin in response to glucose (22). Both glucocorticoids and mineralocorticoids are known to stimulate Sgk1 expression (36). However, no difference in pancreatic Sgk1 expression was seen between null and wt mice.

It has been suggested that mineralocorticoid excess can affect glucose homeostasis by indirect effects on electrolyte balance. Insulin sensitivity of peripheral tissues is reduced by treatment with mineralocorticoids (37,38) and it has been suggested that insulin secretion in patients with hyperaldosteronism is impaired due to hypokalemia (39,40). We speculated that dietary potassium supplementation which is used to mitigate the adverse effects of mineralocorticoids (41) would improve glucose tolerance. Dietary potassium did improve glucose tolerance in null mice but was equally effective in wild type mice indicating perhaps novel effects of potassium which are independent of mineralocorticoid activity.

The effects of glucocorticoid deficiency on known hepatic glucocorticoid-inducible genes were negligible as Pepck, Agt and Tat mRNA levels were unaffected. A similar lack of effect has been described in adult glucocorticoid receptor knockout mice (42) leading to the conclusion that other processes compensate. For example, gluconeogenic enzymes and angiotensinogen are positively and negatively controlled by multiple endocrine factors with insulin’s negative influence dominating over the positive effects of cyclic AMP and glucocorticoids (43). It would seem likely, therefore, that, when glucocorticoids and insulin are both reduced, homeostasis is maintained with no net change in gluconeogenic processes.

Mineralocorticoid excess leading to hypertension is an important characteristic of patients with CAH due to 11β-hydroxylase deficiency. Cyp11b1 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 hypermineralocorticidism (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 since 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 characterised by increased adrenal androgen production with marked virilising 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). Since gonadal 11β-hydroxylase is thought to convert testosterone to 11 hydroxyltestosterone, this would favour 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 towards increased testicular mass. It seems unlikely that androgen excess contributes to abnormalities in glucose metabolism as has been noted in female CAH patients (49). Firstly, glucose metabolism in female null mice was unaffected and, secondly, glucose intolerance has been noted in androgen receptor knockout mice (50). Glucose intolerance is marked in aromatase knockout mice which have high testosterone levels but this is associated with insulin resistance and obesity which 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. Since 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 17 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 which subsequently fail to luteolise 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 as in Cyp11b1 null mice would pre-empt any subtle differences in glucocorticoid metabolism (58).

Whether or not glucocorticoid deficiency or adrenal progesterone excess are responsible for the pathology of ovaries of null mice, it is unlikely that pregnancy will be sustained in the absence of corpora lutea. The corpus luteum is normally the source of high levels of progesterone during pregnancy. Although 3β HSD is expressed in the ill-defined stromal tissue of ovaries from null mice, neither the intensity of immunostaining nor the general appearance of this tissue would suggest significant steroidogenic activity. Failure to produce sufficient ovarian progesterone may be a contributory factor in the infertility of Cyp11b1 null females. A further consideration is whether older female null mice continue to ovulate. Normally, ovulation is triggered by a surge in luteinising hormone, which is controlled by gonadotropin releasing hormone (GnRH). Since GnRH is negatively controlled by progesterone, one could speculate that long term increased adrenal progesterone in Cyp11b1 null mice would dampen GnRH, reduce LH pulsatile secretion and prevent ovulation (59). It is interesting, therefore, that Cyp11b1 null females can be made to ovulate when endogenous gonadotropin release is bypassed with exogenous pregnant mare’s serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) treatment.

In conclusion, we have created a mouse-model of CAH without the insidious virilising effects of adrenal androgen production which characterises CAH in humans. In addition to confirming glucocorticoid deficiency we have demonstrated adverse effects on blood pressure control due to the mineralocorticoid activity of deoxycorticosterone. Glucose intolerance is a novel finding of Cyp11b1 null mice which as far as we are aware, has not been reported for CAH patients. Although contra-indicated for conditions of glucocorticoid deficiency, similar findings have been described for patients with hyperaldosteronism. This would suggest patients with CAH due to 11β- but not 21-hydroxylase deficiency might have impaired insulin sensitivity due to 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 which 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.

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**FIGURE LEGENDS**

**Figure 1. ECFP substitutes Cyp11b1 in null mice**

a) Targeting construct used for BAC recombination
b) Urinary corticosterone and deoxycorticosterone of wt, heterozygous and homozygous male (upper 
panels) and female (lower panels) animals. Values shown are means ± SEM, * - indicates significant 
differences (P < 0.001) compared with wt and heterozygote values.
c) Immunohistochemistry of sections of adrenal glands from wt (left-hand panels) and null (right-hand 
panels). Images show staining using anti-GFP (upper panels) and anti-11β-hydroxylase (lower panels) 
antibodies.
d) Confocal microscopy showing fluorescence in null but not wt females adrenal gland (i) wt; ii) 
heterozygous; iii) homozygous

**Figure 2. Adrenal Hypertrophy**

2a) Gross anatomy of wt (left panel) and null (right panel) adrenal glands
2b) Histological sections of wt (left hand panels and null (right-hand panels) adrenal glands showing 
extent of zona fasciculata (ZF) (upper panels) and degree of hypertrophy (lower panels). Size markers are 
shown in the top left hand corner of each image.
2c) Attenuated stress response in male Cyp11b1-null mice subjected to serial blood sampling protocol 
during glucose tolerance test. Values shown are means ± SEM. * - indicates significant difference (P < 
0.001) of plasma corticosterone values from null compared with wild type mice at each time point.

**Figure 3. Glucose and insulin tolerance tests**

3a) Upper panels show responses of male and female animals to glucose injection (ip; downward arrows). 
Lower panels show response of male and female animals to insulin injection (ip; upward arrows). (Open 
circles – wt; filled circles – null animals). Values shown are means ± SEM. ANOVA with repeated 
measurements 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.
3b) Glucose tolerance tests carried out before and after high K+ diet in male mice. Values shown are 
means ± SEM. When all data were analysed using a General Linear Model of ANOVA, both genotype 
and dietary potassium significantly affected glucose tolerance (P < 0.001) without interacting. When 
analysed 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.
Figure 4. Pathology of ovary and uterus in Cyp11b1 null mice.
4a) Sections through the ovary of 5-month-old virgin wt and homozygote null females. CL marks corpus luteum in wt ovary. Antral follicles are present in the null female ovary but CL are absent.
4b) Sections through uteri from wt (upper panels) and homozygote null (lower panels) females cut in cross-section (left panels) or longitudinally (right hand panels). The null females have severe endometrial hyperplasia.
4c) Gross anatomy of uterus (weight - 1.36g; length each horn – 4cm), taken from null female with severe adenomyosis.
4d) Subgross appearance of uterine serosal surface from a null female with severe adenomyosis. The surface is cobbled by multiple fluid-filled cysts.
Figure 1

Cyp11b2

AC

Cyp11b1

11β-hydroxylase
Report fragment

IRES-EGFP loxP neo

a-GFP a-GFP

a-11β Hydoxylase a-11β Hydoxylase

+/- +/- -/-

Daily Urinary Excretion

nmol / g bwt

Corticosterone

Deoxycorticosterone

MALE

FEMALE

Cyp11b1 Genotype

Daily Urinary Excretion

nmol / g bwt

Corticosterone

Deoxycorticosterone

MALE

FEMALE

Cyp11b1 Genotype

wt null

a-GFP a-GFP

a-11β Hydoxylase a-11β Hydoxylase

(i) (ii) (iii)
Figure 2

a.

![Images of tissue samples](image)

b.

![Microscopic images of tissue sections](image)

c.

![Graph showing plasma corticosterone levels](image)

- **Figure 2**

  a. Images of tissue samples showing wild type (wt) and null (null) samples.

  b. Microscopic images of tissue sections with annotations for 50µm and 10µm scales.

  c. Graph depicting plasma corticosterone levels over time following glucose ip inj for wild type and Cyp11b1 null samples.
Figure 3

MALE

Glucose Tolerance Test

FEMALE

Insulin Tolerance Test

Plasma Glucose (mM)

Time (min)

Time (h)
Figure 4
<table>
<thead>
<tr>
<th>Primers and probes</th>
<th>Homology arm primers</th>
<th>PCR screen primers</th>
<th>RT-PCR primers and probe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ex1for GACATCAGAGTCGACGACAATGGCTCTCAGGGGTGACAACAG</td>
<td>11BWT for AGAGACCTTTGAGGTAGGTGATGACTGC</td>
<td>11Bex3for ATGGACTTTTCAGTCCAGTGTGTC</td>
</tr>
<tr>
<td></td>
<td>ex2rev CAGACTTAATTAATTACATTCCCAAGGGCATGCGGCAAG</td>
<td>11BKO for TTGGCTGGACGTAATCTCTCCTCA</td>
<td>11Bex4rev GCGGTCCCAAAGAAAGA</td>
</tr>
<tr>
<td></td>
<td>ex8for GACTATCGGCGTTCCATAGACAGTCCTCAATGGAATCTG</td>
<td>11B rev GAACTGCCCAGTTGTGAATGTGCTACA</td>
<td>11B34FAM probe 6-FAM-TACCATAGAAGCTAGCC-MGB</td>
</tr>
<tr>
<td></td>
<td>ex9rev GATCAGCGGCGGCGAGGCCATCCGCACATCTCTCTTTC</td>
<td>BAC1 for ACAGATGCGTAAAGGAGAAATAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAC1 rev CGCCCTATAGTGATCGTATTAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAC2 for GCACGACAGGTTCGACTCTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAC2 rev TAAATGCTTTGGGTAAATCAGGTCATA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT-PCR primers and probe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11Bex3for ATGGACTTTTCAGTCCAGTGTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11Bex4rev GCGGTCCCAAAGAAAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11B34FAM probe 6-FAM-TACCATAGAAGCTAGCC-MGB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Tissue weights of wt and null mice.
Values shown are means ± SEM. Genotype effects were tested by one way ANOVA for males and females and by two way ANOVA for overall comparisons.

<table>
<thead>
<tr>
<th>Sex/Genotype</th>
<th>Adrenal mg/g bwt</th>
<th>Heart mg/g bwt</th>
<th>Liver mg/g bwt</th>
<th>Brain mg/g bwt</th>
<th>Kidney mg/g bwt</th>
<th>Mesenteric Adipose mg/g bwt</th>
<th>Gonadal Adipose mg/g bwt</th>
<th>Subcutaneous Adipose mg/g bwt</th>
<th>Brown Adipose mg/g bwt</th>
</tr>
</thead>
<tbody>
<tr>
<td>male/null</td>
<td>0.095 ± 0.01</td>
<td>7.15 ± 0.21</td>
<td>56.0 ± 1.1</td>
<td>15.1 ± 0.5</td>
<td>7.51 ± 0.21</td>
<td>7.08 ± 0.77</td>
<td>15.8 ± 1.9</td>
<td>11.3 ± 0.8</td>
<td>4.21 ± 0.33</td>
</tr>
<tr>
<td>male/wt</td>
<td>0.033 ± 0.004</td>
<td>5.90 ± 0.08</td>
<td>45.6 ± 2.1</td>
<td>12.9 ± 0.7</td>
<td>6.10 ± 0.9</td>
<td>15.15 ± 2.480</td>
<td>24.7 ± 2.7</td>
<td>15.51 ± 2.30</td>
<td>6.70 ± 0.48</td>
</tr>
<tr>
<td>P value male</td>
<td>0.001</td>
<td>0.01</td>
<td>0.03</td>
<td>0.03</td>
<td>0.001</td>
<td>0.02</td>
<td>0.03</td>
<td>0.02</td>
<td>0.005</td>
</tr>
<tr>
<td>female/null</td>
<td>0.289 ± 0.067</td>
<td>7.06 ± 0.52</td>
<td>49.4 ± 2.7</td>
<td>nm</td>
<td>6.69 ± 0.46</td>
<td>6.56 ± 1.21</td>
<td>32.8 ± 1.9</td>
<td>6.5 ± 1.21</td>
<td>2.58 ± 0.31</td>
</tr>
<tr>
<td>female/wt</td>
<td>0.132 ± 0.01</td>
<td>5.90 ± 0.27</td>
<td>40.5 ± 2.7</td>
<td>nm</td>
<td>6.68 ± 0.33</td>
<td>14.46 ± 1.71</td>
<td>32.2 ± 1.71</td>
<td>14.46 ± 1.71</td>
<td>3.39 ± 0.27</td>
</tr>
<tr>
<td>P value female</td>
<td>0.08</td>
<td>0.11</td>
<td>0.05</td>
<td>nm</td>
<td>1.0</td>
<td>0.003</td>
<td>0.8</td>
<td>0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>P value all</td>
<td>0.004</td>
<td>0.02</td>
<td>0.02</td>
<td>nm</td>
<td>0.3</td>
<td>0.001</td>
<td>0.02</td>
<td>0.02</td>
<td>0.003</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Sex/Genotype</th>
<th>Systolic Blood Pressure mmHg</th>
<th>Plasma [Na⁺] mM</th>
<th>Plasma [K⁺] mM</th>
<th>Haematocrit %</th>
</tr>
</thead>
<tbody>
<tr>
<td>male/null</td>
<td>128.7 ± 4.5</td>
<td>146.6 ± 3.9</td>
<td>6.42 ± 0.29</td>
<td>47.3 ± 1.2</td>
</tr>
<tr>
<td>male/wt</td>
<td>115.8 ± 4.7</td>
<td>150.0 ± 1.2</td>
<td>7.36 ± 0.36</td>
<td>47.4 ± 0.9</td>
</tr>
<tr>
<td>P value male</td>
<td>0.05</td>
<td>0.4</td>
<td>0.09</td>
<td>0.9</td>
</tr>
<tr>
<td>female/null</td>
<td>136.9 ± 3.2</td>
<td>146.7 ± 2.0</td>
<td>4.73 ± 0.18</td>
<td>40.3 ± 0.4</td>
</tr>
<tr>
<td>female/wt</td>
<td>113.0 ± 2.2</td>
<td>145.0 ± 2.1</td>
<td>6.34 ± 0.29</td>
<td>40.5 ± 1.4</td>
</tr>
<tr>
<td>P value female</td>
<td>0.001</td>
<td>0.6</td>
<td>0.001</td>
<td>0.9</td>
</tr>
<tr>
<td>P value all</td>
<td>0.001</td>
<td>1.0</td>
<td>0.003</td>
<td>0.8</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Sex/Genotype</th>
<th>Food intake g/g bwt</th>
<th>Fluid intake ml/g bwt</th>
<th>Urine Volume ml/g bwt</th>
<th>Urinary Sodium ml/g bwt</th>
<th>Urinary Potassium m/g bwt</th>
<th>Urinary Na/K</th>
</tr>
</thead>
<tbody>
<tr>
<td>male/null</td>
<td>0.13 ± 0.015</td>
<td>0.39 ± 0.03</td>
<td>0.15 ± 0.02</td>
<td>11.9 ± 1.08</td>
<td>9.4 ± 0.9</td>
<td>1.26 ± 0.06</td>
</tr>
<tr>
<td>male/wt</td>
<td>0.01 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>8.3 ± 0.6</td>
<td>8.7 ± 0.9</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>P value male</td>
<td>0.07 0.03</td>
<td>0.11 0.15</td>
<td>0.7 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female/null</td>
<td>0.17 ± 0.01</td>
<td>0.44 ± 0.04</td>
<td>0.22 ± 0.03</td>
<td>15.5 ± 1.1</td>
<td>15.8 ± 1.3</td>
<td>1.02 ± 0.06</td>
</tr>
<tr>
<td>female/wt</td>
<td>0.18 ± 0.02</td>
<td>0.31 ± 0.03</td>
<td>0.12 ± 0.03</td>
<td>14.2 ± 0.6</td>
<td>18.1 ± 3.7</td>
<td>0.86 ± 0.07</td>
</tr>
<tr>
<td>P value female</td>
<td>0.7 0.05</td>
<td>0.05 0.05</td>
<td>0.5 0.6</td>
<td></td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>P value all</td>
<td>0.9 0.01</td>
<td>0.01 0.13</td>
<td>0.7 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**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.

<table>
<thead>
<tr>
<th>Sex/Genotype</th>
<th>Aldosterone (nmol.g⁻¹.d⁻¹)</th>
<th>Progesterone (nmol.g⁻¹.d⁻¹)</th>
<th>DHEA (nmol.g⁻¹.d⁻¹)</th>
<th>Testosterone (nmol.g⁻¹.d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>male/null</td>
<td>0.016 ± 0.003</td>
<td>2.77 ± 0.75</td>
<td>16.7 ± 4.2</td>
<td>5.21 ± 0.28</td>
</tr>
<tr>
<td>male/wt</td>
<td>0.042 ± 0.01</td>
<td>0.77 ± 0.08</td>
<td>10.7 ± 2.0</td>
<td>2.74 ± 0.4</td>
</tr>
<tr>
<td>P value male</td>
<td>0.02</td>
<td>0.01</td>
<td>0.3</td>
<td>0.01</td>
</tr>
<tr>
<td>female/null</td>
<td>0.57 ± 0.08</td>
<td>1.27 ± 0.02</td>
<td>132.3 ± 28.9</td>
<td>1.78 ± 0.38</td>
</tr>
<tr>
<td>female/wt</td>
<td>2.51 ± 0.51</td>
<td>0.68 ± 0.15</td>
<td>73.6 ± 28.0</td>
<td>0.96 ± 0.3</td>
</tr>
<tr>
<td>P value female</td>
<td>0.001</td>
<td>0.01</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>P value all</td>
<td>0.02</td>
<td>0.01</td>
<td>0.3</td>
<td>0.03</td>
</tr>
</tbody>
</table>
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

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