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Wnt-4 Deficiency Alters Mouse Adrenal Cortex Function, Reducing Aldosterone Production

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Wnt-4 is a signaling factor with multiple roles in organogenesis, a deficiency that leads to abnormal development of the kidney, pituitary gland, female reproductive system, and mammary gland. *Wnt-4* is expressed in the cortical region of the developing adrenal gland from embryonic d 11.5 onward, especially in the outermost part. Expression of *Cyp11B2* and preadipocyte factor 1 is lowered in the glands of *Wnt-4* mutant animals, resulting in significantly reduced aldosterone production in the newborn mutants, suggesting that Wnt-4 may be needed for proper formation of the zona glomerulosa. On the other hand, both proopiomelanocortin-derived peptide β-endorphin and corticosterone concentration levels are elevated in Wnt-deficient mice, and the expression of *Cyp17* is altered in *Wnt-4* mutant females, so that it mimics the pattern specific for males. Finally, some cells that are positive for *Cyp21*, which is normally expressed only in the adrenal gland, are found in the gonads of *Wnt-4*-deficient embryos, indicating that Wnt-4 may play a role in cell migration or in the sorting of adrenal and gonadal cells during early development. In summary, these results point to a role for Wnt-4 in adrenal gland development and function. (*Endocrinology* 143: 4358–4365, 2002)

THE ADRENAL GLAND is composed of two tissues of different embryological origin: a mesodermal cortex and a neural crest ectoderm-derived medulla (1). The cortex forms the major part of the gland and is divided into three layers in mammals: the zona glomerulosa immediately beneath the capsule, followed by the zona fasciculata and the innermost zona reticularis. The last-mentioned zone is replaced in mice by the X-zone, which develops prenatally and begins to degenerate at pubertal maturity in males and during the early phase of the first pregnancy in females (2).

As the cortex maintains water, electrolyte, and carbohydrate balances, its proper functioning is vital. Dozens of steroidal compounds, collectively referred to as corticosteroids, have been isolated from the cortex, but only a few of them have biological activity. The production of these corticosteroids, which can be divided into mineralocorticoids, glucocorticoids, and androgens, is regulated by the renin-angiotensin system and hypothalamic-pituitary-adrenal axis. The main mineralocorticoid, aldosterone, is synthesized in the zona glomerulosa, whereas the zona fasciculata and zona reticularis produce glucocorticoids such as corticosterone and cortisol. The adrenal medulla consists of ganglion and chromaffin cells, the latter producing norepinephrine, epinephrine, chromogranins, and neuropeptides, which are components of the autonomic nervous system (1, 3). Despite the different characteristics of the cortex and medulla, the adrenocortical and chromaffin cells of the medulla are intermingled and interact with each other (3).

The murine adrenal gland starts its development on embryonic day (E) 11, when the anlage of the adrenal cortex is formed (4). At the same time the first medullary cells also appear: two chains of sympathoblasts located on either side of the aorta. The cortex forms by budding from the coelomic epithelium between the mesogastrium and the urogenital fold, and the anlage is further pushed out between the mesonephros and the aorta. On E12 the cortical cells are found close to the adrenal medulla sympathoblasts, and by E14 the medullary cells are located within the adrenal gland. The capsule surrounding the adrenal gland and the cortical capillaries are also formed on E14, and both the cortex and the capsule have completed their development by E15. The murine adrenal gland is fully functional at birth (1). Key factors in adrenal cortex development are steroidogenic factor-1 (Sf1) and Wilm's tumor-1, a deficiency in which will cause a total lack of the adrenal gland (5–7). Development of the cortex is also connected with the functioning of the hypothalamus-pituitary-adrenal axis, in that a lack of CRH and its receptors will cause severe alterations in the structure of the adrenal cortex, and steroidogenic acute regulatory protein knockout mice show abnormal cellular architecture and abundant lipoid deposits in the cortex, for example (8, 9).

Wnt signaling is involved in the development of several organs. Wnts are molecules regulating growth and differentiation, and they mediate their signal via at least three pathways; the β-catenin (10) and the planar cell polarity (11, 12) pathways, which are dependent on frizzled receptor and disheveled, and the disheveled-independent, protein kinase C pathway (13). Wnt-4 is essential for the proper development and functioning of the kidney (14), pituitary gland (15), Mullerian duct, and ovary (16). *Wnt-4*-deficient mice die within 24 h of birth, probably because of kidney dysfunction, as the kidney tubules fail to form without the *Wnt-4* gene (14).

Abbreviations: DHEA, Dehydroepiandrosterone; E, embryonic day; 3β-HSDI, β-hydroxysteroid dehydrogenase type I; Pref-1, preadipocyte factor 1; Sf1, steroidogenic factor-1; wt, wild-type.

The pituitary gland of the *Wnt-4* mutant mouse is also poorly developed and is practically devoid of all cell types other than corticotropes, the cells producing proopiomelanocortin-derived hormones, such as ACTH, MSH, β-endorphin, and β-lipotropin (15). Both male and female *Wnt-4*-deficient mice lack any Mullerian duct, and the females show partial sex reversal, with oocyte degeneration (16). Finally, Wnt-4 is involved in progesterone-dependent mammary gland branching (17). We report here on the expression of Wnt-4 in the adrenal gland and show that lack of the gene leads to altered functioning of the gland as well.

Materials and Methods

The Wnt-4-deficient mouse line and genotyping of the animals

The generation of the *Wnt-4*-deficient mouse line was originally described by Stark *et al.* (14). The mice used here were of a mixed 129SV and CD-1 background, the line showing a similar phenotype to the original *Wnt-4*-deficient line 129SV (data not shown). The $Wnt\text{-}4^{+/-}$ and $Wnt\text{-}4^{+/+}$ mice were considered equal here and are referred to below as being of the wild-type (wt), because no obvious phenotype has been observed in the heterozygotes. The animals originated from crossings of *Wnt-4* heterozygous mice, and wt embryonic and newborn littermates were used as controls for the *Wnt-4* mutants. The experiments described here were approved by the local committee on animal care. The genotypes of the embryos younger than E14.5 were identified by PCR, and those of the older embryos and newborn individuals were determined by morphological criteria, as previously described (14).

Whole mount and section in situ hybridization

Embryos at given developmental stages were obtained by dissection of pregnant mice. The day when the plug was detected was designated d 0.5 of gestation. The tissues collected were fixed in 4% paraformaldehyde overnight, dehydrated in a graded methanol series, and stored in 100% methanol at −20 C for whole mount *in situ* hybridization. For section *in situ* hybridization, the fixed samples were dehydrated in an ethanol series, washed with xylene, and embedded in paraffin. Finally, the tissues were sectioned at 5 or 7 μm and placed on glass slides (Menzel-Glaser, Freiburgh, Germany) treated with triethoxysilylpropylamine (Sigma, St. Louis, MO).

The probes were *Wnt-4* (14), *Cyp21* and *Cyp11B2* (Keith Parker, University of Texas Southwestern Medical Center, Dallas, TX), *Hsd3b1* (Robin Lovell-Badge, National Institute for Medical Research, London, UK), *Sf1* (18) and rat *preadipocyte factor 1* (*Pref-1*; Hiroshi Takemori, Osaka University Medical School, Suita, Japan) cDNAs or parts of them, and the *Cyp17*-expressed sequence tag clone AA822113 (National Center for Biotechnology Information).

The riboprobes for whole mount *in situ* hybridization were labeled with digoxigenin (digoxigenin RNA labeling kit, Roche, Indianapolis, IN), and those for *in situ* sections were labeled with digoxigenin or [^{35}S]UTP (Amersham Pharmacia Biotech, Arlington Heights, IL). The *in situ* hybridizations were performed according to standard procedures at 54 C for Pref-1 and at 65 C for the other probes (19), and hematoxylin eosin staining was performed as described by Hogan *et al.* (20). Both whole mount and *in situ* hybridization analyses were carried out for at least five independent samples in each case, and a representative one is shown here.

Morphometric studies

The adrenal glands were collected from E14.5 embryos and newborn mice and divided into four groups: wt females, wt males, *Wnt-4* mutant females, and *Wnt-4* mutant males. Two pairs of glands from each group were fixed overnight in 4% paraformaldehyde and dehydrated in an ascending series of ethanol solutions and xylenes, and the tissues were embedded in paraffin. Serial sections of 5 μm were cut, placed on microscope slides, and stained with toluidine blue. The total area of the adrenal gland was measured on each section with a digital image analysis system MCID-M4 (Imaging Research, Brock University, St. Catharines, Canada); the images were acquired with a Sony DXC-930P color video camera (Sony, Tokyo, Japan) attached to a Nikon Optiphot II microscope and a ×4 objective (Nikon, Tokyo, Japan). Tissue volume was determined by integrating the section areas over their thicknesses. The volumes of the left and right adrenal glands of each animal were added together, and volume measurements were performed on two animals in each group.

Conventional and quantitative RT-PCR

The adrenal glands of the newborn mice were collected, quickly frozen in liquid nitrogen, and stored at −80 C. Later, 5–8 dissected adrenal glands were pooled by sex and phenotype (wt female, *Wnt-4* mutant female, wt male, and *Wnt-4* mutant male) for quantitative RT-PCR, and 10–20 pieces of gland for the conventional procedure. Total RNA was isolated with the Quick Prep kit from Amersham Pharmacia Biotech, and the reverse transcriptase reaction was carried out using the First Strand cDNA synthesis kit from MBI Fermentas (Hanover, NH) or Ready-To-Go RT-PCR Beads from Amersham Pharmacia Biotech. *Cyp11B2* and *Cyp21* mRNA concentrations in the samples were measured by quantitative RT PCR analysis as described (21). Conventional RT-PCR was carried out twice for *Cyp17* using independent RNA samples and the expression of glyceraldehyde-3-phosphate dehydrogenase was used to assess the quality of the RNA. The forward and reverse primers and probes used are listed in Table 1.

Hormone analyses

Measurements were made of the concentrations of β-endorphin, aldosterone, corticosterone, and cortisol in plasma samples and of testosterone, androstenedione, and dehydroepiandrosterone (DHEA) in adrenal gland tissue samples. Blood from decapitated mice was collected into heparinized glass tubes or Microvette CB 300 KE tubes (Sarstedt, Numbrecht, Germany) and centrifuged for 10 min at 3000 rpm, and the plasma samples were stored at −20 C. The plasma samples were pooled in a manner similar to that described above (four to six samples per tube to provide a sufficient volume). Adrenal tissues were collected from newborn mice, frozen quickly, and stored at −20 C. The tissues were sonicated for 15 sec in 100 μl distilled water and washed twice with 300 μl of a solution containing 9 vol diethyl ether and 1 vol ethyl acetate. After centrifugation, the water phase was taken for protein assays using a kit from Bio-Rad Laboratories, Inc. (Hercules, CA), and the organic phases were combined, evaporated, and assayed for steroids.

The evaporated organic phase was reconstituted with 500 μl RIA buffer (DRG Instruments GmbH, Marburg, Germany), and double aliquots of 25–100 μl were subjected to DHEA and androstenedione RIAs. Duplicate 5- to 25-μl aliquots of the plasma samples were submitted to the following RIAs: aldosterone (ICN Pharmaceuticals, Inc., Costa Mesa, CA), corticosterone (DRG Instruments GmbH), cortisol (Orion DG, Espoo, Finland), and β-endorphin, also depicting the proportion of ACTH (β-endorphin RIA) (22). The detection limits were 2 pg/ml for β-

TABLE 1. Forward and reverse primers and probes used in quantitative and conventional RT-PCR

Gene	Forward
Cyp11B2	5′-TGC TGT GTG GTC CTC CCC-3′
Cyp21	5′-GAA GCA AAG GGA TGG CAA AG-3′
Cyp17	5′-GCC TGA CAG ACA TTC TG-3′
Gapd	5′-AAC GAC CCC TTC ATT GAC-3′
Gene	**Reverse**
Cyp11B2	5′-CTG AGG CCC TTG GAA GTT CA-3′
Cyp21	5′-CCA CCG ACA TGT GCA CGT-3′
Cyp17	5′-TCG TGA TGC AGT TGC CC-3′
Gapd	5′-TTC ACA CCC ATC ACA AAC-3′
Gene	**Probe**
Cyp11B2	5′-TGT TTT CCA ATG GTC ACT CCA GGT GTCC-3′
Cyp21	5′-CGA AGA GCG GCT CCA CGA GGG-3′

endorphin, 0.7 fmol/ml for dehydroepiandrosterone, 1 fmol/ml for androstenedione, 0.6 pmol/ml for aldosterone, 0.13 pmol/ml for corticosterone, 10 pg/ml for cortisol, and 1 μg/ml for total protein.

Statistical analyses

P values for the hormone and RT-PCR samples were calculated by *t* test with a two-tailed distribution and two-sample unequal variance.

Results

Wnt-4 is expressed in the cortex of the developing mouse adrenal gland

The results of the whole mount hybridizations, performed to investigate the expression of *Wnt-4* in the mouse embryo, revealed a circular area with *Wnt-4* expression next to the anterior site of the mouse mesonephros on E11.5 (Fig. 1a), similar to the case of *Sf1* expression (Fig. 1b), an early marker of the developing adrenal gland (23). By E12.5 the developing adrenal cortex was a distinct organ, and expression of *Wnt-4* and *Sf1* could clearly be seen in it (Fig. 1, c and d). Section *in situ* hybridization of samples from E14.5 and E18.5 mice showed that the expression of Wnt-4 was located in the cortical region of the adrenal gland, whereas no expression could be detected in the medulla (Fig. 1, e and f). *Wnt-4* expression in the developing adrenal gland was similar in both sexes (data not shown).

Adrenal glands of Wnt-4 mutant mice do not show distinct morphological alterations

The expression of Wnt-4 in the adrenal cortex of the mouse embryo throughout the development of the gland suggested that this factor plays a role in its development. Investigations into this using the Wnt-4 knockout mouse line (14) nevertheless failed to reveal any distinct morphological changes, such as deviant size or shape, in the adrenal glands of the *Wnt-4* mutants (Fig. 2, a and b). Volume measurements, *i.e.* morphometric studies, performed on a few adrenal glands also suggested that the adrenal glands of the wt and *Wnt-4* mutant mice were similar in size (data not shown), and hematoxylin-eosin staining did not reveal any obvious histologically anomalous structures in the *Wnt-4* mutant adrenal glands (Fig. 2, c and d).

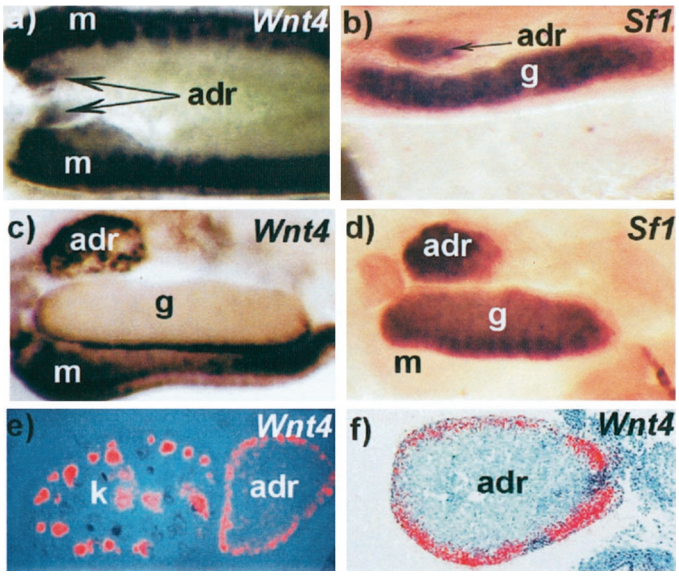

FIG. 1. Whole mount and section *in situ* hybridization results for *Wnt-4* expression in the adrenal gland. As early as E11.5 *Wnt-4* is expressed in a small, circular area close to the anterior ends of the mesonephros (a, *arrows*), similar to *Sf1* (b, *arrow*), marking the future adrenal gland. In b, the mesonephros is located beneath the gonad, which is also stained with the *Sf1* probe. On E12.5 (c), *Wnt-4* expression is localized in the developing adrenal gland, which also expresses *Sf1* (d), and on E14.5 (e) and E18.5 (f), it is detected in the cortical region of the adrenal gland. *Wnt-4* is also intensively expressed in the mesonephros and developing kidney. adr, Adrenal gland; g, gonad; k, kidney; m, mesonephros.

Impaired steroid production in the adrenal cortex of Wnt-4 mutant mice

Expression of adrenal cortical layer markers and the steroidogenic genes *Cyp21* and *Cyp11B2* was analyzed in the adrenal glands of wt and *Wnt-4* mutant newborn mice by section *in situ* hybridization and quantified by quantitative RT-PCR. The genes encode P450c21-hydroxylase (P450c21) and P450 aldosterone synthase (P450c11aldo), respectively, and their expression thus also depicts steroid production in the cortex. *Cyp21* was intensively expressed in the glands of both wt and mutant Wnt-4 mice (Fig. 3, a and b), with no detectable quantitative difference (Table 2). In contrast, both *in situ* hybridization (Fig. 3, c and d) and the real-time PCR results (Table 2) showed the expression of *Cyp11B2* to be reduced in the adrenal cortex of *Wnt-4* mutant mice. As in *Cyp11B2*, the number of cells expressing Pref-1, which is a specific marker of the zona glomerulosa in the cortex (24), was markedly lower in the cortex of *Wnt-4*-deficient mice (Fig. 3, e and f). Pref-1 is also expressed in medullae (24), as seen here in the glands of both wt and *Wnt-4* mutant mice. The male and female mice demonstrated similar expression patterns for these genes (data not shown).

Expression of the third steroidogenic gene, *Cyp17*, was first observed on E12.5 in the future adrenal glands of wt and *Wnt-4* mutant male mice and of wt and mutant female mice

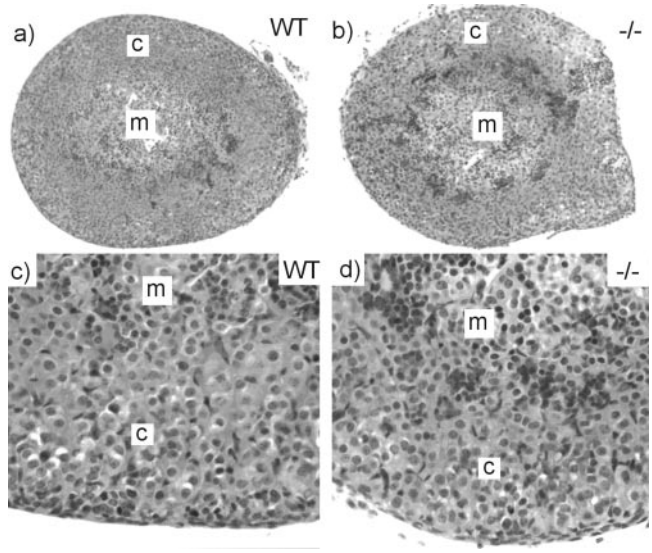

FIG. 2. Hematoxylin-eosin staining shows no striking difference in morphology between the wt (a and c) and *Wnt-4* mutant (b and d) adrenal glands at birth. −/−, *Wnt-4* mutant; c, cortex; m, medulla.

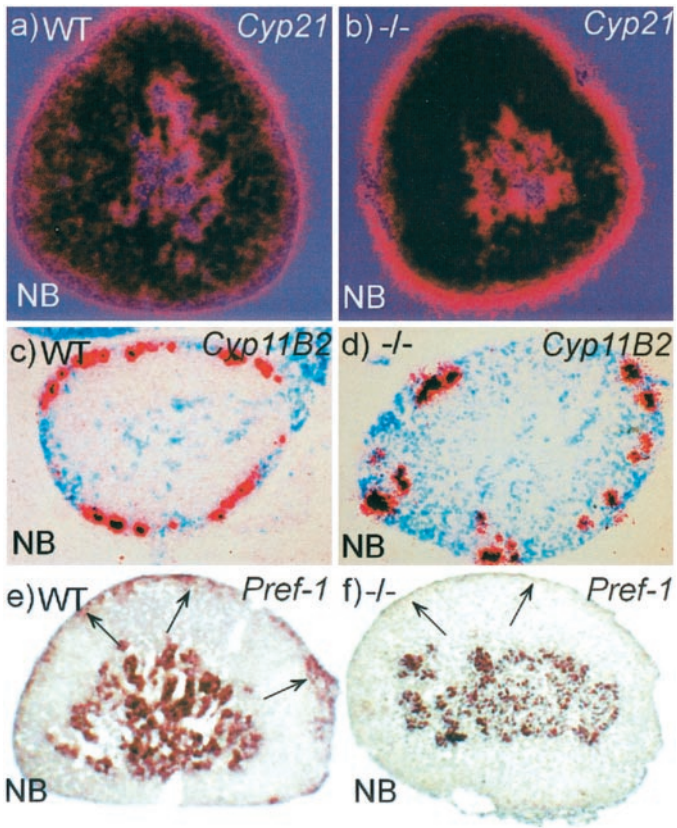

FIG. 3. Expression of *Cyp21*, *Cyp11B2*, and *Pref-1* in the adrenal glands of newborn wt and *Wnt-4 mutant* (−/−) mice. *Cyp21* is expressed throughout the cortical layers (*dark purple*) in both wt (a) and *Wnt-4* mutant (b) adrenal glands. The strong expression also causes scattering of the signal around the organ. On the other hand, fewer cells expressing *Cyp11B2* are seen in the *Wnt-4* mutant adrenal gland (d) than in tissue from a wt mouse (c). Expression of *Pref-1*, a marker of the zona glomerulosa, occurs in a continuous ring around the adrenal glands of wt mice (e, *arrows*), whereas only a few cells express the factor in the glands of *Wnt-4*-deficient mice (f, *arrows*). Pref-1 is also abundant in the medulla of the adrenal gland in both wt and mutant mice. Several successive sections were hybridized to confirm that the results shown in the figures are not due to the location of the section surface.

TABLE 2. *Cyp21* and *Cyp11B2* mRNA levels in mouse adrenal gland measured by quantitative RT-PCR

	Cyp21	*Cyp11B2*
Wnt-4 wild-type ♂	0.70 ± 0.35	2.20 ± 0.98
Wnt-4 wild-type ♀	0.45 ± 0.37	1.24 ± 0.55
Wnt-4$^{-/-}$ ♂	0.54 ± 0.39	0.41 ± 0.26
Wnt-4$^{-/-}$ ♀	0.27 ± 0.13	0.22 ± 0.19
P value ♂	0.58	<0.05
P value ♀	0.48	<0.05

Values in the table are proportional and can be compared only within the group. Three RNA pools, each containing five to eight adrenal glands, were measured in each group.

(data not shown). In all of these cases expression peaked on E14.5, as judged by *in situ* hybridization (Fig. 4, a–d), and was most intensive in the cortex of the wt females (Fig. 4c). Thereafter it continued in the adrenal glands of the wt female animals until birth (Fig. 4g), but was lost between E16.5 and E18.5 in the glands of the wt and *Wnt-4*-deficient males and *Wnt-4*-deficient females (Fig. 4, e, f, and h). Thus, the expression of *Cyp17* in the adrenal cortex of female *Wnt-4* mutant mice followed that in wt male mice. RT-PCR confirmed the results of the whole mount *in situ* hybridization, showing *Cyp17* expression to exist in the glands of newborn female wt mice, but not in those of *Wnt-4*-deficient mice, and to be more abundant on E14.5 than at birth (Fig. 4, i, j, and k). The expression was not seen in samples from adult wt mice (Fig. 4i).

To investigate further the potential dysfunction of the adrenal cortex and pituitary-adrenal axis in *Wnt-4*-deficient mice, the plasma concentrations of aldosterone, β-endorphin, corticosterone, and cortisol and the concentrations of DHEA, androstenedione, and testosterone in the adrenal gland tissue of newborn mice were measured. In agreement with the reduced *Cyp11B2* expression, the production of aldosterone was significantly lower in *Wnt-4* mutant mice, both male and female (Fig. 5a), whereas that of β-endorphin (Fig. 5b), also reflecting ACTH production, was enhanced in these animals. In addition, a statistical difference in the production of corticosterone was seen between the wt and *Wnt-4* mutant mice, both male and female (Fig. 5c). There was a high variation in corticosterone concentrations, which may be due to the effects of the circadian rhythm on corticosterone secretion. This rhythm could not be taken into account when collecting samples, because *Wnt-4*-deficient mice die soon after birth (14, 16). The concentration of DHEA was constant in all the groups measured, and cortisol, androstenedione, and testosterone were below the detection level (data not shown), suggesting that Wnt-4 deficiency does not alter the main routes of steroidogenesis in the adrenal cortex, at least after birth.

Migration and/or sorting of adrenal and gonadal cells may be disturbed in Wnt-4-deficient mice

The development of the adrenal cortex in wt and *Wnt-4* mutant embryos from E11.5 to E13.5 was studied by monitoring the expression of *Cyp21* (Fig. 6, a–f). Expression took place in a clear, circular area in the developing adrenal glands from wt mice (Fig. 6, a, c, and e), whereas in the *Wnt-4* mutant mice, cells expressing *Cyp21* were also dispersed outside the future adrenal cortex (Fig. 6, b, d, and f). Some *Cyp21*-positive cells were seen in the gonadal area (Fig. 6f), which does not normally express the gene (Fig. 6e), suggesting that the cells may be at least partially of adrenal origin. Expression patterns were similar in female and male mice (data not shown).

Hsd3b1, the gene encoding 3β-hydroxysteroid dehydrogenase type I (3β-HSDI), was strongly expressed in the developing adrenal glands of both male and female mice on E14.5 (Fig. 7, a–d), and the expression was intensive in the gonads of wt (Fig. 7a) and *Wnt-4*-deficient (Fig. 7b) males and was detectable in the gonads of female *Wnt-4*-deficient mice (Fig. 7d), but not in female wt embryos (Fig. 7c). Ectopic expression of *Hsd3b1* in the gonads of *Wnt-4*-deficient females was also distinct at birth (Fig. 7e) (16). Detailed examination showed that the 3β-HSDI-positive cells were always located at the anterior end of the ovary, close to the adrenal, in the *Wnt-4* mutant animals (Fig. 7d), as was also the case with *Cyp21* (Fig. 6f). Similarly, *Cyp17* was not ex-

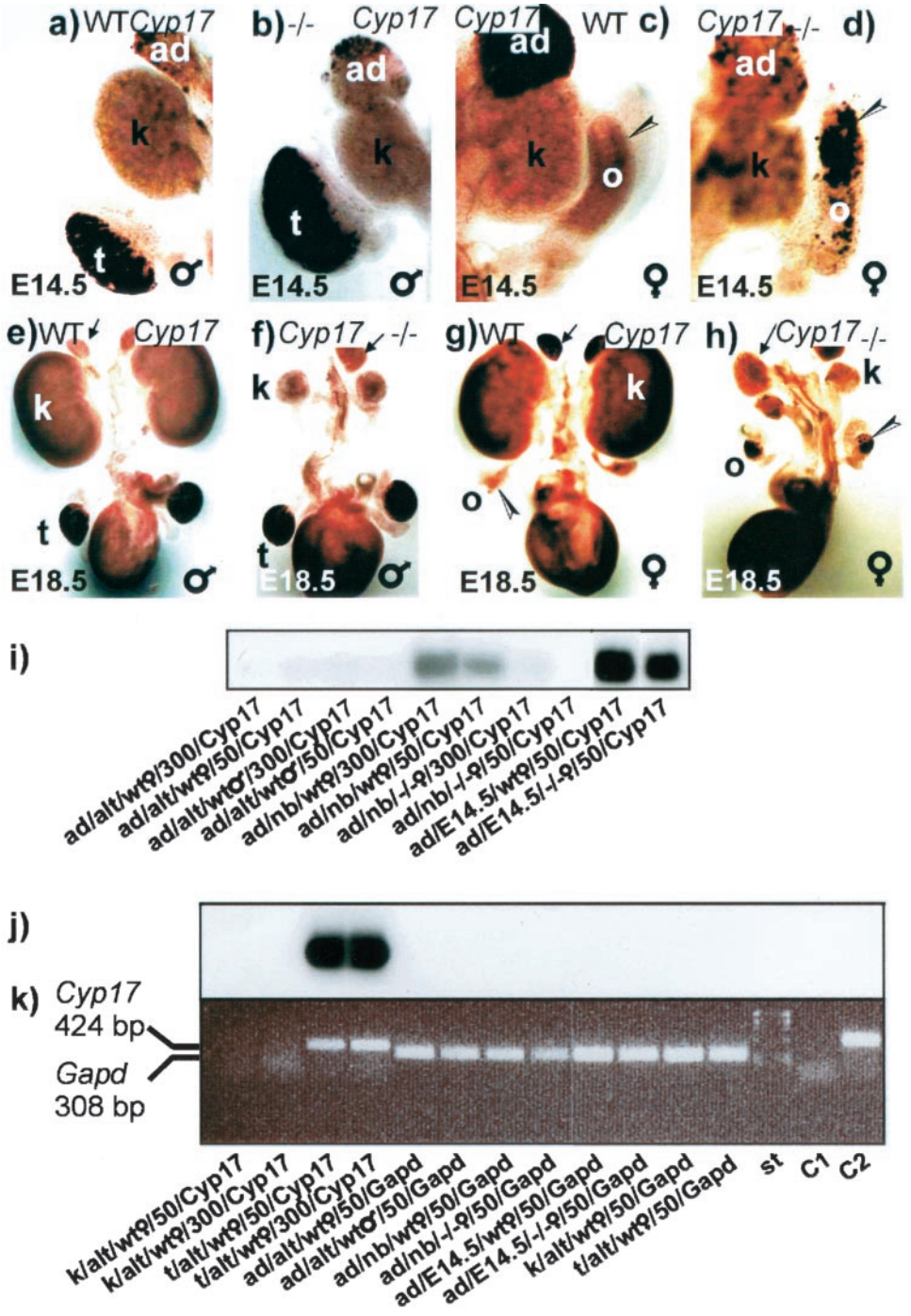

FIG. 4. Expression of *Cyp17* in the developing urogenital systems of wt and *Wnt-4*-deficient mice. On E14.5 *Cyp17* is weakly expressed in the adrenal glands of males (a and b) and *Wnt-4* mutant females (d), but is considerably more intensive in those of wt females (c). On E18.5 strong expression is seen only in the glands of wt females (g, *arrow*), but not in other samples (e, f, and h, *arrows*). *Cyp17* is also expressed in the gonads of males and *Wnt-4* mutant females (*arrowheads* in d and h), but not in those of wt females (*arrowheads* in c and g). RT-PCR also shows *Cyp17* expression in the adrenal glands of wt female mice, but not in those of *Wnt-4*-deficient mice, at birth, whereas it is abundant in both samples on E14.5 (i). Southern blotting and labeling with a *Cyp17* probe have been used to intensify the lanes (i and j). In the control samples used only the lanes amplified with *Cyp17* oligos from the testis sample are recognized (j), whereas ethidium bromide staining (k) demonstrates the quality of the RNA as tested with glyceraldehyde-3-phosphate dehydrogenase oligos and controls. C1 serves as a control for DNA contamination in the RNA sample, and C2 is a positive control for RT-PCR. ad, Adrenal gland; alt, adult; k, kidney; nb, newborn; o, ovary; st, DNA size marker; t, testis; 50, 50 ng RNA used; 300, 300 ng RNA used.

pressed in the developing ovaries of normal mice, but extensive expression was seen in the anterior area of the *Wnt-4* mutant ovary (Fig. 4, d and h).

Discussion

Wnt signaling factors regulate the development of a number of tissues, and the present findings demonstrate the involvement of Wnt-4 in the development of the adrenal cortex. On E11.5, Wnt-4 is expressed adjacent to the anterior site of the mouse mesonephros, correlating with formation of the anlage of the adrenal cortex between the mesogastrium and the urogenital fold and its protrusion between the mesonephros and the aorta. *Wnt-4* expression also correlates with that of *Sf1*, which takes place throughout the adrenal primordium on E11 (23). During further development of the adrenal gland, *Wnt-4* expression becomes established in its outermost cortical region, overlapping with that of *Cyp11B2* and *Pref-1*. Expression of *Wnt-11* has also been detected in the human adrenal cortex (25). The nuclear localization of β-catenin in the glomerulosa cells of the human adrenal cortex (26) further indicates that the Wnt pathway is activated during development of mouse and human adrenal cortex.

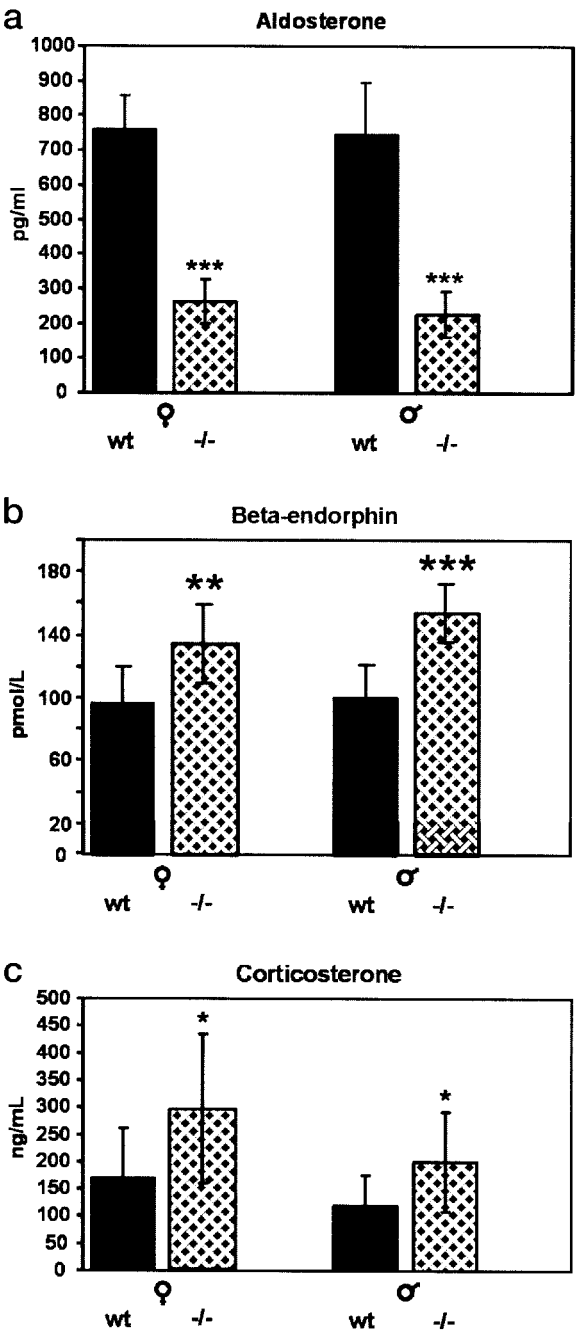

FIG. 5. Concentrations of aldosterone, β-endorphin, and corticosterone measured in the plasma of wt and *Wnt-4*-deficient (−/−) newborn mice. a, The production of aldosterone is considerably decreased in the *Wnt-4* mutant mice, both males and females ($P < 0.0001$; numbers of pools: wt males, n = 14; mutant males, n = 6; wt females, n = 13; mutant females, n = 5; each pool containing blood from six mice). b, β-Endorphin is significantly elevated in both *Wnt-4* mutant males (number of pools, n = 8; $P < 0.001$) and females (number of pools, n = 8; $P < 0.01$) relative to their wt littermates (number of pools: males, n = 7; females, n = 8). c, In addition, the elevation in corticosterone concentration is significant in the mutant females (number of pools, n = 12; $P < 0.05$; each pool containing blood from between four and six mice) when set against the wt mice (number of pools, n = 23), as is also the case in the male mice (number of pools: wt, n = 24; mutant, n = 12; $P < 0.05$). Comparison of all values for wt mice (females and males) with those for *Wnt-4* mutant mice resulted in a significant difference ($P < 0.001$).

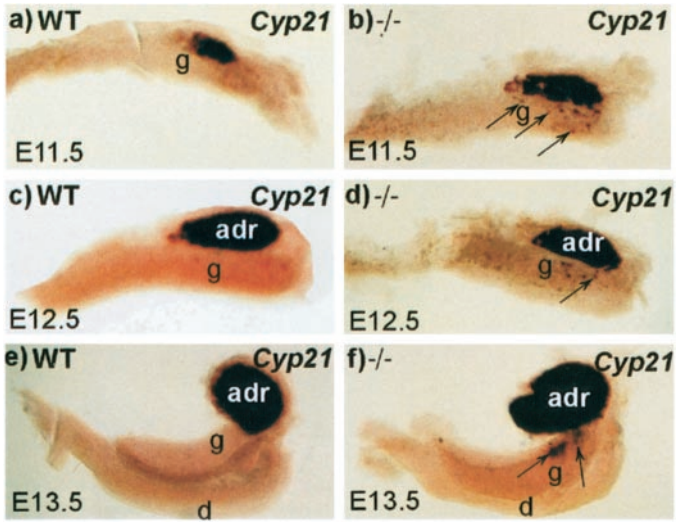

FIG. 6. Expression of *Cyp21* in the urogenital system of wt and *Wnt-4* mutant (−/−) mice, as determined by whole mount *in situ* hybridization. *Cyp21* is expressed only in the adrenal gland of wt embryos on E11.5 (a), E12.5 (c), and E13.5 (e). In the *Wnt-4* mutant urogenital systems (b, d, and f), misplacement of the *Cyp21*-positive cells can also be seen in the gonadal region (*arrows* in b, d, and f). adr, Adrenal gland; d, duct; g, gonad.

Although the adrenal glands of wt and *Wnt-4* mutant mice were similar in shape and size, it is possible that the elevated β-endorphin concentration in the plasma of the *Wnt-4*-deficient animals, also reflecting the ACTH concentration, may lead to hyperplasia and/or hypertrophy, as is the case in Cushing-like syndrome (27), overexpression of CRH (28), and glucocorticoid receptor-deficient mice (29), even though the increase in corticosterone is more conspicuous in the later cases. As *Wnt-4* mutant mice die soon after birth, it is unfortunately not possible to see how the production of corticosterone and ACTH and their effects are balanced out or whether the phenotype later shows adrenal hyperplasia or hypertrophy.

Hsd3b1, *Cyp21*, and *Cyp11B2*, which are needed for mineralocorticoid biosynthesis are expressed in the adrenal cortex of *Wnt-4* mutants, although that of *Cyp11B2* is significantly reduced, resulting in decreased production of aldosterone, the most important mineralocorticoid, which is exclusively synthesized in the zona glomerulosa (30). The principal functions of aldosterone are to stimulate sodium reabsorption in the distal tubule of the kidneys, to increase the total body sodium level, and to control the plasma potassium level. Lack of aldosterone causes reduced fluid volume, leading to hypovolemic shock and death. Hence *Wnt-4* affects control of the electrolyte balance on at least two levels, being necessary for tubule formation in the kidney (14) and for maintaining a proper concentration of aldosterone.

The present section *in situ* hybridization results demonstrated that expression of *Cyp11B2*, the gene encoding P450c11aldo, and *Pref-1*, another marker of zona glomerulosa, is limited to a small number of cells, suggesting that the zona glomerulosa has not developed normally in the adrenal glands of Wnt-4 mutants. P450c11aldo is needed for the 11β-hydroxylation of deoxycorticosterone to corticosterone

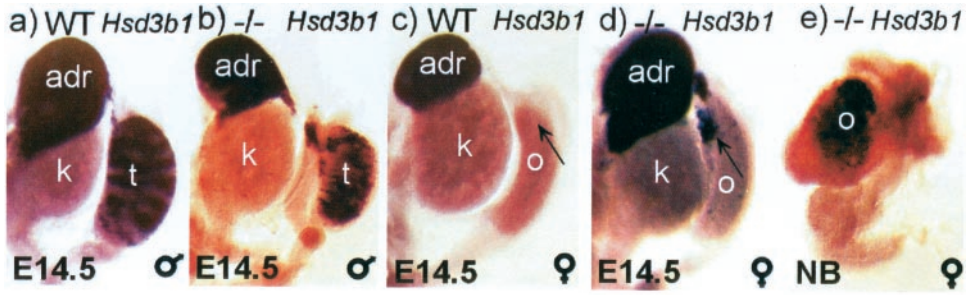

FIG. 7. *Hsd3b1* is expressed intensively in the developing adrenal gland in both sexes (a–d), and is also expressed in the testis (a and b) and in the anterior tip of the gonad in *Wnt-4* mutant females (d, *arrow*) on E14.5. Abundant ectopic expression of *Hsd3b1* is also seen in the gonads of newborn *Wnt-4* mutant females (e). *Hsd3b1* is not expressed in the wt ovary (c, *arrow*). adr, Adrenal gland; k, kidney; nb, newborn; o, ovary; t, testis.

and for the 18-hydroxylation and 18-hydroxydehydrogenation of corticosterone to aldosterone. The corticosterone concentrations in the mutant *Wnt-4* mice suggest that the reduced amount of P450c11aldo does not significantly limit corticosterone biosynthesis, but instead affects the subsequent reactions on the path toward aldosterone, resulting in an accumulation or maintenance of corticosterone and a reduction in aldosterone. The situation is parallel, even though not equal, to that prevailing in human congenital hypoaldosteronism, in which corticosterone levels are increased and aldosterone levels decreased due to specific mutations in P450c11aldo affecting its 18-hydroxylation or 18-hydroxydehydrogenation activities (31, 32).

P450c17 catalyzes the 17α-hydroxylase and 17,20-lyase reactions and is thus necessary for the biosynthesis of cortisol and androgens. Adult mice and rats do not express *Cyp17,* and therefore their main glucocorticoid is corticosterone and not cortisol as in humans (33–35). *Cyp17* expression does take place in the adrenal gland of the wt fetal mouse, however, reaching its peak on E14.5, and showing sex dependency. A pattern of *Cyp17* expression similar to that demonstrated here in the male fetal adrenal gland has also been reported by Keeney *et al.* (36), but their material was not analyzed by sex, and expression in female fetuses at birth was not reported separately. Interestingly, we saw continued expression only in the adrenal cortex of wt females, whereas the pattern in *Wnt-4*-deficient females followed that in wt males. The results suggest that Wnt-4 is also involved in the regulation of steroidogenesis, with *Wnt-4*-deficient female embryos phenocopying the male pathway. Activation of the genes for the androgen biosynthesis pathway in the ovaries, masculinization of the gonads, and partial female to male sex reversal are also typical of mutant Wnt-4 female mice (16). The role of strong *Cyp17* expression in the female adrenal gland remains open, as it was not found to affect cortisol or androgen production in the gland, at least at birth.

Expression of *Cyp21* in the urogenital area is more dispersed in *Wnt-4* mutant mice than in wt mice during embryological development. Some cells expressing *Cyp21* are also seen in the gonadal area in *Wnt-4*-deficient male and female mice, an area not normally expressing the gene, and ectopic expression of the steroidogenic enzymes 3β-HSDI and P450c17 also occurs in the gonads of female *Wnt-4*-deficient mice, leading to activation of the androgen biosynthesis pathway (16). Closer investigation of *Wnt-4* mutant ovaries demonstrated that this ectopic expression always takes place at the anterior end of the gonad. The results suggest that the cells showing such expression are also of adrenal origin, and that Wnt-4 plays a role in cell sorting. The adrenal cortex and the gonadal cells have the same origin (37), and the gonad and future adrenal gland are located immediately adjacent to each other early in their development, on E11, without any clear border. A lack of *Wnt-4* at that stage may thus allow some adrenal cells to migrate into the gonadal region, where they are able to maintain their original identity, still expressing *Cyp17* and *Hsd3b1*.

The results show that Wnt signaling plays a role in the development of the adrenal cortex. The zona glomerulosa does not develop properly in *Wnt-4*-deficient mice, which leads to a decrease in the production of aldosterone. In addition, Wnt-4 may be involved in the regulation of steroidogenesis or at least in the expression of *Cyp17*, and finally, Wnt-4 may shape cell sorting between the adrenal cortex and the gonads.

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