Growth analysis of the mouse adrenal gland from weaning to adulthood: time- and gender-dependent alterations of cell size and number in the cortical compartment

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The adrenal glands are complex endocrine organs regulating multiple physiological processes, such as metabolism, stress response, immune functions, and the cardiovascular system (5, 6, 22). Growth and function of the adrenal glands are regulated by pituitary proopiomelanocortin-derived ACTH, neurotransmitters, neuropeptides, cytokines, and growth factor networks (6, 13, 18).

Genetic engineering (9, 16, 35, 36) and random mutagenesis projects (17, 26) in the mouse provide novel models for dissecting the roles of growth factor systems and their individual components for adrenal growth processes. However, a prerequisite for this approach is detailed knowledge of physiological growth of the adrenal glands in non-transgenic mice (34).

The mammalian adrenal gland is composed of two distinct functional compartments, i.e., the cortex and the medulla. The cortex contains three histologically distinct zones, outmost the “ zona glomerulosa”, followed by the “ zona fasciculata”, and the “ zona reticularis” directly surrounding the medulla (1). These zones play distinct roles in steroid hormone production (19).

In contrast to other mammals, mice and rats do not have a functionally distinct zona reticularis because of the lack of 17α-hydroxylase expression in the adrenal gland. Therefore, adrenals from mice and other rodents are devoid of the secretion of adrenal androgens (19). A specific feature of the mouse adrenal cortex is the so-called X-zone, a putative postpartum remnant of the fetal adrenal zone (37). On the functional level, expression studies provide indirect evidence for an involvement of the X-zone in adrenal progesterone and 11-DOC metabolism (15).

Although some data regarding adrenal weights of mice have been reported in the literature, they are mostly limited to specific points of time or experimental conditions. To our knowledge, there is no systematic analysis of adrenal growth of the mouse from weaning to adulthood. Therefore, we designed a study of adrenal growth in male and female mice from 3 to 11 wk involving 1) quantitative measurements of adrenal weight and volumes of cortex and medulla; 2) quantification of the volumes of the different cortical zones; 3) determination of numbers and volumes of the zona fasciculata cells; and 4) measurements of serum corticosterone levels as a functional readout. Our dynamic quantitative morphological study of the mouse adrenal gland is an important basis for dissecting and modeling the functions of this central organ in mammalian endocrinology by using systems biology approaches.

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MATERIALS AND METHODS

Animals and tissue preparation. Naval Medical Research Institute outbred mice (albino) were crossed with mice from the inbred strain C57BL/6, and F1 offspring harboring 50% of each genetic background were studied. The mice were maintained under specified pathogen-free conditions in a closed barrier system and monitored as recommended (23). All mice had free access to a standard rodent diet (V1534; Sniff, Soest, Germany) and tap water. The diet used contained 0.59% sodium chloride and 0.97% potassium, which represents the range of standard pellet diets. At an age of 3 wk, animals were weaned and separated according to gender. At different points of time (3, 5, 7, 9, and 11 wk after birth), male and female mice (n ≥ 12/sex/age group) were weighed and killed after obtaining a blood sample under ether anesthesia. The abdominal cavity was opened, and the adrenal glands were recovered, carefully freed from adjacent tissues under a stereo dissecting microscope, and weighed individually to the nearest 0.1 mg. For stereological analyses, mice were fixed immediately after death by cervical dislocation by orthoperafusion with 4% paraformaldehyde via the left heart ventricle. After perfusion fixation, internal organs were removed, and the adrenal glands were postfixed in situ for an additional 48 h in the same fixative. Adrenal glands were subsequently excised and weighed as described above. The right adrenal glands were embedded in Epon. Epon blocks were trimmed with a TM60 Reichert-Jung milling machine (Leica, Wetzlar, Germany), and 0.5-μm semithin sections were obtained with a Reichert-Jung “Ultracut E” microtome (Leica).

The left fixed adrenal gland was weighed to the nearest 0.1 mg and embedded in paraffin wax following standard procedures.

Stereological investigations. The paraffin-embedded left adrenal gland was exhaustively sectioned on a nominal thickness of 3 μm on a microtome equipped with a section counter.

Every 20th section of the series was saved, stained with hematoxylin and eosin, and used for morphometric evaluation carried out on a Videoplan image analysis system (Zeiss-Kontron, Munich, Germany) coupled to a light microscope via a color video camera. The total number of paraffin sections sampled per adrenal gland ranged from 19 to 45. On these sections, the cross-sectional areas of cortex and medulla were planimetrically determined; cross-sectional areas of the adrenocortical zones were estimated by point counting at a 10 objective. For calibration, an object micrometer (Zeiss, Jena, Germany) was used. Volume fractions of the cortex and the medulla in the adrenal gland as well as the different zones in the cortex were determined as described in detail by Hoeflich et al. (16).

For determination of zona fasciculata cell numbers and size, the dissector method (29) was applied as described previously (16).

At least eight serial semithin sections (0.5 μm) comprising the whole organ were cut from each Epon-embedded right adrenal gland and stained with toluidine blue and safranin. From the stack of serially cut semithin sections, one section was drawn at random by means of a random number (R) between two and eight (the baseline section was not used for sampling) as a reference section in a dissector. The second section (look-up section) was sampled among numbers 2–8 by means of R ± 3, i.e., the dissector height was equivalent to the thickness of three semithin sections (1.5 μm). Five fields were chosen at random in the zona fasciculata of the reference section, and the corresponding fields were identified in the look-up section. Light microscopic images of the selected fields were acquired with a color video camera using a ×25 objective, and color prints were prepared at the same final magnification (×1,000).

A plastic transparency with equally spaced test points (n = 70) and an unbiased counting frame representing an area of 13,500 μm² was superimposed on the printed images. All profiles of zona fasciculata cell nuclei sampled in the frame in the reference section that were not present in the look-up section were counted (Q⁻). On reference sections, the number of points hitting zona fasciculata cells was counted as well as the points hitting the zona fasciculata. The operation of counting of Q⁻ (cell nuclei) was then repeated by interchanging the roles of the look-up section and the reference section, thereby increasing the efficiency by a factor of two. Given that zona fasciculata cells have only one nucleus, which is documented in the literature (10) and was also observed in this study, no cell is counted two times with this procedure.

On average, 107 nuclei (range: 55–183) were counted with the five dissector pairs per adrenal gland. The numerical density of the epithelial cells in the zona fasciculata was calculated by dividing the total number of cells counted in all dissectors in an adrenal gland by the cumulative volume of the dissectors (area of the unbiased counting frame × dissector height × number of dissectors) sampled in the adrenal gland. Assuming the same numerical density of epithelial cells in the zona fasciculata in both (right and left) adrenal glands and neglecting the small amount of tissue shrinkage when using Epon embedding, the total number of zona fasciculata cells per adrenal gland was calculated as the product of the numerical density of epithelial cells in the zona fasciculata and the volume of the zona fasciculata. The mean volume of zona fasciculata cells was obtained by dividing the volume density by the numerical density of the epithelial cells in the zona fasciculata.

Stored lipids in the adrenal gland. To address the content of stored lipids in the adrenal glands, we prepared frozen sections from the adrenal glands of eight mice (2 male, 2 female 7-wk-old mice and 2 male, 2 female 11-wk-old mice). Four sections were taken from the midregion of each adrenal gland. The sections were stained by Oil Red-O and counterstained with hematoxylin. Stored lipids are visible as red droplets, and cell nuclei appear in blue. Light microscopic images of the stained sections were acquired with a color video camera at a final magnification of ×900.

Expression of side-chain cleavage enzyme and 11β-hydroxylase in the adrenal gland. Adrenal glands from three mice per group were homogenized in extraction buffer as described previously (16), and protein content was quantified using the bicinchoninic acid method. Protein (5 μg) was separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, Eschborn, Germany). Membranes were blocked (5% dry milk and 1% Tween 20 in Tris-buffered saline) and incubated with primary antibodies (rabbit anti-side-chain cleavage enzyme, AB1244; Chemicon, Hampshire, UK; mouse anti 11β-hydroxylase, kindly provided by Dr. E. Gomez-Sanchez) overnight at 4°C at a dilution of 1:1,000. After three washings in Tris-buffered saline containing 1% Tween 20, membranes were incubated with horseradish peroxidase-coupled goat anti-rabbit IgG (1:2,000; Cell Signaling Technology, New England Biolabs, Frankfurt, Germany) or goat anti-mouse IgG (1:10,000; Dianova, Hamburg, Germany) for 1 h at room temperature. Finally, membranes were developed on a Kodak Image Station using an enhanced chemiluminescence (ECL) detection kit (ECL Advance Western Blotting Detection Kit; GE Healthcare, Freiburg, Germany). Band intensities were quantified using the ImageQuant Software package (GE Healthcare). All signal intensities were normalized for the Coomassie blue signal.

Quantification of corticosterone concentrations in blood serum. Blood samples were taken between 1300 and 1600. The animals were anesthetized individually in a glass jar containing saturated ether vapor, and retroorbital blood was collected within 30 s from initial handling within the cage.

Corticosterone was measured by a specific in-house RIA established at the Steroid Laboratory of the Department of Pharmacology using tritiated corticosterone (1,2,4,6-[^3]H)corticosterone; Amersham Biosciences, Freiburg, Germany) and an antibody raised and characterized in the Steroid Laboratory, as described elsewhere (33). Before RIA, a recovery-corrected extraction was performed. In brief, corticosterone was extracted from 10- to 20-μl aliquots of rodent serum samples in 500 μl dichloromethane-cyclohexane (1:2 vol/vol); the organic phase was evaporated to dryness and redissolved in 600 μl
RIA buffer. To determine the recovery of the extraction procedure, a defined amount of tritiated corticosterone was added to each sample before extraction and was measured afterwards in 100 μl eluate aliquots. RIA was performed in 100-μl aliquots of the eluate in duplicates. The standard curve ranged from 0.07 to 14.4 nmol/l (7–1,443 fmol/vial), and the sensitivity was 0.14 nmol/l. Corrected for dilution and recovery factors, the measurable corticosterone concentration ranges from 4 to 866 nmol/l in rodent serum. The intra-assay and interassay coefficients of variation are <10% and <15%, respectively. A reference value calculated from 43 blood samples (different sampling techniques, sex, age) amounted to 13.2 ± 10.7 μg/100 ml.

To estimate the total amount of corticosterone in the circulation, we multiplied the estimated plasma volume by the absolute corticosterone concentration since, according to our results, corticosterone concentrations are identical in serum and plasma samples (data not shown). Mouse plasma volumes were estimated as 0.05 ml/g body wt as proposed by Rippe et al. (28).

**Statistical analysis.** Statistical analysis was performed using the SPSS software package. Time course data (3, 5, 7, 9, and 11 wk) were analyzed separately for male and female mice by using ANOVA followed by Bonferroni (morphological data) or least-significant difference post hoc tests (serum corticosterone levels). Data from male and female mice within age class were compared using the two-sided Student’s t-test. P values <0.05 were considered significant. Data are presented as means ± SD (weight analysis and morphological data) or as means ± SE (corticosterone data). In Figs. 1–7, means labeled with different letters are significantly different (“a” vs. “b” indicates a statistically significant difference; a vs. a or a vs. “ab” means no significant difference). Furthermore, letters (a, b, c for females; x, y, z for males) indicate differences within genders, whereas the asterisks indicate significant differences between genders at specific points of time.

**RESULTS**

**Adrenal weights.** At all points of time, the adrenal glands of female mice were heavier than those of their male counterparts. The gender-dependent weight dimorphism reached its maximum in the 9th wk when female adrenal glands were about two times heavier than those of males (Fig. 1). In both male and female mice, the adrenal weights steadily increased until an age of 7 wk. Female adrenal glands remained at this level until week 11, whereas in male mice adrenal weights decreased between weeks 7 and 9 by 25% and remained at this level afterwards (Fig. 1). Body weights slightly increased between weeks 7 and 9 (data not shown). In all age groups, the body weights of female mice were lower if compared with males. Thus the difference in relative adrenal weights between females and males was higher than that in absolute adrenal weights (data not shown).

**Adrenal zonation.** Because the adrenal glands are composed of different compartments, we determined the compartment volumes at different ages in both genders.

From week 5 onward, females had significantly greater cortex volumes than males. The volume of the adrenal medulla was also larger in female than in male mice (significant at 3, 7, 9, and 11 wk). Adrenal cortex volumes reached their maximum at an age of 7 wk (males: 2.60 ± 0.18 mm³; females: 3.90 ± 0.39 mm³; Fig. 2A) and then decreased by 40 and 20% in male and female mice, respectively.

In male mice, the volume of the adrenal medulla increased between 3 and 7 wk of age and slightly declined thereafter. In female mice, the medullar volume increased steadily between weeks 3 and 7 and showed a further increase by 60% between 9 and 11 wk of age (Fig. 2B).

**Analysis of the cortical compartment.** Because the adrenal cortex is composed of three different histological zones, we examined the growth kinetics of each zone. In spite of a
twofold higher adrenal weight in female mice, the volumes of the zona glomerulosa were similar in both genders with the exception of 9-wk-old mice. In females, the zona glomerulosa reached its final volume at an age of 7 wk. In males, the zona glomerulosa increased in volume until week 7, declined between weeks 7 and 9, and increased again between weeks 9 and 11 (Fig. 3B).

In female mice, the zona fasciculata increased in volume until an age of 5 wk and was from this point of time significantly larger when compared with age-matched male mice. In male mice, a maximum of zona fasciculata volume was reached at an age of 7 wk after birth (2.00 ± 0.17 mm$^3$). In line with the significant reductions of the entire cortex in males between 7 and 9 wk, also the volumes of the zona fasciculata declined during this period (Fig. 3B).

The innermost zone in the murine adrenal cortex is the X-zone. In 3- to 5-wk-old female mice, an almost threefold increase of its volume was observed with no changes at later points of time (Fig. 3C). In males, the total volume of the X-zone was lower in 5-wk-old mice than in 3-wk-old mice. The X-zone was not detectable after week 5 in male mice (Fig. 3C).

**Analysis of zona fasciculata cell size and number.** Because the zona fasciculata contributes more than two-thirds to the total volume of the adrenal gland, and growth is achieved both by cell hypertrophy and hyperplasia, we studied changes of cell sizes and numbers in this zone in mice between 3 and 11 wk of age. Except for 3-wk-old animals, the cell numbers in the zona fasciculata were higher in females than in males in all age groups studied. In both genders, the total zona fasciculata cell number reached a maximum at the age of 7 wk (males: 988 ± 66 × 10$^3$; females: 1,991 ± 301 × 10$^3$) and decreased significantly until week 9 (males: 656 ± 111 × 10$^3$; females: 1,003 ± 249 × 10$^3$; Fig. 4A). The increase and decrease in zona fasciculata cell numbers was much more marked in female than in male mice.

**Fig. 3.** A–C: total volumes of the zona glomerulosa (A), zona fasciculata (B), and X-zone (C) in female (circles) and male (squares) mice. Volumes were quantified by quantitative stereology as described in MATERIALS AND METHODS. Data are presented as means and SD; different superscripts (females: a, b, c and males: x, y, z) indicate significant ($P < 0.05$) differences within genders (by Bonferroni post hoc test, for details, see MATERIALS AND METHODS). *Significant differences between genders at a defined age ($P < 0.05$; $n = 3/sex/age group).

**Fig. 4.** A and B: total numbers (A) and mean volumes (B) of zona fasciculata cells in female (circles) and male (squares) mice. Data are presented as means and SD; different superscripts (females: a, b, c and males: x, y, z) indicate significant ($P < 0.05$) differences within genders (by Bonferroni post hoc test, for details, see MATERIALS AND METHODS). *Significant differences between genders at a defined age ($P < 0.05$; $n = 3/sex/age group).
In male mice, the volume of zona fasciculata cells steadily increased between 3 and 11 wk of age (from 1,377 ± 250 to 2,262 ± 227 μm³). In 3- to 7-wk-old female mice, the zona fasciculata cell volumes were similar; however, between 7 and 9 wk of age, a volume increase of almost twofold was observed ($P < 0.05$). In 7-wk-old male mice, the zona fasciculata cell volume of male mice (1,997 ± 166 μm³) was higher ($P < 0.01$) than that of their female littermates (1,274 ± 154 μm³; Fig. 4B).

Serum corticosterone levels. In female and male mice, the absolute serum corticosterone concentration decreased from 3 to 11 wk of age. In male mice, a significant decrease of serum corticosterone was observed between weeks 3 and 5. Serum corticosterone levels were as a tendency higher in female than in male mice; however, because of the known high interindividual variation in serum corticosterone levels, statistical significance was reached only in 5-wk-old mice (Fig. 5A).

Figure 5B shows the estimated total amounts of corticosterone (ETAC) in the circulation. In female mice, no significant changes occurred between 3 and 11 wk of age. In male mice, the ETAC significantly increased between weeks 5 and 7. In contrast to the serum corticosterone concentrations, the ETAC did not decline between 3 and 11 wk of age. In female mice, the ETAC was significantly higher ($P < 0.05$) at an age of 5 and 11 wk if compared with male littermates (Fig. 5B).

Stored lipids in the adrenal gland. We also investigated the content of stored lipids in adrenal gland sections from male and female mice (7 and 11 wk old). An age-dependent increase in the amount of stored lipids was observed in both genders. Furthermore, adrenal glands of female mice seem to have more lipids stored (Fig. 6, A–D). The lipid droplets in 11-wk-old female mice appear to be larger than in 7 wk-old female mice or in male mice of both age groups, respectively (Fig. 6 A–D). As expected, no staining was seen in the adrenal medulla.

Expression of side chain cleavage enzyme and 11-β-hydroxylase. Corticosterone secretion is also dependent on the expression of steroid-synthesizing and -processing enzymes. Therefore, we have measured the expression of side chain cleavage enzyme and 11-β-hydroxylase in 3-, 7-, 9-, and 11-wk-old mice. An age-dependent increase of both enzymes was observed in male and female mice (Fig. 7; and data not shown).

**DISCUSSION**

Adrenal gland growth. Postnatal growth of the adrenal glands in mice (3) and rats (8, 20, 25, 27) can be divided into an initial rapid and a subsequent slower growth phase. In accordance with the literature, we observed a first phase of rapid growth until week 7. Between weeks 3 and 7, the adrenal glands of both sexes increased twofold in weight. In female mice, the adrenal glands displayed a constant weight after week 7. In contrast to females, male mice exhibited a 25% reduction of adrenal weight between week 7 and week 9. Tanaka and Matsuzawa (30) compared adrenal weights of male and female C57BL/6 and DDD mice at days 70 and 140 and also showed a reduction of adrenal weights exclusively in male mice.

A gender-dependent dimorphism of adrenal weight in rodents has been known for a long time, and a number of authors reported this finding for the mouse (2, 22, 30), although the underlying biological reasons remain to be clarified. In spite of similar adrenal weights of male and female newborn mice (2, 22), adrenal weights of female mice are significantly higher than those of male mice at 3 wk of age, suggesting that a gender-dependent adrenal weight dimorphism is relevant for normal physiology soon after birth. The genetic background of the mouse strain used clearly plays an important role on adrenal gland size and zonal composition. Badr et al. (2, 3) showed several decades ago that there is considerable genetic variation that affects postnatal development of the adrenal gland. Tanaka et al. (31) found differences in the adrenal zonation comparing AJJ and SMJ mice. Thus the findings of the present study cannot be simply extrapolated to other genetic backgrounds, and similar dynamic studies of adrenal growth processes will be required at least for the most common mouse strains.

Growth dynamics of the adrenal cortex and medulla. The adrenal cortex and the adrenal medulla both contribute to the higher weight of the female adrenal gland, but, because the cortex accounts for almost 80% of the whole adrenal gland, especially this compartment is relevant regarding the gender-dependent weight differences. Our results show a difference in the adrenocortical volume between male and female mice from...
week 5 onward. Because there was no significant volume reduction in the adrenal medulla of male mice, the decrease in adrenal weight is mainly because of reductions in the cortical compartment. Adrenal weight losses resulting from cortical volume reductions have been described in male mice (30). A higher volume of the adrenal medulla in female than in male mice was already observed in 3-wk-old animals. The fact that cortical volumes were not significantly different between male and female mice at this age underlines differential growth regulation of the two adrenal compartments. Moreover, we observed a sudden rise of the medullar volume by 60% in the adrenal gland of 11-wk-old vs. 9-wk-old female mice, whereas in the male adrenal gland even a slight volume reduction is present after week 7. The underlying mechanisms and biological consequences of this volume expansion in female mice deserve further investigation.

Analysis of the cortical compartment. The volume of the outmost mineralocorticoid synthesizing zona glomerulosa was similar in both genders. However, in 9-wk-old mice, the zona glomerulosa was significantly larger in female mice. This difference is a result of a zona glomerulosa volume loss in male mice between week 7 and week 9 ($P = 0.059$). Similar longitudinal growth studies of the zona glomerulosa have only been performed in rats. Pignatelli and coworkers (25) followed zona glomerulosa growth in up to 90-day-old rats using equatorial sections. In this study, a similar growth kinetic of the zona glomerulosa has been found in male and female mice.

The zona fasciculata, accounting for 60–65% of the total adrenal volume and for 70–75% of the adrenal cortex, is the largest zone of the cortex. Because of its high contribution to total adrenal volume, the gender-dependent adrenal volume dimorphism is mostly caused by zona fasciculata volume differences. In accordance with our findings, Malendowicz (21) identified the zona fasciculata as the primary cause for the gender-dependent weight dimorphism.

To our knowledge, a detailed longitudinal growth study of the zona fasciculata in mice has not been reported so far.

It is of note that, in males, the zona glomerulosa and zona fasciculata kept identical proportions of total adrenal volumes with ~18–20% (zona glomerulosa) or 60–65% (zona fasciculata) despite the cortical volume loss between 7 and 9 wk.
In contrast to most mammals, there is no functionally distinct zona reticularis in the mouse, but instead it is replaced by the so-called X-zone, which is unique to mice. The functional relevance of the X-zone is still unclear or under controversial debate (11, 14, 15, 19, 24). In female mice, the X-zone contributes maximally 8% to the adrenal gland volume. Therefore, in our mouse strain, the X-zone has only little impact on the gender-dependent adrenal weight dimorphism. In males, we and others have found complete degeneration of the X-zone during puberty, whereas in females this zone persists until the first pregnancy (30). Tanaka et al. did not detect the X-zone in 5-wk-old male C57BL/6J or DDD mice. Contribution of the X-zone to the total cortical area in 5-wk-old female mice was 15% in C57BL/6J mice and 63% in DDD mice (7, 30). In adrenal sections from 3-wk-old male Swiss Albino mice, the area of the X-zone corresponded to 17 and 26% of the total adrenal area in males and females, respectively (7). The different observations on the X-zone contribution to adrenal gland volume/section areas might be because of different methods used, since in those studies the adrenal zones were measured as linear or area values. We conclude that the X-zone morphology is tightly regulated by genetic effects and modified by endogenous hormones, as has also been stated by Tanaka et al. (30, 31). One of the factors that dictate the growth dynamics of this zone are androgens. It has been demonstrated that testosterone and dihydrotestosterone injections lead to X-zone regression in female mice, whereas gonadectomy results in X-zone regrowth (15). However, androgen-induced X-zone regression might also be mediated through downregulation of pituitary gonadotropin secretion (4). We found the largest X-zone volumes in males at an age of 3 wk. Hershkovitz et al. (15) also found a peaking 20α-hydroxysteroid dehydrogenase enzyme activity (X-zone specific marker) at an age of 3 wk in male mice that disappeared thereafter.

**Zona fasciculata cells and corticosterone as a functional parameter.** Both cell size and number contribute to the actual size of an organ or compartment. We quantified zona fasciculata cell volumes and numbers between weeks 3 and 11. Furthermore, as a functional parameter of zona fasciculata cells, we have quantified corticosterone concentrations in blood serum and estimated the total amounts of corticosterone in the circulation. Our results are comparable to the reference values from a laboratory routinely measuring corticosterone in the circulation. Our results are comparable to the reference values from a laboratory routinely measuring corticosterone in the circulation. The reference values was calculated as the mean from 43 blood samples (different sampling techniques, sex, age) and amounts to 13.2 ± 0.7 μg/100 ml. At a closer look discriminating younger (up to 3 wk: 16.5 ± 13.8 μg/100 ml) and elder (10 wk old and older: 11.7 ± 8.3 μg/100 ml) mice, it is found that the values of the present study very nicely fit also with results from other studies. However, the very low serum corticosterone levels in 5-wk-old male mice are unexpected and deserve further investigation. We exclude the possibility that anesthesia or sampling may have affected our results for several reasons as follows: 1) our serum corticosterone concentrations are similar to reference values; 2) blood sampling in our hands is achieved in clearly <1 min and an ACTH-triggered corticosterone response is not detected within the first 3 min after anesthesia (32); and 3) serum corticosterone levels in mice, after ACTH administration, are two- to threefold higher than the concentrations shown here (Ref. 16 and data not shown).

From week 5 on, the zona fasciculata of female mice comprised significantly more cells than the zona fasciculata of male mice. In females, zona fasciculata cell numbers increased almost threefold between 3 and 7 wk and then showed a marked decrease by ~50%. This decrease in cell number was associated with a steep increase in zona fasciculata cell volume. In males, the age-related changes in adrenocortical cell number and volume were less pronounced. Importantly, changes in zona fasciculata cell number and volume, both in male and in female mice, did not result in corresponding changes of serum corticosterone concentrations. In a previous study (16), we used a panel of transgenic mice to investigate consequences of overexpression of growth hormone (GH) and/or insulin-like growth factor-binding protein-2 (IGFBP-2) on adrenal growth processes. Overexpression of GH resulted in an increase of both size and number of zona fasciculata cells in 11-wk-old male mice. IGFBP-2 completely abrogated the hypertrophic effect of GH excess but did not affect adrenocortical cell numbers. The reduction in cell size was associated with a significant decrease in serum corticosterone levels, suggesting that the size of zona fasciculata cells is more relevant for corticosterone secretion than their number (16). The findings of the present study of nontransgenic mice, partially confirm this assumption. To more appropriately compare absolute numbers and sizes of zona fasciculata cells with corticosterone in the circulation, we have estimated the total amounts of corticosterone in the circulation (ETAC; Fig. 5B). Notably, a positive association was found between the means of the ETAC and the means of the total number of zona fasciculata cells throughout the different age groups, indicating a functional role of zona fasciculata cell numbers for corticosterone secretion over time in male and female mice. No association was present between the means of the ETAC and the means of the cell sizes. However, the sharp decline of cell numbers in 7- to 9-wk-old females was not accompanied by a significant reduction of the amount of corticosterone in the circulation. This lack of decrease seems to be compensated by the strong increase of zona fasciculata cell volume exclusively in 9-wk-old female mice. Thus we conclude that both cell number and cell volume are important parameters for corticosterone secretion.

Besides histological parameters, biochemical parameters also need to be addressed in future experiments dealing with age- and gender-specific characteristics of adrenal functions. The majority of stored lipids is composed of cholesterol ester. Furthermore, during steroid biosynthesis, a plethora of processing enzymes (e.g., side chain cleavage enzyme and 11-β-hydroxylase) is involved. Preliminary data on the amount of stored lipids or the expression of side chain cleavage enzyme and 11-β-hydroxylase in the adrenal glands indicate that the amount of stored lipids but also the expression of both enzymes in the zona fasciculata are increased between 3 and 11 wk of age (Figs. 6 and 7 and data not shown). Notably, in 11-wk-old female mice, the lipid droplets appear to be larger than in 7-wk-old female or male mice of both age groups, respectively. These differences are reflected by higher ETAC levels in 11-wk-old female vs. male mice.

In conclusion, our results indicate that 1) the different compartments and zones of the adrenal gland display differential nonlinear growth patterns; 2) number and volume of the zona fasciculata cells, particularly in females, undergo marked antagonistic changes between 7 and 9 wk; and 3) gender- and age-dependent aspects need to be carefully considered in studies using mouse models to define molecular mechanisms in-
involved in growth and function of the adrenal glands. Quantitative stereological methods are the only reliable way to obtain information about the contribution of cell size and number to organ growth. To our knowledge, we performed the first analysis of adrenal gland growth from weaning to adulthood in the mouse using unbiased stereological methods (12). These data may serve as a basis for further studies, e.g., concerning the influence of genetic constitution on adrenal gland growth in mice.

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