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The mouse adrenal gland: age- and gender- dependent alterations of growth and function

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Dedicated to my family

"The great tragedy of science is the slaying of a beautiful hypothesis by an ugly fact."

Thomas Henry Huxley
(1825-1895)

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The adrenal gland is of critical importance for a plethora of biological processes such as stress response, immune function, cardio vascular regulation and metabolism. To date, growth and function of the mouse adrenal gland have predominantly been assessed only at single developmental phases or under distinct experimental conditions, thus the current picture of physiological adrenal gland growth and function is fragmentary. A detailed timeline of the growing mouse adrenal gland including unbiased stereological results has never been rendered. In this work, postnatal mouse adrenal gland growth and function was investigated in a systematic and longitudinal way. Therefore, adrenal gland growth was analyzed at the organ, zonal and cellular level from weaning to adulthood. In addition, functional parameters of the adrenal gland have been included in this study since serum concentrations of aldosterone and corticosterone were measured. As a perspective, preliminary results on factors that influence steroidbiosynthesis in the adrenal gland are presented.

1. Historical Context

In the early literature, adrenal glands were first described and illustrated in detailed accuracy by the anatomist Bartholomeus Eustachius in the year 1563 (LENARD 1951; Carmichael 2001). He described the adrenal glands in humans and recognized them as individual organs. However, the function of the adrenal glands remained obscure. In 1716 the "L´académie des Sciences de Bordeaux" offered a prize for the answer to the question: "Quel est l´usage des glandes surrénales?". No prize was awarded as no conclusive answers had been submitted at that time (Hiatt & Hiatt 1997). Detailed investigations on adrenal gland function had been initiated, when disorders of this organ were associated with diseases. In 1855 Thomas Addison first described the clinical signs of the homonymous disease and linked it to adrenal disorders (Addison 1855). In the year 1856, the importance of the adrenal glands was further established, as Brown-Sequard showed, that bilaterally adrenalectomized guinea pigs were not able to survive (Brown-Sequard 1856). The German physiologist Arnold first distinguished different zones in the adrenal cortex and named them according to their appearance zona glomerulosa, zona fasciculata and zona reticularis (Arnold 1866). Until the beginning of the 20th century, very little was known about the substances produced by the adrenal glands. In 1901 Takamine and Abel independently isolated and crystallized a substance derived from the adrenal medulla and named it epinephrine (Hiatt & Hiatt 1997). The "opposite" disorder to Addison's syndrome (adrenocortical insufficiency) was discovered almost 80 years after Addison´s description, in 1932. Harvey W. Cushing first described hyperadrenocorticism and related it to disorders of the hypothalamic-pituitary-adrenal axis

(Cushing 1932). Later in the 1930´s the adrenocortical hormones corticosterone, deoxycorticosterone and cortisol were isolated and synthesized by Kendall, Pfiffner, Wintersteiner and Reichstein (Horton 2003). Thereby they paved the way for new therapeutic approaches and the widespread use of steroids in medicine (Orth & Kovacs 1998; Carmichael 2001; Horton 2003).

2. Structure of the mammalian adrenal gland

The adrenal glands are a paired organ located at the cranial pole of the kidneys. Nagel first described two independent parts of the adrenal gland: the adrenal cortex and the adrenal medulla (Nagel 1836). During embryogenesis the adrenal gland develops from two distinct germ layers: the adrenal cortex derives from the mesoderm and the adrenal medulla emerges from the ectodermal neural crest (Rüsse & Sinowatz 1998). In mammals, these two fractions merge to a single organ during the course of embryological development. The cortex itself can be divided into three morphological and functional distinct zones. According to Arnold's terminology, the outmost zone is called zona glomerulosa, followed by the zona fasciculata and the innermost zone, the zona reticularis.
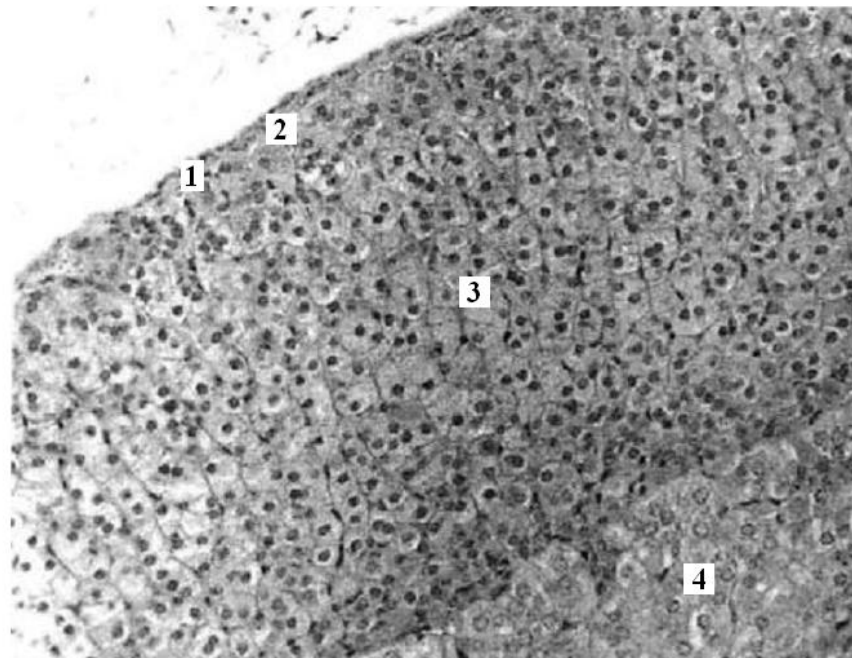

Fig. 1: Cross section of the mouse adrenal gland. 1: capsule; 2: zona glomerulosa; 3: zona fasciculata; 4; medulla. Picture taken from Hedrich et al., the laboratory mouse (Hedrich 2004).

3. Function of the adrenal gland

The adrenal gland is an endocrine organ, and thus its function is mediated through its secreted hormones. Cholesterol or cholesterol esters respectively are the basic substrate for cortical steroids. Through stepwise conversion and modification of cholesterol or its esters, the characteristic steroid hormones of each zone are synthesized (Fig. 2).
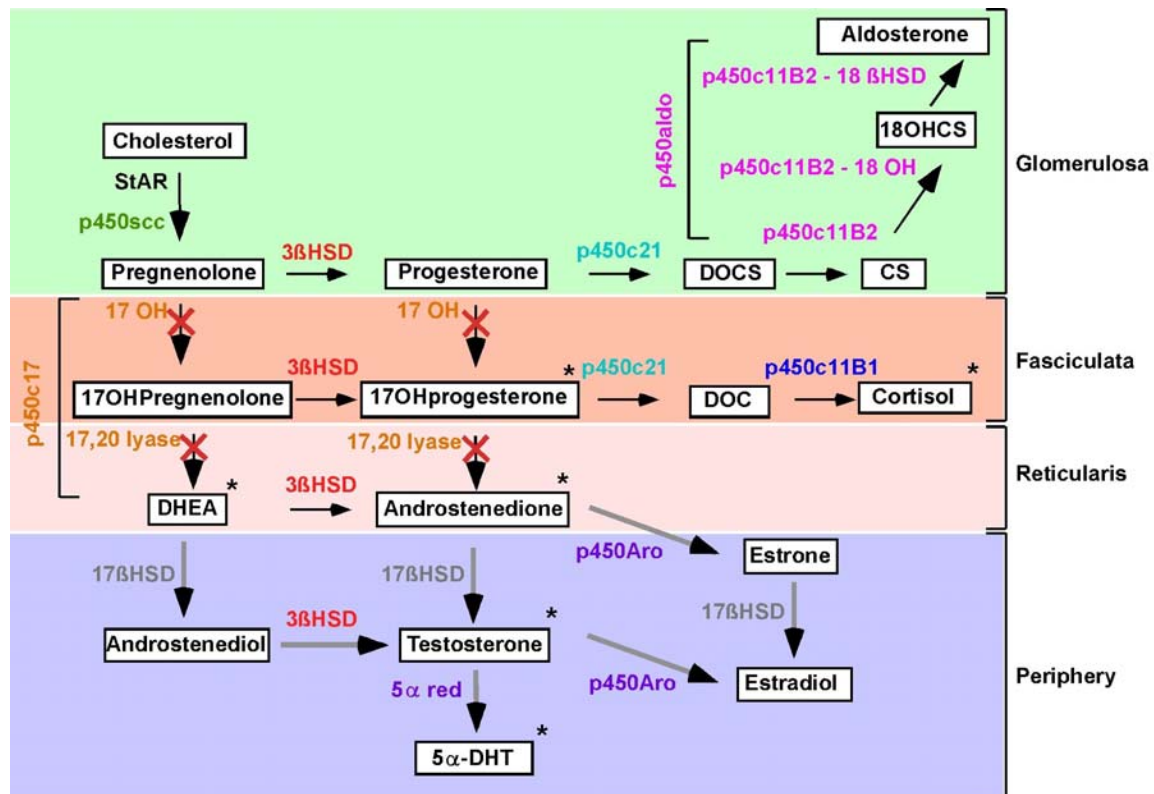

Fig. 2: The steroidogenesis in the human adrenal cortex. Crossed out arrows illustrate, which pathways can not be used by mice. Diagram modified from Keegan and Hammer (Keegan & Hammer 2002).

The zona glomerulosa is the origin of aldosterone, the primary mineralocorticoid. Aldosterone functions at two main locations of the kidney: in the distal kidney tubulus and in the collecting duct. In the distal kidney tubulus aldosterone binds to mineralocorticoid receptors. It thereby increases the permeability of the luminal membrane to potassium and sodium and activates their basolateral Na^+/K^+ ion pumps, thus reabsorbing sodium ions and water into the blood, and secreting potassium ions into urine. Hence, aldosterone is an important regulator of salt homeostasis and fluid balance. Aldosterone and its primary regulatory system, the renin-angiotensin system, can also potently influence the blood pressure through this mechanism. At the collecting duct aldosterone stimulates hydrogen and ammonium ion secretion. Thus, aldosterone furthermore is a major control unit of the acid/base balance (Williams 2005).

The glucocorticoids cortisol, corticosterone and deoxycorticosterone are secreted by the zona fasciculata. Glucocorticoid actions are diverse and glucocorticoid receptors are found on every nucleated cell in mammals (Silverman *et al.* 2005). The name glucocorticoid derives from the observation, that glucocorticoids influence the glucose metabolism. Glucocorticoids enhance gluconeogenesis through different mechanisms: they a) stimulate the synthesis of glucose from amino acids and lipids through an increased expression of the involved enzymes, b) mobilize amino acids in order to supply a substrate reservoir, c) inhibit glucose uptake in fat or muscle tissue and d) stimulate fat breakdown (lipolysis) in adipose tissues. In addition to their metabolic functions, glucocorticoids also influence the immune system where they have anti-inflammatory and immunosuppressive properties and are crucial for host defence against infection (Weber 1998; Bornstein & Chrousos 1999; Padgett & Glaser 2003; Zacharowski *et al.* 2006). This pharmacological effect is widely applied in medicine since their discovery in the 1930´s. Therapeutic application of glucocorticoids is advantageous for the treatment of allergies, autoimmune diseases and even in different forms of cancer. In addition, modern transplantation medicine would not be conceivable without glucocorticoids. Glucocorticoids also affect fetal development since effects for the developing lungs or the formation of the gonads, have been shown (White 2006; Gnanalingham *et al.* 2006). Deoxycorticosterone has a weaker glucocorticoid potency than corticosterone but it can also bind to mineralocorticoid receptors and thus exert additional functions as a mineralocorticoid (Farman & Rafestin-Oblin 2001). A subset of the androgens is secreted by the zona reticularis. The two main adrenal androgens are dehydroepiandrosterone ("DHEA" and its sulphated form "DHEA-S") and androstenedione ("Andro"). Both can serve as substrates for the conversion into testosterone and estradiol in the periphery (Keegan & Hammer 2002). The two medullar born catecholamines epinephrine and norepinephrine are classical acute stress hormones and function as "fight or flight" hormones. They modulate the cardiovascular system (increased cardiac output, vasoconstriction in the skin and guts, vasodilation of the arterioles in leg muscles), increase oxygen uptake and supply the body with energy in the form of glucose. Norepinephrine has a further, non-hormonal function and can act as a neurotransmitter (Engelhardt & Breves 2000). Considering the multiple functions of the adrenal glands it is particularly astonishing, that bilateral totally adrenalectomized human patients survive in most cases and adapt well to the post-adrenalectomy state with daily medication (Telenius-Berg *et al.* 1989).

4. Species-specific properties of the mouse adrenal gland

A characteristic of rodent adrenal glands in general is the pronounced gender-dependent difference in size which was first discovered by Hatai (Hatai 1914). In female mice, the adrenal glands are markedly larger than those of male mice. Frith stated, that in mice the zona fasciculata is indistinguishable from the zona reticularis (Frith 1983). From more recent studies it became clear, that mice do not have a functionally distinct zona reticularis due to the lack of 17α-hydroxylase expression in the adrenal gland. Therefore, adrenals from mice as well as other rodents are devoid of the secretion of adrenal androgens (Keegan & Hammer 2002). In mammals, cortisol is the predominant steroid of the adrenal cortex. The lack of 17α-hydroxylase expression in mice also leads to the inability to synthesise cortisol. Thus, in mice, corticosterone is the principal steroid of the adrenal gland. A specific feature of the mouse adrenal cortex is the so-called X-zone, a putative post-partal remnant of the fetal adrenal zone (Zubair *et al.* 2006). The functional relevance of the X-zone is still unclear or under controversial debate (Gersh & Grollmann 1939; Nyska & Maronpot 1999; Heikkila 2002; Keegan & Hammer 2002; Hershkovitz *et al.* 2006). In males, a complete degeneration of this zone is found during puberty, whereas in females this zone persists until the first pregnancy and disappears thereafter (Tanaka & Matsuzawa 1995).

5. Regulation of growth and function of the adrenal gland

Adrenal gland growth and function is under the control of a variety of factors. The principal regulator of adrenal gland growth and function is pituitary proopiomelanocortin (POMC)-derived adrenocorticotropic hormone (ACTH). However, a whole subset of regulatory factors including neurotransmitters, neuropeptides, cytokines and growth factor networks have been described (Bornstein & Chrousos 1999; Karpac *et al.* 2005; Hammer *et al.* 2005). Also the growth hormone (GH) / insulin-like-growth factor (IGF) system plays an important role on adrenal gland growth and function (Wolf *et al.* 1994; Weber *et al.* 1999; Hoeflich *et al.* 2002; Fottner *et al.* 2004). GH and IGF-I overexpression both lead to an increase in adrenal weight (Brem *et al.* 1989; Cecim *et al.* 1991), whereas suppression of the IGF system results in decreased adrenal gland weights (Lewinski *et al.* 1986; Merola *et al.* 1992). Apparently, the complexity of these regulatory networks needs to be further unraveled, as interferences of these networks frequently lead to adrenal gland neoplasias and consecutive diseases.

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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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Abstract

The adrenal gland is of critical importance for a plethora of biological processes. We performed the first systematic analysis of adrenal gland growth using unbiased stereological methods in male and female mice from weaning to adulthood (weeks 3, 5, 7, 9 and 11) at the organ, compartment, and cellular levels. Adrenal weights increased from week 3 to week 7 in male and female mice, remained at this level in females, but decreased by 25% between week 7 and week 9 in males. Female adrenal glands displayed a higher weight at any stage investigated. The volume of the zona fasciculata was consistently higher in female vs. male mice. In both genders, the number of zona fasciculata cells reached a maximum at the age of 7 weeks and decreased significantly until week 9. Serum corticosterone concentrations decreased from 3 to 11 weeks of age both in male and female mice. However, the estimated total amounts of corticosterone in the circulation were similar in 3-and 11-week-old mice. Furthermore, total circulating corticosterone was higher in females than in males at an age of 5 and 11 weeks. In the zona glomerulosa and in the X-zone time- and gender-dependant growth effects were observed. In conclusion, our results demonstrate that growth and function of the adrenal glands are markedly influenced by gender and age. These factors require careful consideration in studies aiming at the functional dissection of genetic and environmental factors affecting adrenal growth and function.

Introduction:

The adrenal glands are complex endocrine organs regulating multiple physiological processes, such as metabolism, stress response, immune functions and the cardio-vascular system (5; 6; 22). Growth and function of the adrenal glands are regulated by pituitary proopiomelanocortin (POMC)-derived adrenocorticotropic hormone (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 normal 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 due to the lack of 17α-hydroxylase expression in the adrenal gland. Therefore, adrenals from mice as well as 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 post-partal 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-deoxycorticosterone metabolism (15).

Although some data regarding adrenal weights of mice have been reported in the literature, they are mostly limited to specific points in 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 weeks involving i) quantitative measurements of adrenal weight and volumes of cortex and medulla; ii) quantification of the volumes of the different cortical zones; iii) determination of numbers and volumes of the zona fasciculata cells; and iv) 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.

Materials and methods:

Animals and tissue preparation

NMRI (Naval Medical Research Institute) outbred mice (albino) were crossed with mice from the inbred strain C57BL/6 mice, and F1 offspring harboring 50% of each genetic background were studied. The mice were maintained under specified pathogen-free (SPF) conditions in a closed barrier system and monitored as recommended (23). All mice had free access to a standard rodent diet (V1534; Ssniff, 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 three weeks, animals were weaned and separated according to gender. At different time points (3, 5, 7, 9, 11 weeks after birth) male and female mice (N≥12 / sex / age group) were weighed and sacrificed 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 sacrifice by cervical dislocation by orthograde perfusion with 4% paraformaldehyde (PFA) 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 semi-thin sections were obtained with a Reichert-Jung "Ultracut E" microtome (Leica, Wetzlar, Germany).

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

Stereological investigations

The paraffin-embedded left adrenal gland was exhaustively sectioned at 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 340 x final magnification provided by a 10-x objective. For calibration, an object micrometer (Zeiss, Jena, Germany) was used. Volume fractions of the cortex $V_{V(cortex/adrenal\ gland)}$ and the medulla $V_{V(medulla/adrenal\ gland)}$ 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 disector method (29) was applied as described previously (16).

At least 8 serial semi-thin sections (0.5 µm) comprising the whole organ were cut from each Epon-embedded right adrenal gland and stained with toluidine blue and safranine. From the stack of serially cut semi-thin sections, one section was drawn at random by means of a random number (R) between 2 and 8 (the baseline section was not used for sampling) as a reference section in a disector. The second section (look-up section) was sampled among number 2-8 by means of R±3, i.e., the disector height was equivalent to the thickness of three semi-thin sections (1.5 µm). Five fields were systematically sampled 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-x objective and color prints were prepared at the same final magnification

(1000x). A plastic transparency with equally spaced test points (N=70) and an unbiased counting frame representing an area of 13,500 μm^2 was superimposed on the printed images. All profiles of zona fasciculata cell nuclei sampled in the frame that were present in the reference section and not 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 twice with this procedure.

On average, 107 nuclei (range: 55–183) were counted with the five disector 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 disectors in an adrenal gland by the cumulative volume of the disectors (area of the unbiased counting frame x disector height x number of disectors) 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 8 mice (2 male, 2 female 7-week-old mice and 2 male, 2 female 11-week-old mice). Four sections were taken from the mid region of each adrenal gland. The sections were stained by Oil-Red-O and counterstained with haematoxylin. Stored lipids are

visible as red droplets, cell nuclei appear in blue. Light microscopic images of the stained sections were acquired with a color video camera at a final magnification of 900x.

Expression of side-chain cleavage enzyme and 11-beta-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. Twenty micrograms of protein were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Eschborn, Germany). Membranes were blocked (5% dry milk and 1% Tween20 in Tris buffered saline) and incubated with primary antibodies (rabbit anti side-chain cleavage enzyme, AB1244, Chemicon, Hampshire, UK; mouse anti 11-beta-hydroxylase, kindly provided by Dr. E. Gomez-Sanchez) over night at 4°C at a dilution of 1/1000. After three washings in Tris buffered saline containing 1% Tween20 membranes were incubated with horse radish peroxidase coupled goat anti rabbit IgG (1/2000; Cell Signaling Technology, New England Biolabs, Frankfurt, Germany) or goat anti mouse IgG (1/10000; Dianova, Hamburg, Germany) for one hour at room temperature. Finally, membranes were developed on a Kodak Image Station using an 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 activities were normalized for the Coomassie blue signal.

Quantification of corticosterone concentrations in blood serum

Blood samples were taken between 1300 and 1600 h. The animals were anesthetized individually in a glass jar containing saturated ether vapor and retro-orbital blood was collected within 30 s from initial handling within the cage.

Corticosterone was measured by a specific in–house radioimmunoassay (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). Prior to RIA a recovery-corrected extraction was performed. In brief, corticosterone was extracted from 10 to 20µl 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 prior to 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 -1443 fmol per 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 intraassay and interassay C.V.s 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.

In order to estimate the total amount of corticosterone in the circulation we multiplied the estimated plasma volume with the absolute corticosterone concentrations which, according to our results, are identical in serum and plasma samples (data not shown). Mouse plasma volumes were estimated as 0.05 ml/g body weight 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, 11 weeks) were analyzed separately for male and female mice by using ANOVA followed by Bonferroni (morphological data) or LSD 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 ± standard deviations (SD; weight analysis and morphological data) or as means ± standard errors of the means (SEM; corticosterone data). In the figures means labeled with different letters are significantly different ("a" versus "b": indicates a statistically significant

difference, "a" versus "a" or "a" versus "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 week, 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 weeks. Female adrenal glands remained at this level until week 11, whereas in male mice adrenal weights decreased between week 7 and 9 by 25 % and remained at this level afterwards (Fig. 1). Body weights slightly increased between week 7 and 9 (data not shown). In all age groups the body weights of female mice were lower if compared to 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

Since the adrenal glands are composed of different compartments we determined the compartment volumes at different ages in both genders.

From week 5 onwards, females had significantly higher 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 weeks). Adrenal cortex volumes reached their maximum at an age of 7 weeks (males: 2.60 ± 0.18 mm^3; females: 3.90 ± 0.39 mm^3; 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 weeks of age and slightly declined thereafter. In female mice, the medullar volume increased steadily between week 3 and 7 and showed a further increase by 60% between 9 and 11 weeks of age (Fig. 2B).

Analysis of the cortical compartment

As the adrenal cortex is composed of three different histological zones, we examined the growth kinetics of each zone. In spite of a two-fold higher adrenal weight in female mice the volumes of the zona glomerulosa were similar in both genders with the exception of 9-week-old mice. In females, the zona glomerulosa reached its final volume at an age of 7 weeks. 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, respectively (Fig. 3A).

In female mice, the zona fasciculata increased in volume until an age of 5 weeks and was from this point of time significantly larger when compared to age-matched male mice. In male mice a maximum of zona fasciculata volume was reached at an age of 7 weeks after birth (2.00 ± 0.17 mm^3). In line with the significant reductions of the entire cortex in males between 7 and 9 weeks, 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-week-old female mice an almost 3-fold 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-week-old mice than in 3-week-old mice. The X-zone was not detectable after week 5 in male mice (Fig. 3C).

Analysis of zona fasciculata cell size and number

Since 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 between 3 and 11 weeks age. Except for 3-week-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 absolute zona fasciculata cell number reached a maximum at the age of 7 weeks (males: $988 \pm 66 \times 10^3$; females: $1991 \pm 301 \times 10^3$) and decreased significantly until week 9 (males: $656 \pm 111 \times 10^3$; females: $1003 \pm 249 \times 10^3$; Fig. 4A). The increase and decrease in zona fasciculata cell numbers was much more marked in female than in male mice.

In male mice, the volume of zona fasciculata cells steadily increased between 3 and 11 weeks of age (from $1377 \pm 250 \ \mu m^3$ to $2262 \pm 227 \ \mu m^3$). In 3- to 7-week-old female mice the zona fasciculata cell volumes were similar, however, between 7 and 9 weeks of age, a volume increase of almost two-fold was observed ($p<0.05$). In 7-week-old male mice the zona fasciculata cell volume of male mice ($1997 \pm 166 \ \mu m^3$) was higher ($p<0.01$) than that of their female littermates ($1274 \pm 154 \ \mu m^3$; Fig. 4B).

Serum corticosterone levels

In female and male mice the absolute serum corticosterone concentration decreased from 3 to 11 weeks of age. In male mice a significant decrease of serum corticosterone was observed between week 3 and 5. Serum corticosterone levels were as a tendency higher in female than in male mice, however – due to the known high inter-individual variation in serum corticosterone levels – statistical significance was reached only in 5-week-old mice (Fig. 5A). Fig. 5B shows the estimated total amounts of corticosterone (ETAC) in the circulation. In female mice, no significant changes occurred between 3 and 11 weeks 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 weeks of age. In female mice ETAC was significantly higher ($P<0.05$) at an age of 5 and 11 weeks if compared to 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- week-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 6A-D). The lipid droplets in 11-week-old female mice appear to be larger than in 7-week-old female mice or in male mice of both age groups respectively (Fig 6A-D). As expected, no staining was seen in the adrenal medulla.

Expression of side-chain cleavage enzyme and 11-beta-hydroyxlase

Corticosterone secretion is also dependent on the expression of steroid synthesizing and processing enzymes. Therefore, we have measured expression of side-chain cleavage enzyme and 11-beta-hydroyxlase in 3-, 7-, 9- and 11-week-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 gland weight between week 7 and week 9. Tanaka et al. (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 is known for a long time and a number of authors reported this finding for the mouse (3; 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 weeks of age, suggesting that 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. showed several decades ago that there is considerable genetic variation that affects post-natal development of the adrenal gland (2;3). Tanaka et al. found differences in the adrenal zonation comparing A/J and SM/J mice (31). 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 as 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 onwards. Since there was no significant volume reduction in the adrenal medulla of male mice, the decrease in adrenal weight is mainly due to reductions in the cortical compartment. Adrenal weight losses due to 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-week-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-week-old vs. 9-week-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. Only in 9-week-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 followed zona glomerulosa growth in up to 90 days old rats using equatorial sections. In this study a similar growth kinetic of the zona glomerulosa has been found in male and female mice (25).

The zona fasciculata, accounting for 60% - 65% of the total adrenal volume and for 70% to 75% of the adrenal cortex, is the largest zone of the cortex. Due to 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 about 18 –20 % (zona glomerulosa) or 60-65% (zona fasciculata) despite the cortical volume loss between 7 and 9 weeks.

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 by maximally 8 % to the adrenal gland volume.

Therefore, in our mouse strain, the X-zone has only little impact on gender-dependent adrenal weight dimorphism. In males, others and we 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-week-old male C57BL/6J or DDD mice. Contribution of the X-zone to the total cortical area in 5-week-old female mice was 15% in C57BL/6J mice and 63% in DDD mice. (7; 30). In adrenal sections from 3-week-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 due to different methods used as 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 DHT injections lead to X-zone regression in female mice while gonadectomy results in X-zone regrowth (15). However, androgen induced X-zone regression might also be mediated through down-regulation of pituitary gonadotropin secretion (4). We found the largest X-zone volumes in males at an age of 3 weeks. Hershkovitz also found a peaking 20αHSD enzyme activity (X-zone specific marker) at an age of 3 weeks in male mice which disappeared thereafter (15).

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 value from a laboratory routinely

measuring corticosterone concentrations. The reference value was calculated as the mean from 43 blood samples (different sample techniques, sex, age) and amounts to 13.2 ± 10.7 μg/100 ml. At a closer look discriminating younger (up to 3 weeks: 16.5 ± 13.8 μg/100 ml) and elder mice (10 weeks old and elder: 11.7 ± 8.3 μg/100 ml) 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-week-old male mice are unexpected and deserve further investigation. We exclude the possibility that anaesthesia or sampling may have affected our results for several reasons: 1) our serum corticosterone concentrations are similar to reference values; 2) blood sampling in our hands is achieved in clearly less than one minute and an ATCH triggered corticosterone response is not detected within the first three minutes after anaesthesia (32); 3) serum corticosterone levels in mice, after ACTH administration are 2- to 3-fold higher than the concentrations shown here (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 three-fold between 3 and 7 weeks and then showed a marked decrease by about 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-week-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 non-transgenic mice partially confirm this assumption. In order 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-week-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-week-old female mice. Thus, we conclude that both cell number and cell volume are important parameters for corticosterone secretion.

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

In conclusion, our results indicate that i) the different compartments and zones of the adrenal gland display differential non-linear growth patterns; ii) number and volume of the zona

fasciculata cells, particularly in females, undergo marked antagonistic changes between 7 and 9 weeks; and iii) gender- and age-dependent aspects need to be carefully considered in studies using mouse models to define molecular mechanisms 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.
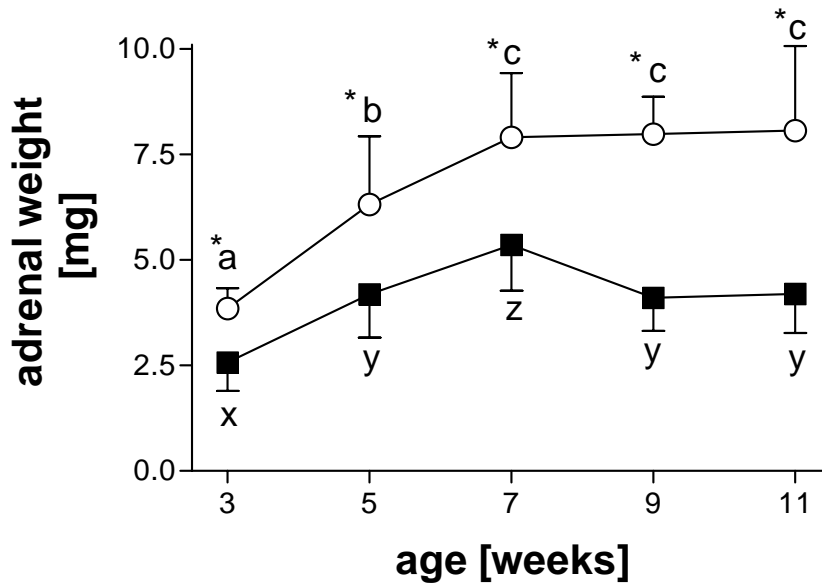
Fig. 1

Fig. 1: Absolute weights of the adrenal glands in female (circles) and male (squares) mice from weaning to adulthood. The growth phase of the adrenal glands ends at 7 weeks in females and at 9 weeks in males. In males, adrenal glands undergo a significant weight loss between 7 and 9 weeks of age. Data are presented as means and standard deviations (SD); different superscripts (females: a,b,c males: x,y,z) indicate significant ($P<0.05$) differences within genders (by Bonferroni post-hoc test, for details see materials and methods). Asterisks indicate significant differences between genders ($P<0.05$; $N \geq 12$ per sex and age group; $p<0.001$).
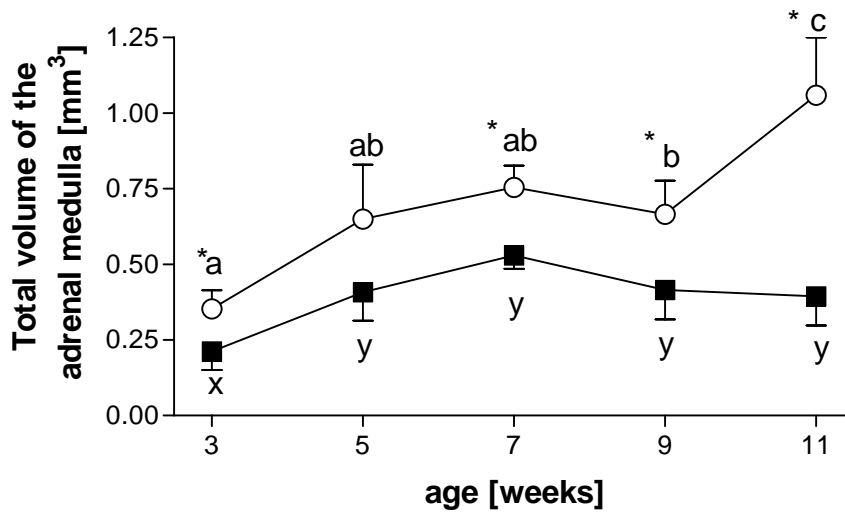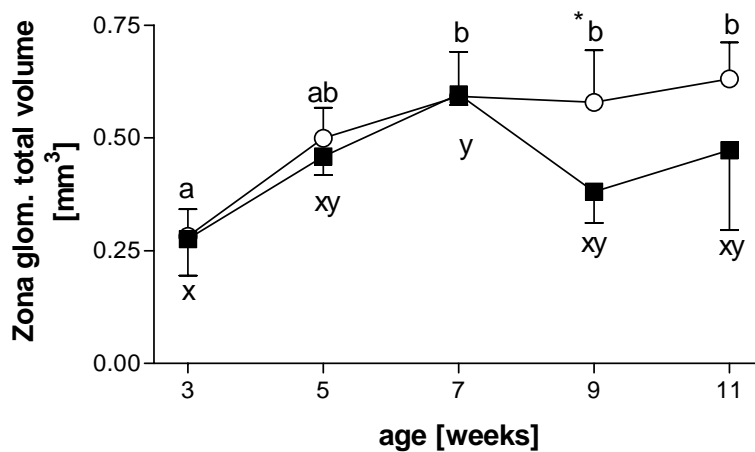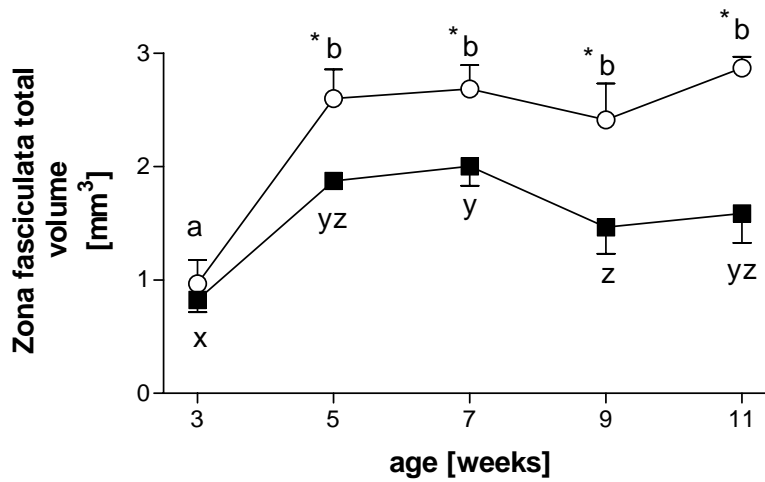
A
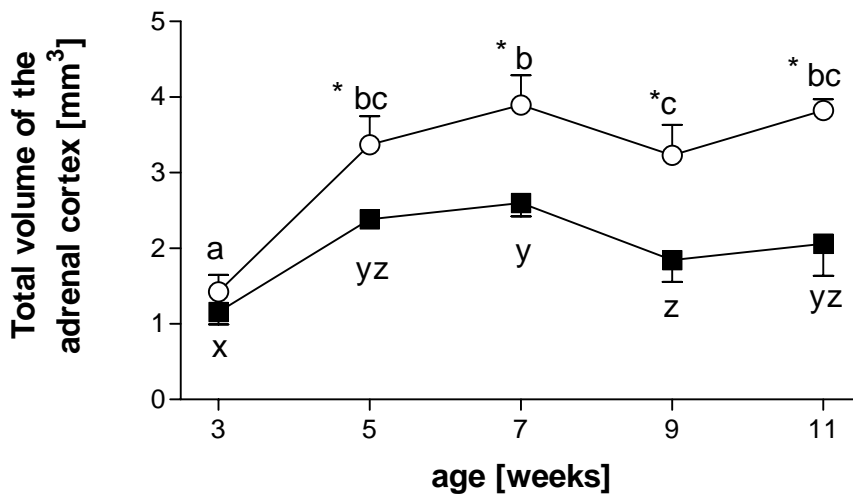
B

Fig. 2 A and B

Fig. 2 A and B: Total volumes of the adrenal cortical (A) and medullar (B) compartments 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 standard deviations (SD); different superscripts (females: a,b,c, males: x,y,z) indicate significant ($P<0.05$) differences within genders (by Bonferroni-post-hoc test, for details see materials and methods). Asterisks indicate significant differences between genders at defined ages ($P<0.05$; N≥3/sex/age group).
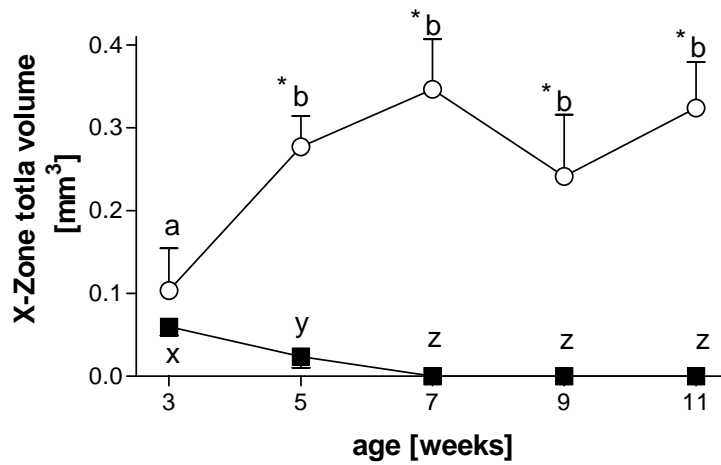
A

B

C

Fig. 3 A, B and C

Fig. 3 A, B and C: Total volumes of the zona glomerulosa (A), zona fasciculate (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 standard deviations (SD); different superscripts (females: a,b,c males: x,y,z) indicate significant (P<0.05) differences within genders (by Bonferroni post-hoc test, for details see materials and methods). The asterisks indicate significant differences between genders at a defined age (P<0.05; N≥3/sex/age group).
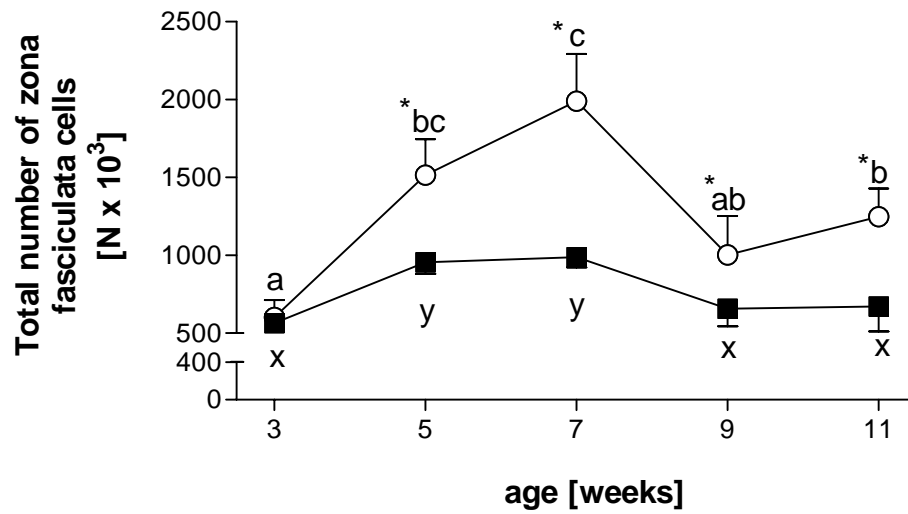
A

B

Fig. 4 A and B

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 standard deviations (SD); different superscripts (females: a,b,c males: x,y,z) indicate significant (P<0.05) differences within genders (by Bonferroni post-hoc test, for details see materials and methods). The asterisks indicate significant differences between genders at a defined age (P<0.05; N≥3/sex/age group).
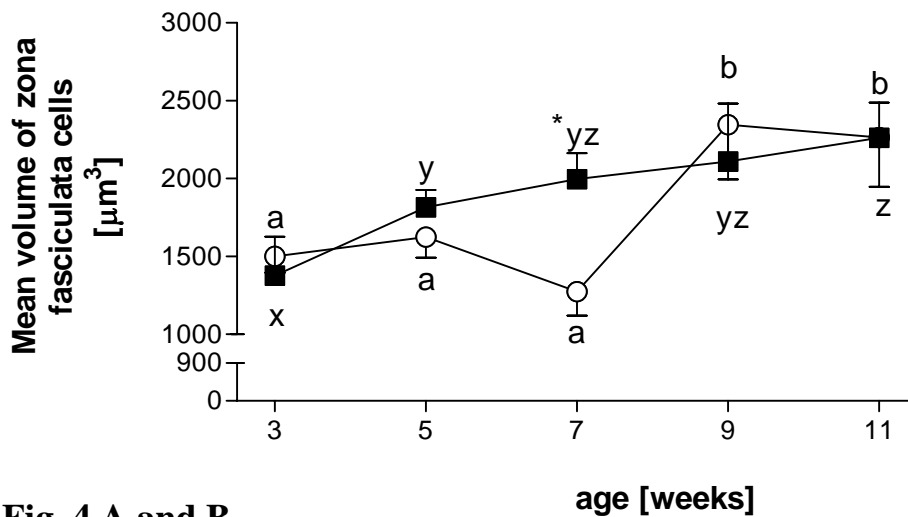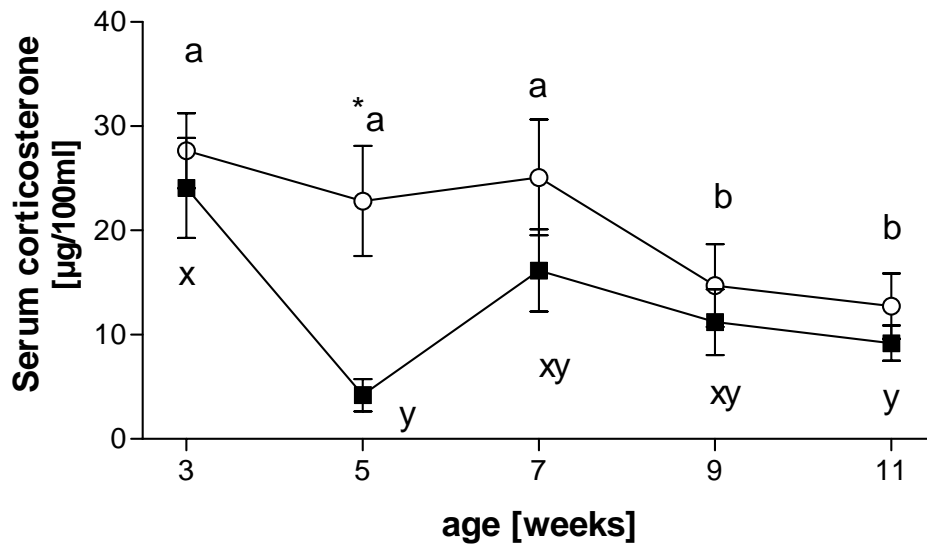
A

B
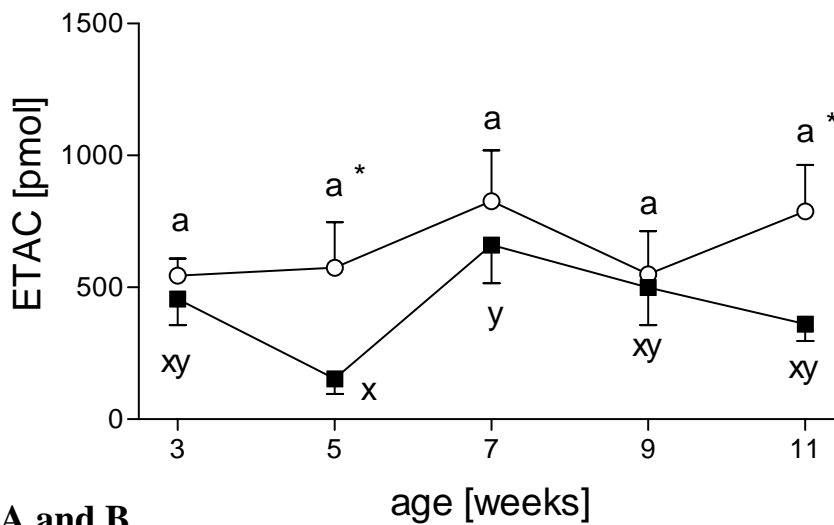
Fig. 5 A and B

Fig. 5 A: Absolute serum corticosterone levels in female (circles) and male (squares) mice from 3 to 11 weeks of age. Data are presented as means and standard errors of the means (SEM); different superscripts (females:a,b males:x,y) indicate significant (P<0.05) differences within genders (by LSD post-hoc test, for details see materials and methods). The asterisk indicates a significant difference between genders at an age of 5 weeks (P<0.05; N ≥8 sex/age group).

Fig. 5 B: Estimated total amount of corticosterone in circulation (ETAC) from of female (circles) and male (squares) mice from 3 to 11 weeks of age. The values were calculated by multiplying the absolute corticosterone concentration with the estimated plasma volume. Data are presented as means and standard deviations (SD); different superscripts (females: a males: x, y) indicate significant ($P<0.05$) differences within genders (by ANOVA and subsequent Bonferroni post-hoc test, for details see materials and methods). Asterisks indicate significant differences between genders at a specific point of time ($P<0.05$; $N \geq 8$ per sex and age group).
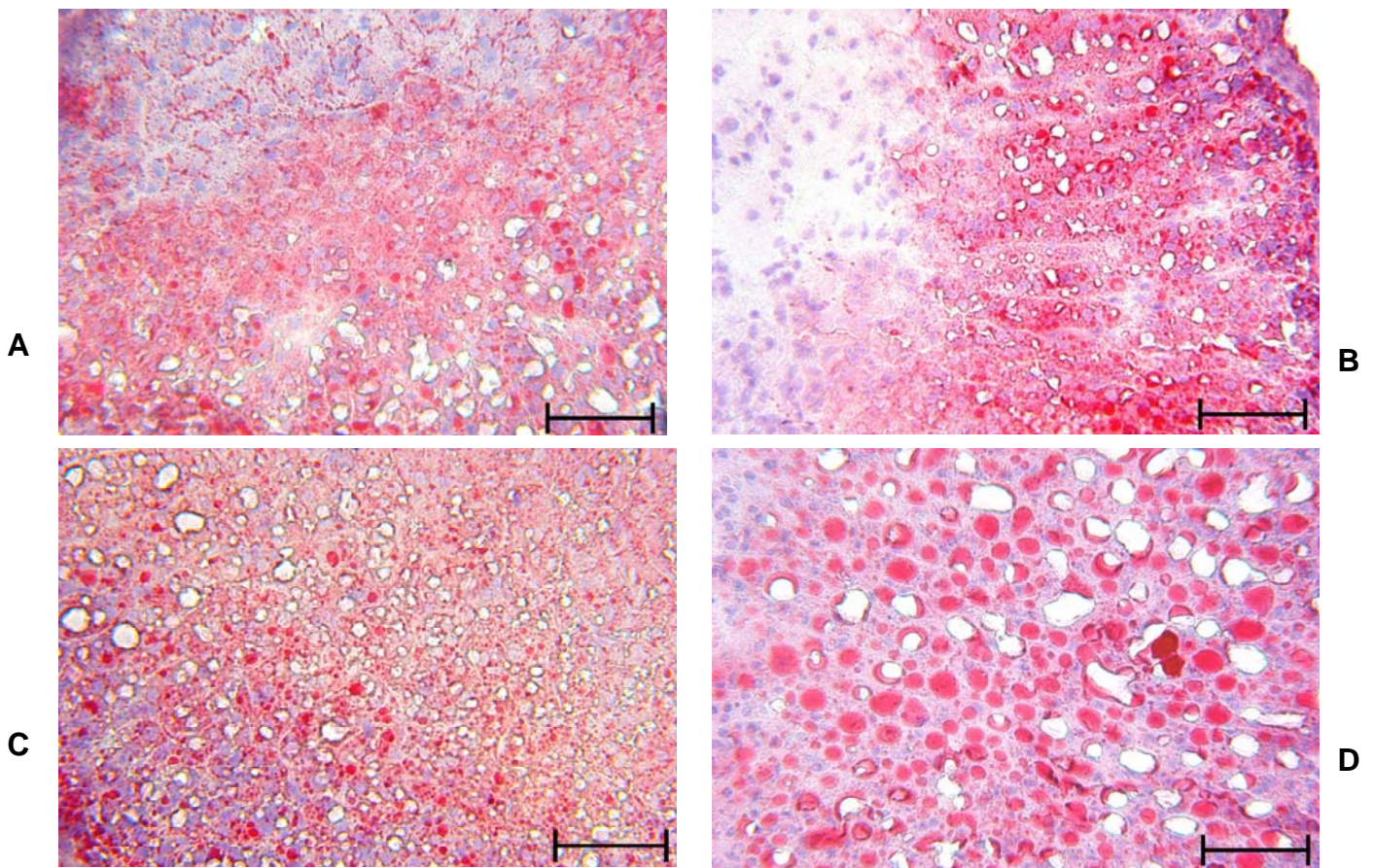
Fig. 6 A-D

Fig. 6 A-D: Stored lipids in frozen adrenal gland sections. A and B: 7- and 11-week-old male mice, respectively; C and D: 7- and 11-week-old female mice, respectively. The stored lipids are visible as red droplets, cell nuclei appear in blue. The scale corresponds to 25 μm. The pictures were taken at a final magnification of 900x (representative pictures from 4 stained sections per adrenal gland; N=2 per sex and age group).
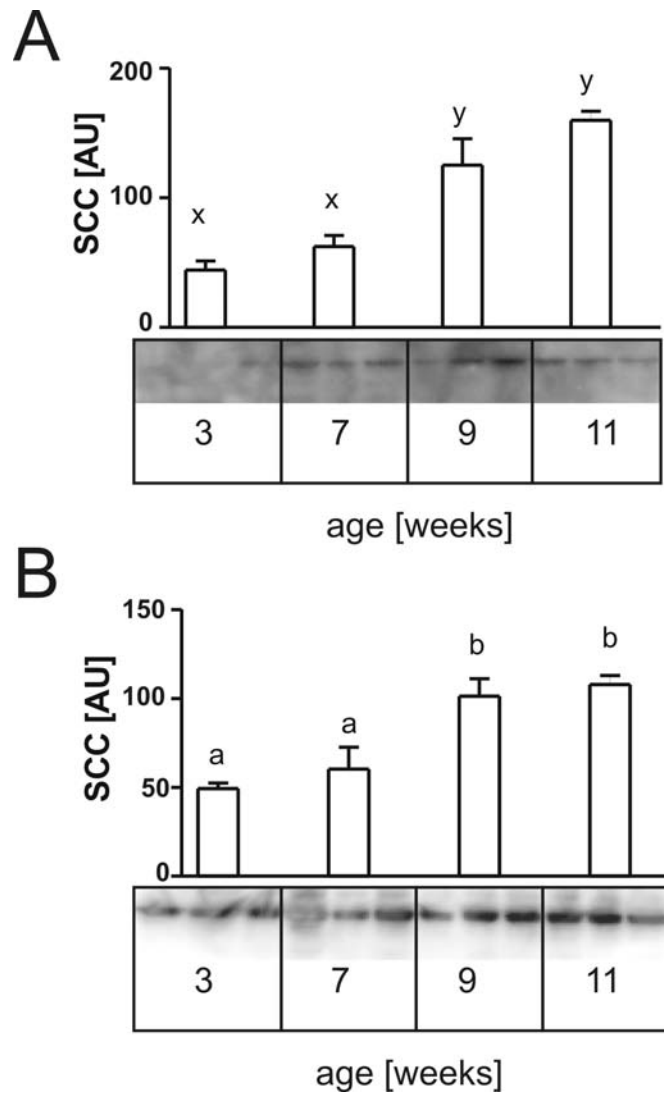
Fig. 7 A and B

Fig. 7 A and B: Protein expression of side-chain cleavage enzyme in the adrenal glands from male (A) and female (B) mice from 3 to 11 weeks of age. Data are presented as means and standard errors of the means (SEM); different superscripts (females: a ,b, males: x, y) indicate significant (P<0.05) differences within genders (N=3 per sex and age group).

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A Highly Sensitive Immunofluorometric Assay for the Measurement of Aldosterone in Small Sample Volumes: Validation in Mouse Serum

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Abstract

Data on the involvement of aldosterone in the regulation of the Renin-Angiotensin-Aldosterone System in rodents is still scarce. Partly due to the high volumes needed by commercially available assays and to the lack of sensitivity to detect the very low aldosterone concentrations. For this purpose, we have developed a highly sensitive and non-isotopic, time-resolved fluorescence, competitive immunoassay requiring a volume of only 50 µl serum for a duplicate measurement. A highly specific monoclonal antibody against aldosterone with negligible cross-reactivity with potentially interfering steroids is used. The assay was validated in human and mouse samples and exhibited a linear working range from $10 - 1000$ pg/ml. For determination of basal aldosterone values in mice, serum was collected from F1 hybrid mice (C57BL/6 x NMRI; n = 75). Values were found within the linear range of the assay (173 ± 21 pg/ml), with no significant difference between males and females. Additionally, we show an increase in serum aldosterone in mice from 3 to 11 weeks of age. Mice of the same genetic background were treated with dexamethasone i.p. (n = 7), resulting in significantly decreased aldosterone concentrations (35 ± 3 pg/ml vs. 114 ± 33 pg/ml in control mice; $p<0.001$). In contrast, adrenocorticotropic hormone given i.p. resulted in significantly increased serum aldosterone (603 ± 119 pg/ml; n = 7; $p<0.001$).

In conclusion, we have developed and validated an extremely sensitive assay for determination of aldosterone concentrations from very small serum samples, which could be especially useful in pharmacological intervention studies in rodent models.

Introduction

An increasing body of work to examine adrenocortical zonation and to define factors involved in adrenal steroidogenesis has been performed in the rat model or other species such as guinea pig and the bovine model (Peters B. *et al.*, 2007, Peters J. *et al.*, 1999, Takaya *et al.*, 2001, Vinson, 2003, 2004). However, genetically altered animal models which are of increasing importance for intervention studies and to establish a genotype phenotype relationship are restricted to the mouse (Berger *et al.*, 2000, Billet *et al.*, 2006, Cao *et al.*, 2006, Cole *et al.*, 2003, Jensen *et al.*, 2004, Kessler *et al.*, 2005, Lee *et al.*, 2005, Makhanova *et al.*, 2006, Peters B. *et al.*, 2007, Peters J. *et al.*, 1999, Takaya *et al.*, 2001, Vinson, 2004). Currently there is a lack of longitudinal and systematic studies addressing adrenal gland growth and function as well as circulating aldosterone concentrations in mouse models. Plasma concentrations of corticosterone are relatively high and are therefore unproblematic to measure in mice (Coll *et al.*, 2004, Davies *et al.*, 2007, Karpac *et al.*, 2005, Lee *et al.*, 2005, Makhanova *et al.*, 2006, Sausbier *et al.*, 2005, Schmidt *et al.*, 2006, Wang *et al.*, 2004, Weber *et al.*, 1999). However, measurements of aldosterone levels in mice are problematic due to the tighter dynamic range and the large amount of plasma required for the commonly available aldosterone assays. Moreover, aldosterone is the "minority hormone" of the adrenal cortex and plasma cortisol concentrations in humans are 2000 times higher than plasma aldosterone concentrations (Muller, 1995). There is a discrepancy in the levels of aldosterone which have been reported so far in mice (Cao *et al.*, 2006, Cassis *et al.*, 2005, Cole *et al.*, 2003, Coll *et al.*, 2004, Davies *et al.*, 2007, Kessler *et al.*, 2005, Makhanova *et al.*, 2006, Takaya *et al.*, 2001). This problem arises from the variability in the assays commercially available to measure aldosterone and the antibodies utilized. The antibodies used in commercial assays are mostly polyclonal and differ in affinity and specificity in recognising aldosterone. Moreover, polyclonal antibodies against aldosterone are mostly incorporated in radioimmunoassays (RIAs), but these require large volumes and are associated with the

obvious hazards and limitations of using radioactivity. In the present article, we present a non-isotopic, competitive immunoassay which utilizes a highly specific monoclonal antibody against aldosterone. The assay requires a small volume of serum or plasma which is especially desirable if an experimental procedure involves repeated in vivo sampling or if a variety of different parameters need to be determined. The assay is based on the competition principle with non-radioactive biotinylated aldosterone tracer in combination with Streptavidin-Europium for time-resolved fluorescence measurement (TR-FIA).

Studying the Renin Angiotensin Aldosterone System (RAAS) in rodents has been hampered by the fact that the rodent adrenal gland is a complex organ with morphological differences found not only between species but also between different strains as well as at different ages; this poses obstacles in the assessment and comparison of data arising from transgenic and non-transgenic animal experiments. Mineralocorticoids are secreted by the outermost zone of the adrenal gland, the zona glomerulosa. Though it is known that the function of this zone is controlled both by the RAAS via angiotensin II and III (Williams, 2005) and by pituitary hormones, especially adrenocorticotropic hormone (ACTH) (Muller, 1995, Seely *et al.*, 1989), sodium chloride (NaCl) and K^+ levels also play a major part (Lee *et al.*, 2005, Mortensen RM, 2001). However, the complexity of the regulatory mechanisms involved in the synthesis and secretion of aldosterone is not well understood.

In this study, we present a new assay to study the RAAS and validate the assay by measuring aldosterone levels after stimulation and suppression of the hypothalamic pituitary adrenal (HPA) axis in mice. Additionally, we show levels of serum aldosterone in mice from 3 to 11 weeks of age in order to address age- and gender-dependent alterations of serum aldosterone concentrations.

Materials and Methods

Materials   All solvents, reagents and commercially available steroids were of analytical reagent grade or highest percent purity. Steroids, organic and other materials were purchased from Fluka and Sigma-Aldrich (Taufkirchen, Germany). Streptavidin labeled with Europium was purchased from Perkin-Elmer Life Sciences (Turku, Finland). Buffers were prepared in distilled, deionized water.

Anti-aldosterone antibody

The monoclonal antibody against aldosterone used in the immunoassay was prepared as previously described (Gomez-Sanchez *et al.*, 1987). Cross-reactivities for cortisol and corticosterone were found at <0.003% and were at <0.1% for other potentially cross-reacting steroids. The antibody was tested in our laboratory at a series of dilutions in sodium phosphate (Na_2HPO_4 x$2H_2O$) buffer, pH 7.4, in order to determine the optimum concentration producing highest counts and highest displacement capability of the tracer used in the final assay procedure (Figure 1).

Aldosterone tracer

Biotinylated aldosterone, used as tracer in the immunoassay, was prepared by coupling aldosterone 3-O-carboxymethyloxime (3-CMO) with a commercially available biotin hydrazide derivative according to a procedure previously described (Dressendorfer *et al.*, 1992). Briefly, aldosterone 3-CMO active ester derivative was prepared on the first day as follows: 11.5 μmol of aldosterone3-CMO (Steraloids IInc., Newport, USA, No. Q2010-000) was dissolved in dry, amine-free N,N-Dimethylformamide (DMF) (Sigma, No. D8654) and coupled with 5.6 mg N-hydroxysuccinimide (NHS) (Fluka, No. 56480) and 10.3 mg N,N′-Dicyclohexylcarbodiimide (DCC) (Fluka, No.36650). The reaction mixture was stored at room temperature under light-exclusion for 24 hours. Coupling of the aldosterone-NHS ester

to biotin was then carried out as follows: 25 μmol Biotinamidocaproyl hydrazide, (Sigma, No. B3770) was dissolved in Dimethyl Sulfoxide (DMSO) (Sigma, No. D5879), added to the initial reaction mixture and left at room temperature for another 24 hours under light exclusion.

Aliquots of the aldosterone 3-CMO-biotin conjugate were further diluted in 50 mM Tris-HCl buffer (pH 7.8) and applied to a reverse phase chromatography column for purification, (250 x 4.6 mm, Synergi 4u Fusion-RP 80A, Phenomenex, No.00G-4424-E0).

Mice / Experimental

Mice (C57BL/6 x NMRI) were maintained under specified pathogen-free (SPF) conditions in a closed barrier system and monitored as recommended (Nicklas *et al.*, 2002). All mice had free access to a standard rodent diet (V1534; Ssniff, Soest, Germany) and tap water ad libitum. The diet used contains a standard concentration of salts, 0.59 % v/v NaCl and 0.97 % v/v K^+. At an age of three weeks, animals were weaned and separated according to gender. At different time points (3, 5, 7, 9, 11 weeks after birth) male and female mice (n $\geq$ 8 / sex / age group) were weighed and then sacrificed after obtaining a blood sample under ether anesthesia.

Blood samples were taken between 1300 and 1600 h. The animals were anesthetized individually in a glass jar containing saturated ether vapor and retro-orbital blood was collected with heparinized microcapillaries in clearly less than 1 minute from initial handling within the cage(Hoeflich *et al.*, 2002, Weber *et al.*, 1999).

All animal experiments were carried out according to the German Animal Protection Law.

Assay Procedure

a)   *Sample extraction*

50 μl of the serum sample collected from each animal was thawed and extracted using 2 ml of

Dichloromethane (DCM)/ Polyethylene glycol 10000 (PEG; 50 mg/l) according to a modified version of that described earlier (Tschop *et al.*, 1998). Each 50 µl sample was pipetted in a glass tube and vortexed at a low speed for 30 minutes and after the two phases separated, the organic phase (1.7 ml) was removed, transferred to another tube and left to evaporate overnight. On the following day samples were reconstituted with pure methanol and assay buffer.

b)   Assay procedure

After reconstitution, 50 µl of each of the samples were pipetted in duplicate, directly onto microtitre-plates previously coated with the monoclonal mouse anti-aldosterone capture antibody along with aldosterone calibrators and controls. Calibrators were prepared from an ethanolic stock of 10 mg/ml (Fluka 05521, Taufkirchen Germany) by serial dilution. Subsequently, 100 µl of biotinylated aldosterone tracer were pipetted into each well (5 pg/well), the plate was sealed with self-adherent foil to avoid evaporation and incubated overnight at 4°C. The following day, the incubation was terminated by washing three times on an automatic microtiter plate washer (TECAN, SLT; Crailsheim, Germany) using an in-house prepared 0.05% v/v PBS/Tween-20 buffer. 200 µl per well of a Streptavidin coupled to Europium conjugate (PerkinElmer/Wallac, Turku, Finland) were then added and incubated for thirty minutes on a horizontal shaker at room temperature. After a 6-fold wash of the plate, an in-house „enhancement solution" was added and incubated on the shaker for fifteen minutes, used to transfer the Europium conjugate into a fluorescent complex. Amount of biotinylated aldosterone bound was then measured using a fluorometer (VICTOR[3], PerkinElmer).

Plasma aldosterone in RIA compared to TR-FIA

A series of eighteen human plasma samples taken from daily profiles in two patients with Conn's syndrome was assayed for aldosterone content using a commercially available RIA kit

(200 μl required) (Aldosterone Coat-A-Count, Diagnostics Product Corporation Biermann) and compared to the results obtained by the in-house TR-FIA (50 μl) after extraction with dichloromethane and reconstitution according to the aforementioned procedure.

TR-FIA Validation   All validation experiments carried out to assess the performance of the assay (see also *Results* section) were completed using human plasma samples which were extracted accordingly, due to lack of ease of obtaining large volumes of serum or plasma from rodents.

Stimulation / suppression tests

Seven mice (aged 11 weeks) were injected intraperitoneally (i.p.) for 10 consecutive days with a daily dose of 250 μl ACTH solution (Synacthen ®, Novartis Germany, 0.25 mg/ml). A second group (n = 7) received daily i.p. injections of 200 μl Dexamethasone solution (Vetoquinol, Firma, Germany; 4 mg/ml). A control group (n = 7) received 250 μl vehicle only per day (0.9 % v/v NaCl; B. Braun, Melsungen, Germany) i.p.. On day 10, 3 hours after the last injection, mice were sacrificed and blood samples were collected in the afternoon as described above.

Statistical analysis

Since the data presented did not meet the assumptions for parametric analysis all data was analysed using non-parametric tests. In order to examine differences in sex within each age group and overall Mann-Whitney U test was performed. Kruskal-Wallis was used to examine age-dependent differences in aldosterone levels in control mice and to examine differences in aldosterone levels between dexamethasone-treated, Synacthen®-treated, and non-treated control mice. Significant differences in the multi-group test were specified by subsequent pair-wise post-hoc Mann-Whitney U tests.

Results

TR-FIA Validation - Performance of assay

a) Antibody titration

Amount of aldosterone antibody immobilised on the microtiter-plates was adjusted in order to give optimum sensitivity. Figure 1 shows the dilution curves, each representing the results obtained with decreasing amounts of antiserum. Although the points of 50% maximal binding, ED_{50}, were very similar in the two upper curves, total counts decreased significantly at a 1:5000 titration so that 1:2500 was finally chosen as a compromise between highest total counts and lowest possible concentration of antiserum, and was used in all following experiments. A typical standard curve for the aldosterone TR-FIA is shown in Figure 2.

b) Analytical sensitivity

The analytical sensitivity or lower limit of detection (LOD) of the assay was determined by assessing the zero standard in a typical assay a total of 20 times and the mean (± standard deviation; SD) value of the counts was calculated (50791 ± 3444). A statistically significant difference from the zero standard was taken to be more than 2 times the standard deviation from this repeated mean (i.e. 43903 counts). The corresponding aldosterone value was thereafter determined from its intercept with the displacement curve and corresponded to 8 pg/ml.

c) Precision

Intra-assay coefficients of variation were determined by 20-fold measurements of pooled plasma samples with aldosterone concentrations of 17 pg/ml, 37 pg/ml, and 208 pg/ml which were extracted, reconstituted, pooled again and then added as samples on the same plate and found to be 21.3%, 12.3%, and 10.3% respectively. Inter-assay coefficients of variation were determined by 20-fold measurements, on consecutive days, of plasma samples of 15 pg/ml, 33 pg/ml, and 158 pg/ml and were found to be 15.8 %, 14.6%, and 7.1 % respectively.

d) Linearity and recovery

A plasma sample which was measured above the highest standard point of the linear range of the assay was diluted (1:2) in the matrix medium used to produce the calibrators and measured at 963 pg/ml. This sample was then further serially diluted (1:4, 1:8, 1:16, 1:32) and the average recovery rate was found to be 105% (Table 1). Two plasma samples, measured at 29 pg/ml and 20 pg/ml, were each extracted and reconstituted a total of nine times and each of the standard points was then added at a 1:1 dilution to the sample. Average recovery for each sample was found at 102% and 115% (Table 2).

Clinical validation

Plasma aldosterone in RIA compared to TR-FIA

Plasma samples measured by the TR-FIA after extraction showed a high correlation to the values obtained by measurement with the commercially available radioimmunoassay kit (Figures 3, 4) as described by the equation ($y = 0.66x - 3.69$; $R^2 = 0.932$). The TR-FIA values mirrored the fluctuations seen in the patients' aldosterone plasma levels throughout the day as measured by the RIA kit. Values measured by the in-house TR-FIA were at approximately 66% of the concentration given by the RIA.

Serum aldosterone concentrations in male and female mice

Values from all wild-type animals used in the study were examined in order to determine gender differences which may be present in mice. There was no overall significant difference ($p = 0.396$) found between female ($n = 35$) and male ($n = 40$) mice, whose serum values had mean ($\pm$ SEM) levels of 163 ± 34 pg/ml and 181 ± 27 pg/ml, respectively (Figure 5). In addition, there was no sex difference in any particular age group ($p > 0.05$ at 3, 5, 7, 9 and 11 weeks, Table 3).

Serum aldosterone levels during development

Since no gender-dependent differences in serum aldosterone levels were observed throughout the studied points of time (3, 5, 7, 9 and 11 weeks of age) aldosterone values for female and male mice were averaged at the different points of time (week 3: n = 8, week 5: n = 8, week 7: n = 27, week 9: n = 12 and week 11: n = 20; Figure 6) and found to have mean (± SEM) aldosterone concentrations of 52 ± 9 pg/ml, 57 ± 11 pg/ml, 272 ± 45 pg/ml, 107 ± 33 pg/ml, and 174 ± 34 pg/ml respectively. The Kruskal-Wallis non-parametric test displayed an overall significance between the groups (χ^2 = 18,98; df = 4; p = 0.001). No significant difference in aldosterone levels was found between weeks 3 and 5 (p = 0.753). At an age of 7 weeks aldosterone rose by 377% compared to the 5-week old mice (p = 0.003). Although there was a drop at week 9, serum aldosterone concentrations remained high overall and were not significantly different between 9 and 11 weeks (p = 0.115). At week 11 the aldosterone levels were still significantly higher than at both weeks 3 and 5 (p = 0.007 and p = 0.011).

Serum aldosterone during suppression and stimulation tests

Kruskal-Wallis non-parametric test showed an overall significance between the three groups of control, dexamethasone suppressed, and ACTH stimulated mice (χ^2 = 15.866; df = 2; p < 0.001; Figure 7). Animals treated with dexamethasone had significantly lower concentrations of aldosterone (35 ± 3 pg/ml) than the control mice (114 ± 33 pg/ml; p = 0.021). 6 out of the 7 mice treated with dexamethasone suppression had values below the LOD (8 pg/ml). For the calculation of the mean, all these values were assigned a concentration of 8 pg/ml. Mice stimulated with ACTH showed significantly elevated values compared to the control group (603 ± 119 pg/ml; p = 0.003).

Discussion

In the present study we describe the development and application of an immunoassay designed to detect levels of aldosterone in small volumes of human or mouse serum or plasma samples. Assays previously developed for the measurement of aldosterone in human serum or plasma and modified for use in animals, are mostly radioactive, require high volumes and employ polyclonal antisera. To our knowledge only two studies report the use of a monoclonal antibody (de Lauzon *et al.*, 1987, Hanquez *et al.*, 1988). In the first study, however, the assay required samples of 2 ml for extraction purposes, the limit of quantification was 20 pg/well and this assay was not validated in serum or plasma of rodents. In the second case, application of the assay using biological fluids was only suggested but not demonstrated or applied.

Assay validation

In the assay described and validated here we utilize a monoclonal antibody which allows the measurement of concentrations in a very sensitive range and with reproducible results. The assay works in a competitive manner with the advantage of incorporating a non-isotopic, biotin-labeled aldosterone tracer, so that the amount of aldosterone present in the sample correlates inversely with the amount of antibody immobilised in each well and bound to the biotin tracer. Although the exact amount of immobilized aldosterone per well is unknown, the results from the dilution curves in Figure 1 indicated that the optimum amount of antiserum was that yielded by a well-coating at a 1:2500 titration of the original supernatant retrieved from cell culture. The validation criteria for the assay have been met accordingly, and the assay shows good linearity and reproducibility following extraction of samples with dichloromethane. The main advantage presented here is the very small amount of only 50 µl of serum or plasma necessary for a duplicate measurement due to modification

of a previously established extraction procedure. This is especially useful for procedures requiring repeated blood drawing, for example that recovered by tail-vein bleeding. Furthermore, the assay allows detection of aldosterone levels at low concentrations as may be the case in pharmacological studies or in genetically modified mice in which circulating aldosterone concentrations are altered and may be extremely low.

As a further step towards validating the assay we have correlated the values obtained with the TR-FIA to a commercially available RIA commonly used in many laboratories carrying out animal studies, albeit with conflicting results, for baseline values in wild-type rodent plasma and serum samples. Although our TR-FIA measures at a lower level of approximately 66% that of the RIA, most probably due to the fact that a monoclonal antibody is more selective in the species of aldosterone within a sample that it recognizes, there was good correlation between the two assays (R^2 = 0.93; Figures 3 and 4). The difference in measurement can also be explained by the fact that the commercial RIA we used for comparison does not incorporate an extraction step in the assay procedure, a step which excludes the possibility of potentially interfering substances giving falsely high results. Major differences in mean aldosterone values despite high inter-assay correlations are common and have been described previously (Schirpenbach *et al.*, 2006). Differences in the specificity of antibodies as well as several other factors may contribute, one important issue arising from the difference in calibration of assays, which often generates higher values in some compared to lower values in others.

Gender- and age-related differences in serum aldosterone concentrations of mice

In the present study we report values for serum aldosterone in mice, which are well within the measurable range of this assay. After assessment of samples taken from seventy-five C57BL/6 x NMRI mice we found no gender-dependent difference. This observation included male and female mice aged between 3 and 11 weeks after birth. We exclude the

possibility that anaesthesia or blood sampling may have influenced steroid levels in our experiments since, as has been shown previously (Vahl *et al.*, 2005), rapid sampling carried out in less than 3 minutes does not trigger a stress response. To our knowledge so far, there has been no in-depth study describing gender-related levels of aldosterone in these or any other strain of mice. In one recent study (Sausbier *et al.*, 2005), carried out to evaluate the influence of large conductance, voltage- and Ca^{2+}-dependent K^+ (BK) channels, BK mutant mice exhibited higher serum aldosterone levels than wild-type mice of the same SV129 x C57BL/6 genetic background, but no differences between male and female mice were observed in either group.

Other authors have previously found (Zhang *et al.*, 2003), and it is stated in Jensen et al. (Jensen *et al.*, 2004), that aldosterone does not change significantly in rodents during postnatal development, however, these experiments were carried out in rats. The importance of aldosterone in developmental processes, such as growth of the kidney, has been addressed by many studies. For instance, targeted disruption of aldosterone and the components of the RAAS in mice leads to diminished kidney growth, vascular wall thickening and low blood pressure (Jensen *et al.*, 2004). In the present investigation we observed a highly significant increase in the levels of aldosterone between five and seven weeks of age and, though these levels decreased at weeks 9 and 11, they remained at a significantly higher level when compared to 3- and 5-week-old mice. In a current study we have also analysed the zonal composition in the adrenal glands of exactly the same strain of mice (Bielohuby *et al.*, in press). Although total cortical volume was clearly higher in female mice when compared to their male littermates, the zona glomerulosa did not contribute to this gender dependent phenotype. These findings confirm our measurements on aldosterone concentrations comparing male and female mice. We also found a significant rise of total zona glomerulosa volume comparing 3 and 7 weeks of age with no further significant changes until week 11, similar to what we observed in serum aldosterone concentrations. Changes in aldosterone

levels accompanied by changes in adrenal weights have not been systematically studied yet and though these observations merit further attention.

No difference in plasma aldosterone concentrations between neonate and adult 3-month-old mice (C57BL/6 strain) were reported by Cao et al (Cao *et al.*, 2006). As we did not include mice older than 11-weeks in our study, it is possible that the aldosterone levels decline thereafter. In another study showing aldosterone levels in 1-week- and 1-month-old wild-type and proopiomelanocortin (POMC) null animals, the authors reported levels which were approximately twice as high in the wild types at 1 month compared to 1 week (793 ± 103 vs. 357 ± 145 pg/ml) and significantly decreased at one month (203 ± 39 pg/ml) in the POMC null mice, while no difference occurred between wild-type and mutant mice at week 1 (89 ± 5 pg/ml) (Karpac *et al.*, 2005). However, the different strain, assay, and blood sampling techniques used hamper a comparison between the studies.

Aldosterone and ACTH in mice

Research on the actions of ACTH on the adrenal cortex have focused, so far, mainly on its maintenance of glucocorticoids in plasma, for example its role in aiding steroidogenesis via transfer of cholesterol across the mitochondria in conjunction with steroid acute regulatory protein (StAR). But also on its chronic effects to enhance the gland's steroidogenic enzymes in the biosynthetic pathway and to make more cells capable of conducting steroidogenesis (Vinson, 2003). Although assessment of adrenal function in human patients for diagnostic purposes is carried out by standardized ACTH tests (Alia *et al.*, 2006), there is little direct evidence available for the actions of ACTH on the production of aldosterone in rodents. Aldosterone has primarily been studied in the context of its function within the RAAS. One study has shown diminished, though detectable, levels of aldosterone with no distinguishable architecture of the zona glomerulosa in POMC deficient mice (Coll *et al.*, 2004) and the authors in that study report that aldosterone levels do not increase in response to ACTH

injection. Others report aldosterone levels below limits of detection in mice with a complete lack of ACTH (Yaswen *et al.*, 1999), which illustrates the need for standardisation in order to avoid discrepancies in assay measurement as an interfering factor. It was our purpose to indicate the applicability and relevance of the current assay by showing that adrenocortical function can be monitored effectively in small sample volumes. To demonstrate this we carried out a study including pharmacological intervention with dexamethasone suppression as well as stimulation with an ACTH analogue (Synacthen). ACTH is thought to be an acute regulator of aldosterone synthesis in stress situations (Davies *et al.*, 2007, Muller, 1995). To demonstrate that there were clear increases and decreases in the production of aldosterone under the circumstances of the study and to make sure that there was definite hyper- and hypoplasia of the adrenal gland under stimulation and suppression we used a previously established protocol (Coll *et al.*, 2006).

Conclusion

In the present article we describe the development of a highly sensitive TR-FIA for the determination of aldosterone levels in small volumes of serum or plasma. The assay employs a highly specific monoclonal antibody against aldosterone and requires a sample of only 50μl. We have validated the system in human plasma and mouse serum samples and shown its potential to be used in pharmacological intervention studies. Furthermore, we have shown that in mice there is an increase in aldosterone levels around puberty. As mice aged between 3 and 11 weeks are frequently used to carry out studies, our results may serve as a reference point for aldosterone concentrations in future experiments. We have thus shown that the assay presented is a reliable technique which can potentially be used to measure the mineralocorticoid aldosterone in a variety of experimental settings and mouse models where endocrine changes are being investigated.
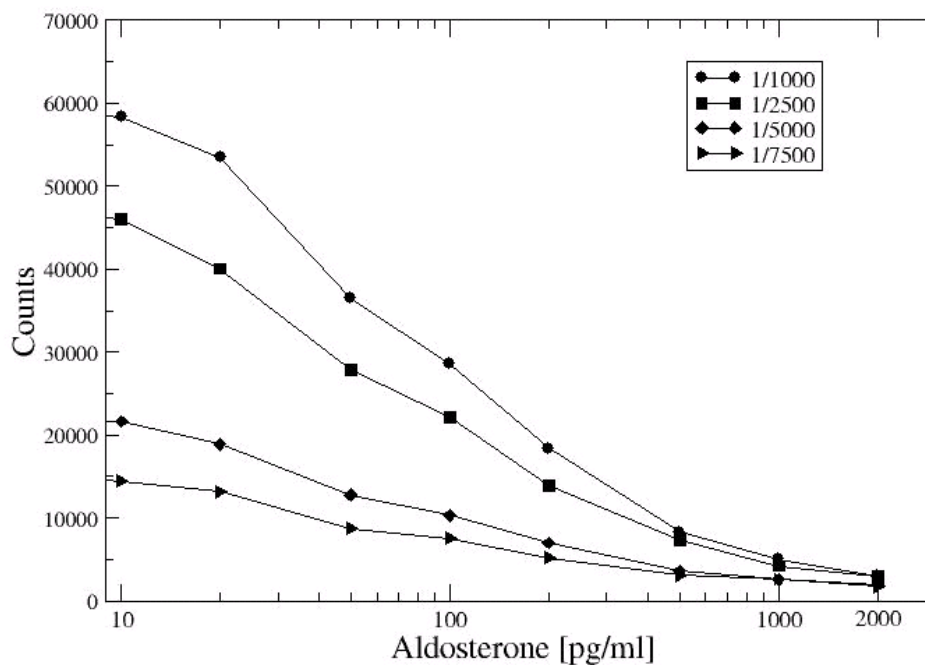
Figures:

Figure 1. Curves for the displacement of aldosterone obtained by different titrations of anti-
aldosterone antiserum used for coating of microtiter-plates. The curves show counts at
maximal binding when no un-labeled aldosterone is present in the calibrators and decreasing
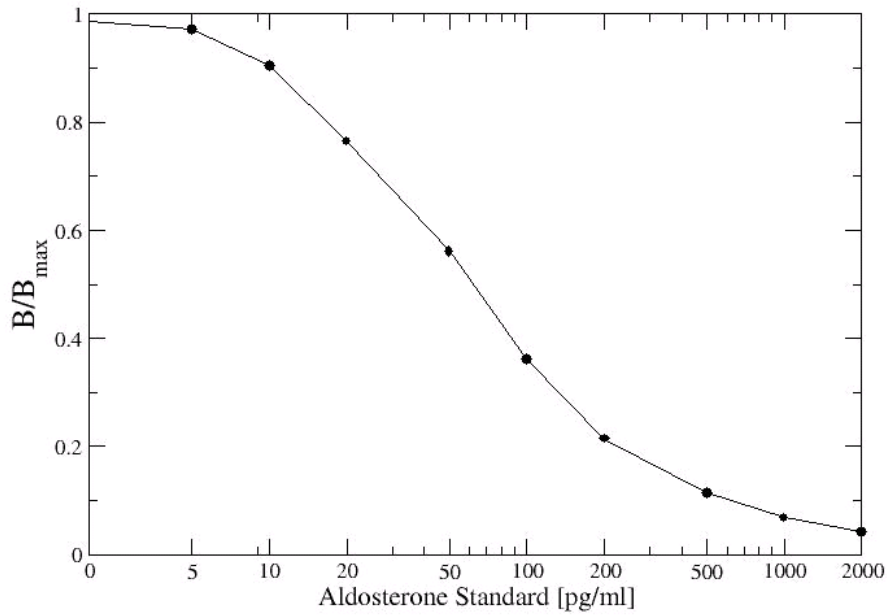counts with reduced binding due to increasing concentrations of labeled aldosterone.

Figure 2. Binding of aldosterone in standards by monoclonal antibody for assay's displacement curve. Amount bound is expressed as a fraction of maximal binding in the standard containing no labeled aldosterone.
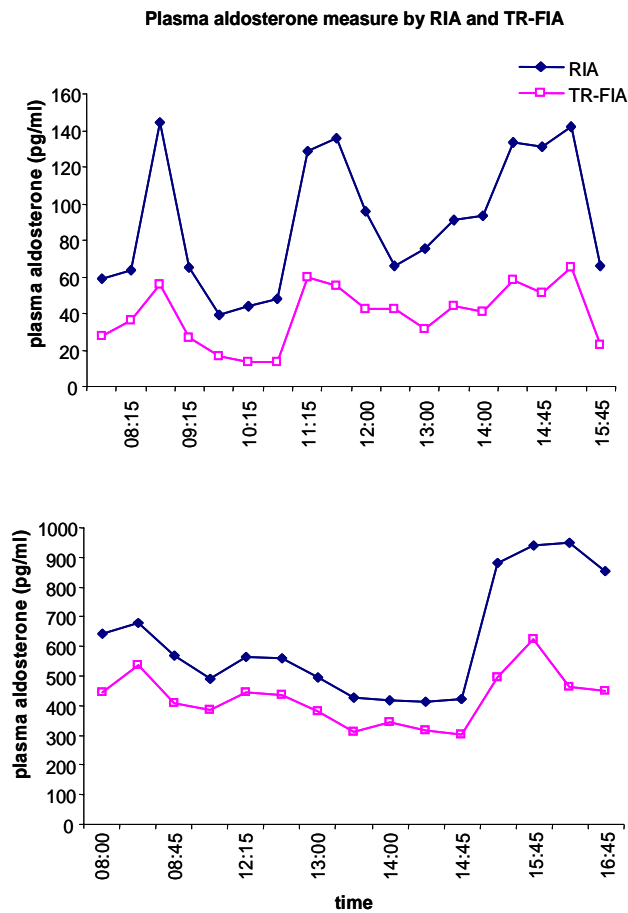

Figure 3. Comparison of plasma aldosterone as measured by the TR-FIA and by a commercially available RIA (DPC coat-a-count).
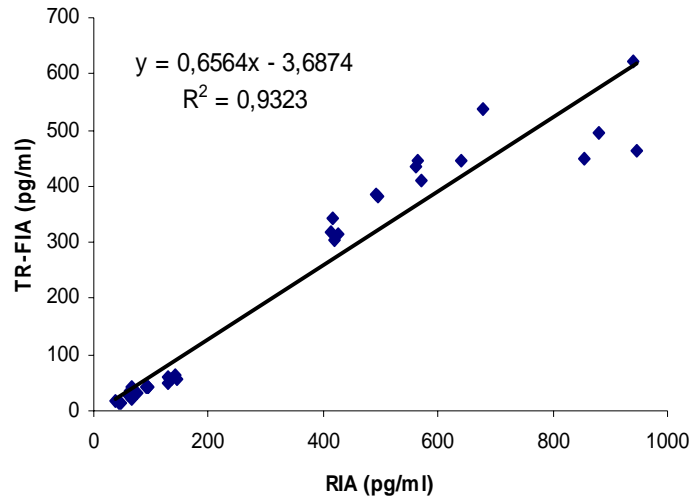
Figure 4. Correlation of plasma samples analysed by both the commercial RIA and TR-FIA. The correlation was found to be extremely high ($R^2 = 0.886$) even at lower concentrations. TR-FIA measured at levels at approximately 42% of the RIA.
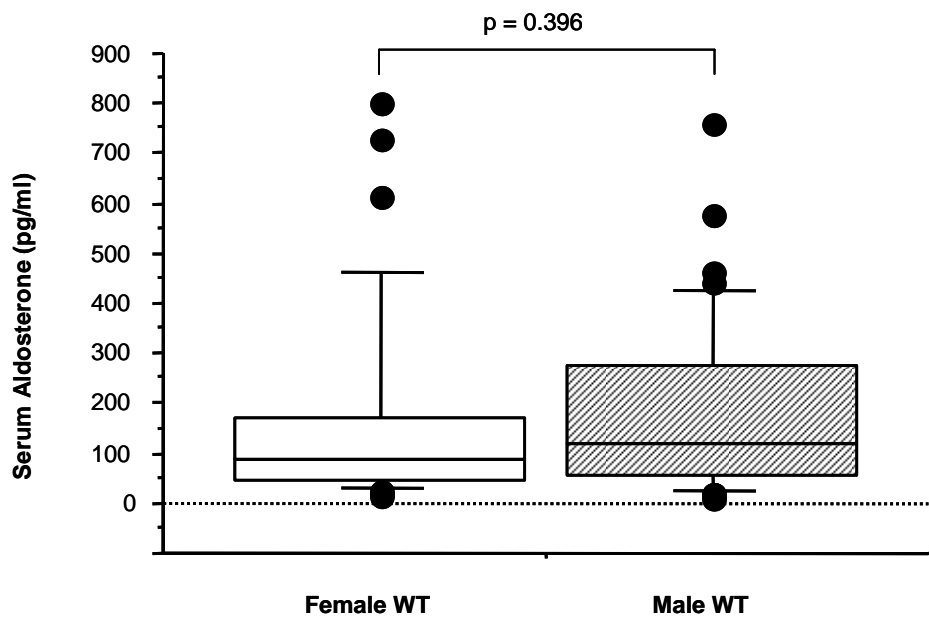

Figure5. Mean serum aldosterone levels in female and male WT mice (n = 35 and n = 40 respectively). Bars indicate 25th and 95th percentiles for each group and significance was calculated by unpaired t-test.
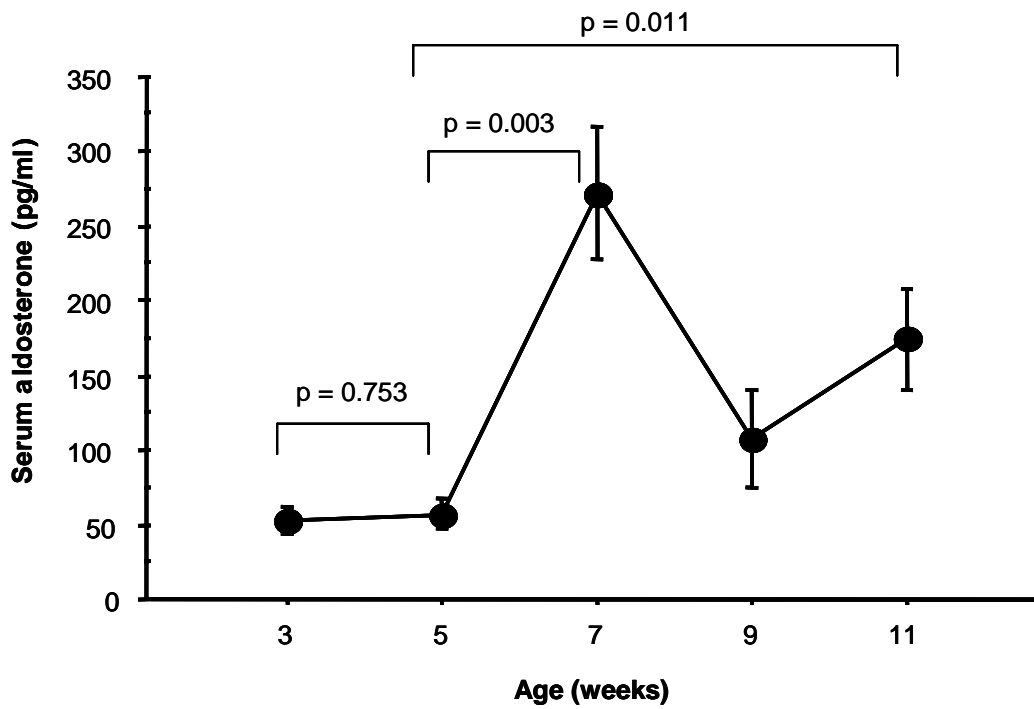
Figure 6. Mean (±SEM) serum aldosterone levels in grouped male and female WT mice at indicated weeks of development. 3 weeks (n = 8), 5 weeks (n = 8), 7 weeks (n = 27), 9 weeks (n = 12) and 11 weeks (n = 20). Significances were determined by Kruskal-Wallis and unpaired t-test.
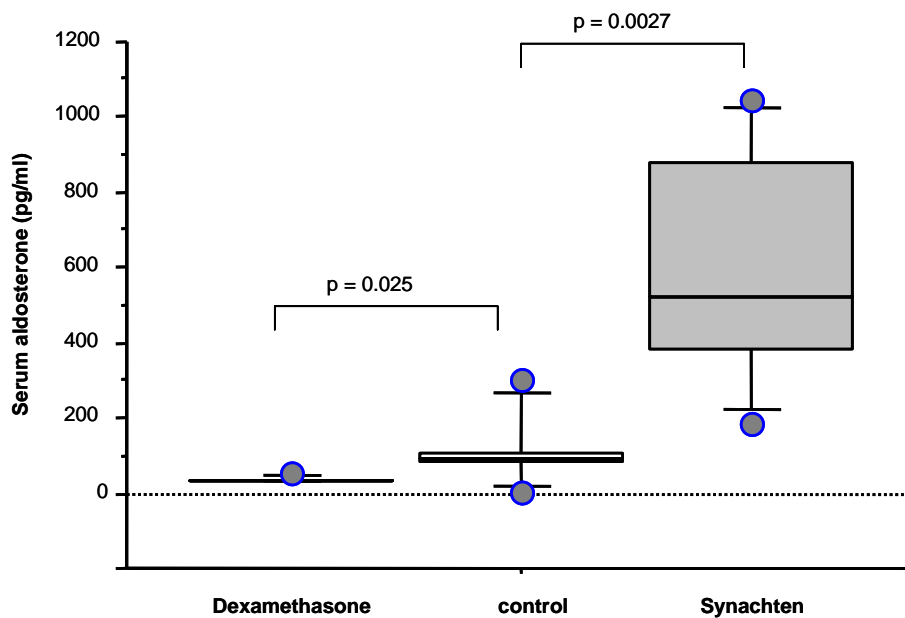

Figure 7. Mean serum aldosterone levels in grouped male and female WT mice treated with 10days dexamethasone, 10 days NaCl (control group), and 10 days Synacthen ACTH. Significance compared to baseline control group were determined by unpaired t-test.

	Dilution factor	Measured value	Expected value	(%) Expected value
Dilution in 0 std	original sample	>std	--	--
	1:2	963	--	100
	1:4	509	481.5	106
	1:8	272	240.8	113
	1:16	125	120.4	104
	1:32	62	60.2	103
Avg % expected				105%

Table 1 Linearity of plasma sample serially diluted in the "0" standard after extraction.

Table 1. Linearity of a plasma sample serially diluted in the "0" standard after extraction.

Table 2. Recovery of samples after extraction and spiked at a 1:1 dilution with each of the standard points.

1:1 Dilution with std (pg/ml)	Measured value	Expected value	% expected value
Sample A	29	--	100
+ 5	20	17	117.6
+ 10	20	19.5	104.1
+ 20	27	24.5	108.6
+ 50	46	39.5	115.4
+ 100	65	64.5	100.3
+ 200	119	114.5	103.5
+ 500	239	264.5	90.4
+ 1000	476	614.5	77.5
+ 2000	1053	1014.5	103.8
Avg % expected			**102%**
Sample B	20	--	100
+ 5	18	12.8	140.6
+ 10	21	12.3	170.7
+ 20	18	20.3	88.7
+ 50	42	35.3	119.0
+ 100	55	60.3	91.2
+ 200	120	110.3	108.8
+ 500	316	260.3	121.4
+ 1000	560	510.3	109.7
+ 2000	1011	1010.3	100.1
Avg % expected			**115%**

Table 2. Recovery of two plasma samples, reconstituted after extraction and spiked at a 1:1 dilution with each of the standard points.

Age (weeks)	Female		Male		p
	n	Aldosterone (pg/ml)	n	Aldosterone (pg/ml)	
3	4	54 ± 9	4	51 ± 17	0.773
5	4	73 ± 16	4	42 ± 10	0.149
7	12	277 ± 77	15	268 ± 54	0.807
9	6	61 ± 15	6	154 ± 62	0.174
11	9	170 ± 62	11	178 ± 37	0.569

Table 3. Serum aldosterone concentrations in female and male mice at indicated weeks of age.

Table 3. Serum aldosterone concentrations in female and male mice at increasing weeks of age. There was no significant difference between the male and female mice throughout the weeks of development.

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8. *Discussion & future prospects*

Adrenal gland studies - rats versus mice

So far, numerous studies on the adrenal glands have been performed in rats, whereas in the present work, mouse hybrids (C57BL/6 x NMRI) were used. In literature, two explanations for the frequent use of rats are found: first, the mouse adrenal gland is relatively small and second, the incomplete understanding of the X-zone poses a further obstacle (Vinson 2003). Although much research has been done in even smaller tissues, such as mouse ovaries (Barnett *et al.* 2007), the small size of the mouse adrenal gland can be a problem regarding e.g. the yield of the total protein amount from homogenized tissue. Nevertheless, the mouse is the most common laboratory animal used (Bundesministerium für Ernährung 2005) and some techniques, e.g. genetic engineering or ENU mutagenesis projects are simplified in mice (Weinreb & Lindsey 2005; Hanley & Arlt 2006; Rathkolb *et al.* 2000). Thus, detailed knowledge of physiological growth and function of the mouse adrenal glands are an important basis for these approaches.

The aldosterone assay

In the second part of this study, by use of a new, highly sensitive imunoassay for the determination of aldosterone in rodent and human serum or plasma, serum aldosterone concentrations were measured in mice. Aldosterone is the "minority" hormone of the adrenal gland. Its plasma concentration is about 2000 times smaller than that of corticosterone (Muller 1995). Therefore, much sensitiver assays are needed if compared with e.g. corticosterone assays.

The assay requires only a small sample volume and is highly specific

Previous assays were primarily developed for the use in humans and not validated for the measurement of rodent samples. Amounts of 250-500 µl of plasma or serum are commonly needed for the measurement of aldosterone (Parth *et al.* 1976; Nichols Institute Diagnostics 2003; Adaltis Italia S.p.A. 2004). In humans, relatively large amounts of plasma or serum are rather easy to obtain, whereas in mice, large quantities of serum or plasma are not always available. Therefore, the establishment of a sensitive assay that utilizes small sample volumes is beneficial also for future studies. The assay described, utilizes only 50 µl for a duplicate measurement of aldosterone. A small sample volume is especially desireable, if more than 50 µl can not be obtained from a single sampling procedure. This is the case when methods such

as tail clipping are used for blood sampling in mice. On the other hand, it can be usefull to measure a whole panel of different serum or plasma parameters. Using the majority of the serum or plasma sample for aldosterone thus would restrict analyses of additional parameters. Furthermore, the assay uses a highly specific monoclonal antibody. In contrast, most commericially available assays use polyclonal antibodys which results in a weaker sensitivity. In the developed assay, cross reactivity with other, potetially interfering steroids was found to be negligible, which generates reliable and reproducible results, even of extremely low, suppressed aldosterone concentrations. Furthermore, the use of a non radioactive labeling technique faciliates the aquaintance with this assay, as the obvious hazards of radioactivity are purged.

Stereological methods

Stereology provides practical techniques for extracting quantitative information about a three-dimensional object from measurements made on two-dimensional planar sections of the object (Gundersen & Jensen 1987). The stereological methods used for the morphometrical analyses of the adrenal glands are model-free and allow a precise estimation (Gundersen *et al.* 1988; Mattfeldt 1990) of the measured zonal volumes or the cell numbers / sizes, respectively. In the examined period, between weaning and adulthood, by use of stereological methods, absolute values of adrenal gland zonal compostion and total cell numbers / mean cell volumes were obtained for the first time. A major problem of previous studies was, that morphometrical results generated by use of different techniques, such as the areal measurements of adrenal gland mid-sections or measured cortical diameters, were relevant only for the study used and were not applicable to other studies. In contrast, absolute values do allow a direct comparison between studies using unbiased stereology. Thus the benefits of using quantitive stereological methods can only be emphaszied.

The systems biology approach

In numerous studies, specific animal models at either singular points in time or defined artificial conditions are used to elucidate biological features. However, biological processes are subject to permanent modifications and alterations, thus dynamic and systematic studies promote a more comprehensive picture of these processes. Systems biology approaches on organ systems are aimed at a more comprehensive and integrative characterization and understanding of biological systems. The combination and integration of different data sets, methods or time axes can lead to a larger body of biological information (Quackenbush

2006). Furthermore, the increased understanding of molecular processes together with now available advanced techniques enable the re-examination of earlier concepts, also of adrenal gland growth and function (Vinson 2003). In order to meet the requirements of systems biology approaches, the presented study was conducted by systematically analysing adrenal gland growth and function from weaning to adulthood.

Adrenal gland growth in mice is affected by gender and age

Adrenal glands in female mice are markedly enlarged if compared to male mice (Hatai 1914; Bastida *et al.* 2006). With the exception of the zona glomerulosa, also the different zones display a gender-dependent growth behaviour. Therefore, in adrenal gland studies, the gender issue needs to be addressed. In many cases, a combination of data that arise from both genders is questionable. Unfortunately, even in recent studies concerning the adrenal glands, some authors do not discriminate between male and female mice.

The adrenal glands of mice do not grow in a consistent or linear way, they rather display an inhomogeneous growth pattern from weaning to adulthood. Consequently, the timing for future studies on adrenal glands needs to be considered and defined carefully. Moreover, analyses of more than one age group might help in the interpretation and verification of complex data sets.

Serum aldosterone concentrations and zona glomerulosa volumes

No differences in the absolute zona glomerulosa volumes of male and female mice were found, with the exception of 9-week-old mice. In accordance with these stereological results, there was also no difference between the genders on the functional side (i.e. the serum aldosterone concentrations), which argues for a similar physiological condition in male and female mice. However, there is a discrepancy among the surprisingly scarce literature found on gender-dependent aldosterone levels in mice so far: a recent study reported a significant difference between male and female Swiss CD1 mice. In that study, female mice had markedly higher plasma aldosterone levels than male mice (Bastida *et al.* 2006). In contrast, a study by Heikkila et al. in 2002 reported no gender-dependent differences in plasma aldosterone concentrations. Therefore they used mouse hybrids with a 129SV x CD1 background (Heikkila *et al.* 2002). Apart from the influence of genetics and blood sampling techniques, probably also the variability in the assays commercially available to measure aldosterone and the antibodies utilized play an important role on the obtained plasma aldosterone concentrations.

Serum corticosterone concentrations: effects of sex, age and sampling methods

Bastida et al. reported higher corticosterone levels in female mice if compared to male littermates (Bastida *et al.* 2006). Also in different rat and mouse studies, females had higher corticosterone concentrations than males (KITAY 1961; Le Mevel *et al.* 1979; Lesniewska *et al.* 1990; Atkinson & Waddell 1997). Contrasting these studies, Badr analysed three different mouse strains and did not find marked differences between male and female mice regarding the corticosterone concentrations (Badr 1971). In the presented study, female mice had higher serum corticosterone concentrations, however, statistical significance was reached only in 5-week old mice.

Age has also been found to influence the serum corticosterone concentrations, as they decreased significantly in both genders between 3 and 11 weeks of age. Rather conflicting results regarding the age-related alterations of serum corticosterone concentrations are found in literature: in different mouse models, age has been found to decrease (Hess & Riegle 1970; Britton *et al.* 1975), not to affect (Barnett *et al.* 1974; Tang & Phillips 1978) or to increase (Landfield *et al.* 1978; DeKosky *et al.* 1984), the blood corticosterone levels. Most probably, serum corticosterone concentrations depend at least in part on the genetic background as well as on the blood sampling method used. However, in this study, the corticosterone analyses were repeated with three independent mouse cohorts and in all experiments the results were similar.

Zona fasciculata volume, mean cell volumes/total cell numbers and corticosterone secretion

In different transgenic animal models, areal changes of the zona fasciculata were reflected by respective changes in the corticosterone concentrations. In growth hormone transgenic mice, which had markedly enlarged zona fasciculata volumes (Hoeflich *et al.* 2002), as well as in animal models in which the zona fasciculata volumes were reduced (Coll *et al.* 2004; Karpac *et al.* 2007; Patchev *et al.* 2007), the corticosterone concentrations were correlated with the areal changes of the zona fasciculata. Interestingly, in the present study, the enlarged zona fasciculata in female mice compared to male mice was not fully reflected by corresponding higher serum corticosterone levels. Female mice had higher corticosterone concentrations, but those were not that high as would have been expected from the clearly larger zona fasciculata volumes. However, the differences between transgenic and non-transgenic studies might also be due to genetically derived alterations of factors, which regulate corticosterone secretion, independent of the zona fasciculata volume and this study shows, that adrenal gland size or

zona fasciculata volume respectively is not a definite indicator for the serum corticosterone levels present.

To further clarify the relationship between the corticosterone concentration and the zona fasciculata volume, zona fasciculata mean cell volumes und total cell numbers were measured. From the 5th week 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 three-fold between 3 and 7 weeks and then decreased by about 50%. This decrease in cell number was associated with a steep increase in zona fasciculate cell volume. In males, age-related changes in zona fasciculata cell number and cell volume were less pronounced. However, in male and female mice, changes in zona fasciculata cell number and volume did not result in corresponding changes of serum corticosterone concentrations. The coherency between zona fasciculata cell hyperplasia or cell hypertrophy respectively and plasma corticosterone levels is discussed by several authors. In a study by Patchev et al., targeted deletion of the transcription-intermediary-factor-2 (TIF-2) in mice, a co-activator of the glucocorticoid receptor, led to reduced basal morning corticosterone levels. Interestingly, they also found a reduction of the cellular densities in the zona fasciculata of these mice (Patchev *et al.* 2007). In 2002, Hoeflich et al. showed an interrelation between zona fasciculata cell sizes and corticosterone levels. Therefore they studied transgenic mouse models overexpressing growth hormone (GH) and GH transgenic mice co-expressing increased levels of insulin-like-growth-factor-binding-protein-2 (IGFBP-2). The reduction in zona fasciculata cell sizes of the GH / IGFBP-2 double transgenic mice was accompanied by a reduction of the corticosterone levels. Total zona fasciculata cell numbers and ACTH levels were unaffected in both transgenic models (Hoeflich *et al.* 2002). A recent study published by Ulrich-Lai et al., links both, zona fasciculata cellular hypertrophy and hyperplasia, to increased plasma corticosterone levels (Ulrich-Lai *et al.* 2006). In that study in the rat, adrenal gland growth and function were analyzed using a chronic stress model. These data support the idea, that the increased adrenal weight in chronically stressed animals is due to an increase in cell size and in cell number. The increase in cortical function (i.e. corticosterone) was paralleled by hyperplasia and hypertrophy. In order to better understand the relation between absolute numbers and mean volumes of zona fasciculata cells with corticosterone secretion, the total amounts of corticosterone in the circulation (ETAC) were estimated (AJP paper, Fig. 5 B). Although a direct statistical correlation analysis of the parameters ETAC, cell number and cell volume is difficult to obtain, as the parameters were gained in different mice, 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. This indicates a functional role of zona fasciculata cell numbers for corticosterone secretion over time in male and female mice. In contrast, no association between the means of the ETAC and the means of the cell sizes was found. However, the sharp decline of cell numbers in 7- to 9-week old female mice 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-week old female mice. Thus, it might be concluded from this study, that both cell number and cell volume are important for physiological corticosterone secretion.

Control of steroid-biosynthesis

Steroidogenesis, and thus corticosterone synthesis, is also regulated by a) the amount of stored lipids, which are in the case of the adrenal glands predominantly cholesterol or cholestrerol esters (such as cholesteryl palmitate and cholesteryl adrenate) respectively (Cheng & Kowal 1994), as well as by b) steroidogenic enzymes (Williams 2005; White 2006). To address the stored lipids content in the course of this study, adrenal gland sections were stained by the Oil-Red-O staining method. Although absolute quantification of the stored lipids in the sections used is not possible and the experimental animal number is yet too small, differences appear to be present. In fact, the amount of stored lipids seems to be higher in female and male 11-week old mice if compared to 7-week old female or male mice, respectively. Furthermore, the amount of stored lipids appears to be higher in female than in male mice. Also different steroidogenic enzymes are regulated during adrenal gland growth. The first, and rate-limiting step in the steroid synthesis is catalyzed by cytochrome P450scc, the cholesterol side-chain cleavage enzyme (SCC), which converts the common precursor cholesterol into pregnenolone (Miller 1988; Hu *et al.* 1999). After two further enzymatic conversion steps, 11-β-hydroxylase (isoforms CYP11B 1 and 2) converts 11-deoxycorticosterone to corticosterone (White 2001; Williams 2005) and thus also determines the final corticosterone concentration (see also Fig. 2). In humans, 11-β-hydroxylase furthermore transforms 11-deoxycortisol to cortisol (White 2001). In this study, an increased expression of both enzymes, SCC and 11-β-hydroxylase, was found in 3- vs. 11-week old mice. These results, together with the data on the stored lipids indicate, that 11-week-old mice have an increased capacity of steroid biosynthesis than younger mice.

Systematic and longitudinal *in vivo* studies of steroidogenesis regulating factors are rare. In a rat study by Hatano et al., SCC expression also gradually increased with age (Hatano *et al.*

1994). Bruder et al. analysed zona glomerulosa cells from 7 and 42 days old rats. They found an increased activity of SCC and 11-β-hydroxylase in the cells taken from the older rats (Bruder *et al.* 2002). Thus, age and probably also gender affect the biochemical control of steroidogenesis. As a perspective, the time- and gender-dependency of steroidogenesis controlling enzymes together with the stored lipid amounts in the adrenal gland, should receive detailed investigation.

Concluding remarks

In the last two decades, advanced techniques and new experimental animal models gave privileged insights into specific aspects of adrenal gland physiology. The challenge in the future will be to generate also more dynamic views on the control of adrenal growth and functions during the whole life-span. As a perspective multi-dimensional insights into the complex functions and interactions of tissues are needed in order to fulfil the demands of systems biology.

9. Summary

The adrenal gland is a complex endocrine organ and its secreted hormones affect a multitude of essential systems, including stress response, immune function, cardio vascular regulation and anabolic or catabolic processes. In order to define age-dependent changes within the adrenal gland in the mouse, a systematic analysis of functional and growth-related parameters was performed by investigating the adrenal glands of male and female mice from weaning to adulthood (weeks 3, 5, 7, 9 and 11) at the organ, compartment, and cellular level. Therefore, an adrenal weight analysis was performed and the absolute volume of every adrenal zone was determined using unbiased stereological methods. At the cellular level, zona fasciculata (ZF) mean cell volume and total cell number were analysed at five distinct points in time. Furthermore, the amount of stored lipids and the expression of two enzymes involved in steroidogenesis, side-chain-cleavage enzyme (SCC) and 11-β-hydroxylase, were explored in the adrenal glands at selected points in time. Serum aldosterone and corticosterone concentrations were monitored throughout the investigation period as functional readouts of the zona glomerulosa and the ZF, respectively.

Results: Increasing weights of the adrenal glands were observed until week 7. Of note is a significant adrenal weight reduction by 25 % exclusively in male mice, between weeks 7 and 9. Adrenal glands in female mice principally displayed higher weights. At an age of 9 weeks,

this weight difference reached a maximum, with female mice characterized by two-fold higher adrenal gland weights than male mice. The weight dimorphism was in particular due to an enlarged ZF in female mice. Also the X-Zone and the medulla, showed increased volumes if compared to male littermates. No gender-dependent volume difference was found in the zona glomerulosa. In addition to the gender-dependent growth effects, time-dependent growth features were observed in each zone, and every zone, with the exception of the X-zone in males, gradually increased in volume between weeks 3 and 7 of age. Time- and gender-dependent effects were also studied at the cellular level in the ZF, where effects of sex and age on the mean cell volumes as well as on the total cell number were present. From week 5 onwards, the ZF of female mice comprised significantly more cells if compared to their male counterparts. In both genders, the number of ZF cells reached a maximum at the age of 7 weeks and decreased significantly until week 9. In male mice, the mean volume of ZF cells increased between 3 and 11 weeks of age. In 3- to 7-week-old female mice ZF mean cell volumes were similar, however, between 7 and 9 weeks of age, a volume increase by approximately 50 % was observed. A positive association of ZF total cell number with the estimated total amounts of corticosterone in the circulation (ETAC) could be established, in contrast, no association of ZF mean cell volume and the ETAC was found.

Serum aldosterone concentrations were similar in both genders, in contrast serum corticosterone levels were higher, at least as a tendency, in female mice. Moreover, dynamic changes of both steroid hormone concentrations were observed in a time-dependent manner. The aldosterone concentrations increased over time, whereas the corticosterone levels decreased significantly between weeks 3 and 11. The amount of stored lipids in the adrenal glands appeared to be higher in female mice if compared to male mice and the adrenal glands of 11-week-old male and female mice seem to store more lipids than 7-week-old mice. Also the expression of the two analysed enzymes, SCC and 11-β-hydroxylase, increased between 3 and 11 weeks of age in both sexes, indicating that 11-week-old mice might have a higher steroidbiosynthesis capacity than the younger mice.

In conclusion, this study demonstrates that, between weaning and adulthood dynamic processes are present on multiple levels of adrenal gland growth. Specific adrenal growth regulation occurs through the influence of age, sex, cell type, cell number and cell size. A particular finding of the study is, that growth is not a unidirectional process by constant increases of cell size and cell number and that decreases of cell number may be compensated by increases of cell volumes. Certainly, these dynamic processes can be assumed to affect also functional parameters, however the final quantity of corticosterone secreted can only be

explained in parts as a function of ZF cell size or ZF cell number, respectively. In general, this work supports the systemic approach integrating multiple information from various levels being more and more relevant for future research projects.

10. Zusammenfassung

Die Nebenniere ist ein komplexes, endokrines Organ, und die von ihr freigesetzten Hormone beeinflussen eine Vielzahl lebenswichtiger Systeme: Sie leiten eine adäquate Stress-Antwort ein, modulieren die Immunfunktion und das Herz-Kreislauf-System sowie anabole und katabole Stoffwechselvorgänge. Um altersabhängige Veränderungen der Nebennieren in Mäusen zu untersuchen, wurde eine systematische Analyse funktionaler und wachstumsbedingter Parameter durchgeführt, indem die Nebennieren von männlichen und weiblichen Mäusen zu verschiedenen Zeitpunkten (im Alter von 3, 5, 7, 9 und 11 Wochen) auf Organ-, Kompartiment- und Zell-Ebene analysiert worden sind. Hierzu wurde eine Wachstumsanalyse der Nebennieren durchgeführt und die Volumina der verschiedenen Nebennierenzonen wurden mit erwartungstreuen stereologischen Methoden bestimmt. Auf Zellebene wurde zu den fünf genannten Zeitpunkten das mittlere zona fasciculata (ZF) Zellvolumen sowie die ZF Gesamtzellzahl untersucht. Darüber hinaus wurde die Menge an gespeicherten Lipiden sowie die Expression von zwei an der Steroidbiosynthese beteiligten Enzymen, side-chain-cleavage enzyme (SCC) und 11-β-Hydroxylase, in der Nebenniere analysiert. Serumkonzentrationen von Aldosteron und Kortikosteron sind über den gesamten Untersuchungszeitraum als funktionale Parameter der zona glomerulosa bzw. der ZF gemessen worden.

Ergebnisse: Bis zum Alter von 7 Wochen nahmen die Nebennieren an Gewicht zu. Interessanterweise reduzierte sich ausschließlich in männlichen Mäusen das Nebennieren-gewicht signifikant um 25% im Alter zwischen 7 und 9 Wochen. Prinzipiell wiesen die Nebennieren weiblicher Mäuse ein höheres Gewicht auf. Im Alter von 9 Wochen erreichte dieser Gewichtsunterschied sein Maximum und die Nebennieren weiblicher Mäuse wogen doppelt soviel wie die der männlichen Wurfgeschwister. Dieser Gewichtsdimorphismus resultierte vor allem aus dem vergrößerten ZF Volumen der weiblichen Mäuse. Auch die Volumina der X-Zone und des Nebennierenmarks waren im Vergleich zu den männlichen Wurfgeschwistern bei den weiblichen Mäusen größer. Keine geschlechtsabhängigen Volumenunterschiede zeigten sich in der zona glomerulosa. Zusätzlich zu den geschlechtsabhängigen Wachstumseffekten wurden altersbedingte Volumenveränderungen der Zonen beobachtet. Jede Zone, außer der X-Zone in männlichen Mäusen, nahm zwischen 3

und 7 Wochen stetig an Volumen zu. Die geschlechts- und zeitabhängigen Veränderungen konnten bis auf die zelluläre Ebene der ZF zurückverfolgt werden, da beide Faktoren das mittlere ZF Zellvolumen und die ZF Gesamtzellzahl beeinflussten. Ab der fünften Woche beinhaltete die ZF der Weibchen signifikant mehr Zellen im Vergleich zu gleichaltrigen Männchen. In beiden Geschlechtern ereichte die ZF Gesamtzellzahl ihr Maximum im Alter von 7 Wochen, gefolgt von einer signifikanten Zellzahl Reduktion zwischen 7 und 9 Wochen. Das mittlere ZF Zellvolumen nahm in männlichen Mäusen im Alter zwischen 3 und 11 Wochen kontinuierlich zu. In 3 bis 7 Wochen alten Weibchen zeigten sich ähnliche mittlere ZF Zellvolumina, zwischen 7 und 9 Wochen dagegen nahmen die mittleren ZF Zellvolumina um etwa 50 % zu. Zwischen der ZF Gesamtzellzahl und der geschätzten Gesamtmenge an frei zirkulierendem Kortikosteron (ETAC) konnte eine positive Assoziierung hergestellt werden, jedoch konnte diese Verbindung nicht zwischen den mittleren ZF Zellvolumina und jener ETAC gefunden werden.

Die Serum Aldosteron-Spiegel ähnelten sich in beiden Geschlechtern zu jedem Untersuchungszeitpunkt, im Gegensatz dazu waren die Serum Kortikosteronkonzentrationen in weiblichen Mäusen, zumindest tendenziell, höher. Die Serum Konzentrationen beider Steroidhormone veränderten sich altersabhängig. Während die Aldosteron Konzentrationen im Serum mit zunehmendem Alter anstiegen, verringerten sich die Kortikosteron-Spiegel signifikant im Zeitraum zwischen 3 und 11 Wochen.

Die in der Nebenniere gespeicherte Lipidmenge erschien in weiblichen Mäusen größer als in männlichen, und die Nebennieren 11 Wochen alter Mäuse beiden Geschlechts erweckten den Anschein, dass sie eine größere Lipidmenge als die Nebennieren der 7 Wochen alten Tiere speichern. Auch die Expression der beiden Enzyme SCC und 11-β-Hydroxylase stieg in beiden Geschlechtern zwischen 3 und 11 Wochen an, sodass 11 Wochen alte Mäuse möglicherweise eine erhöhte Steroidbiosynthese Kapazität aufweisen als jüngere Mäuse.

Letztlich zeigt diese Studie deutlich, dass in wachsenden Mäusen eine Vielzahl dynamischer Prozesse während der postnatalen Nebennierenentwicklung ablaufen. Alter, Geschlecht, Zelltyp, Zellzahl und Zellvolumen üben einen entscheidenden Einfluss auf das Nebennierenwachstum aus. Ein wesentlicher Befund dieser Arbeit ist, dass Wachstum kein unidirektionaler Prozess ist, der alleinig durch eine kontinuierliche Zunahme der Zellzahl und der Zellgröße gekennzeichnet ist, da Reduktionen der Zellzahl durch Erhöhung des Zellvolumens kompensiert werden können. Es kann sicherlich angenommen werden, dass diese dynamischen Vorgänge auch funktionale Parameter beeinflussen, jedoch kann die

Menge des freigesetzten Kortikosterons nur teilweise als Funktion der ZF Zellvolumina bzw. der ZF Zellzahlen erklärt werden.

Generell unterstützt diese Arbeit systemische Ansätze, die verschiedenste Informationen unterschiedlicher Untersuchungsebenen integrieren, da dies für zukünftigen Forschungsprojekte immer wichtiger werden wird.

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