Regeneration of Functional Adrenal Tissue Following Bilateral Adrenalectomy

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It is assumed that after complete bilateral adrenalectomy (ADX), no adrenal tissue will redevelop and adrenal hormone levels will remain low and unaffected by stress. However, anecdotal observations in animals and in patients suggest that under some unknown circumstances the opposite can occur. Herein, we studied whether adrenalectomized rats can develop an alternative source of systemic corticosterone after complete bilateral ADX with minimal replacement therapy. Male and female rats underwent either a standard ADX, in which the glands were removed with minimal surrounding adipose tissue, or an extensive ADX, in which glands were removed with most surrounding adipose tissue. Excised glands were histologically tested for completeness, and corticosterone replacement was nullified within 1 to 3 weeks postoperatively. In four experiments and in both excision approaches, some rats gradually reestablished baseline corticosterone levels and stress response in a time-dependent manner, but differences were observed in the reestablishing rates: 80% in standard ADX vs 20% in extensive ADX. Upon searching for the source of corticosterone secretion, we were surprised to find functional macroscopic foci of adrenocortical tissue without medullary tissue, mostly proximal to the original location. Chronic stress accelerated corticosterone level reestablishment. We hypothesized that underlying this phenomenon were preexisting ectopic microscopic foci of adrenocortical-like tissue or a few adrenal cells that were pre-embedded in surrounding tissue or detached from the excised gland upon removal. We concluded that adrenalectomized animals may develop compensatory mechanisms and suggest that studies employing ADX consider additional corticosterone supplementation, minimize stress, and verify the absence of circulating corticosterone. (***Endocrinology*** **159: 248–259, 2018)**

The adrenal glands, located above the kidneys, secrete several prominent hormones, including epinephrine and norepinephrine, from its medulla and glucocorticoids, mineralocorticoids, and androgens from its cortex (1, 2). The medulla is of a neural crest origin, innervated by the sympathetic nervous system that regulates its secretions, whereas the cortex is of a mesodermic origin and is controlled mainly through several endocrine factors, including pituitary factors [*i.e.*, adrenocorticotropic hormone (ACTH)] and factors of the renin-angiotensin-aldosterone system (2–5).

Complete bilateral adrenalectomy (ADX) is conducted clinically because of various medical conditions (including Cushing disease, pheochromocytoma, and adrenal hyperplasia) and is accompanied by replacement therapy for cortisol and aldosterone. In animal studies, complete bilateral ADX is often conducted to prevent adrenal-mediated stress responses, and adrenalectomized animals are commonly subjected to corticosterone replacement therapy and drink saline to overcome a lack of aldosterone that normally regulates sodium and potassium homeostasis.

Interestingly, layers of the adrenal cortex that produce glucocorticoids and mineralocorticoids (*i.e.*, the zona fasciculata and zona glomerulosa) are continuously regenerating throughout life, and their volume exhibits

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Abbreviations: ACTH, adrenocorticotropic hormone; ADX, adrenalectomy; ADX-CORT, adrenalectomy-corticosterone; ANOVA, analysis of variance; BAT, brown adipose tissue; F344, Fischer 344; HPA, hypothalamic-pituitary-adrenal; RIA, radioimmunoassay; s.c., subcutaneously.

dynamic changes (2, 4, 6–10). Moreover, when substantial portions of the adrenal capsule or cortex are retained or transplanted elsewhere in the host, they regrow, potentially differentiating into other zones (4, 6, 11–14). However, it is assumed that after complete bilateral ADX, no adrenal tissue will redevelop and adrenal hormone levels will remain low and unaffected by stress.

However, in a recent study (15), 11 of 16 patients were able to nullify their glucocorticoid supplementation within 2 years of bilateral ADX, but it was not ascertained whether some adrenal tissue had been spared by surgery. More surprising, a case study reported regeneration of functional adrenal cortex 12 years after a verified total bilateral ADX in a 11-year-old German boy (16). In our laboratory, we have occasionally observed elevated levels of corticosterone in stressed adrenalectomized rats that were maintained for >3 weeks post operation, although we did verify (by visual observation) complete removal of intact original glands. Several alternative sources for the production of adrenal hormones are known, and glucocorticoids can be produced locally by the skin, heart, gonads, and thymus (17–22). However, such local secretion is believed to be insufficient to maintain normal systemic baseline levels of epinephrine, corticosterone, or aldosterone after ADX, let alone to increase these levels under stress conditions or ion imbalance (17, 18, 22, 23).

In this study, we aimed to systematically test whether adrenalectomized rats developed an alternative source of corticosterone secretion when partly or completely weaned from corticosterone replacement, and if they did, to identify the kinetics of this phenomenon and the tissues underlying such secretion.

Materials and Methods

Animals

In all experiments, rats [male/female Fischer 344 (F344) and male Lewis] were housed in our vivarium, two to four per cage, with *ad libitum* access to food and water on a 12:12 light:dark cycle at 22°C ± 1°C. Animals were handled daily during the last week before experimentation to reduce potential procedural stress. Housing conditions were monitored by the Institutional Animal Care and Use Committee of Tel Aviv University, which also approved all studies described herein. In all experiments, rats were 2 months old at the time of ADX/sham operation, and in the age experiment (described later) 6-month-old rats were also used.

Experiment 1: restoration of corticosterone plasma levels after ADX

This study was designed to address in a systematic and controlled manner our previous anecdotal observations suggesting restoration of corticosterone plasma levels and stress responses in adrenalectomized rats. Two-month-old male F344 rats underwent ADX (n = 21) and a sham (n = 20) operation (as described later). Adrenalectomized rats received saline supplemented with corticosterone as a replacement for their drinking water for 3 weeks, which was then replaced by saline alone (gradually reduced within a week). Five of the 21 adrenalectomized animals were euthanized over the following weeks because of loss of 20% of their initial body weight. At 10 to 11 weeks after ADX/sham operation, four blood draws from each animal were conducted (as described later), two during the dark phase before and after 15 minutes of restraint stress, and a week later, two during the light phase before and after 15 minutes of restraint stress. The order of light/dark blood collection was counterbalanced.

Experiment 2A: time dependency of corticosterone restoration after ADX

Given the surprising results of experiment 1, we sought to replicate the findings and test whether they could be generalized to both sexes and to another strain of rats while examining the time-dependent elevation in corticosterone plasma levels after ADX. Should the presence of adrenal glands be merely masking low or moderate levels of corticosterone that were continuously secreted from extra-adrenal tissue (*e.g.,* skin, thymus), corticosterone would be detected immediately upon the removal of the adrenal glands. Baseline and stress levels of corticosterone were assessed at 2, 6, and 12 weeks after ADX/sham operation during the light and dark phases in each time point. This entire study was conducted in (1) female F344 rats, (2) male F344 rats, and (3) male Lewis rats. F344 and Lewis rats were chosen because they are known to have high and low baseline and stress-induced hypothalamic-pituitary-adrenal (HPA) responses, respectively (24, 25). Both sexes of F344 rats were studied because of known sexual dimorphism in baseline and stress-induced HPA responses.

Two-month-old rats were subjected to either ADX or sham operation. Adrenalectomized rats received saline (0.9% NaCl) supplemented with corticosterone (15 mg/L) as a replacement for their drinking water for 1 week, which was then replaced with saline alone. Blood was withdrawn at 2, 6, and 12 weeks after operations, four times at each of these time points (before and after stress during light/dark phases as in Experiment 1) (as described later).

Experiment 2B: the search for the origin of corticosterone in adrenalectomized rats and the discovery of regenerated functional adrenocortical-like tissue

Prominent candidates for corticosterone production include the gonads, brown adipose tissue (BAT), and skin (17–22). Thus, we sought to harvest these organs to assess their corticosterone secretion *in vitro*. Two sham and two adrenalectomized male F344 rats and two adrenalectomized female F344 rats that exhibited high corticosterone levels were exposed to stress (15 minutes of restraint stress, as described later) and were euthanized. The testicles, BAT (2 × 2 cm), and skin (2 × 2 cm) were harvested from the males are the ovaries were excised from the females. Tissues were cut into 1-mm pieces and placed within standard medium (RPMI-1640 supplemented with 50 μg/mL of gentamicin, 2 mM of L-glutamine, 0.1 mM of nonessential amino acids, and 1 mM of sodium pyruvate; purchased from Biological Industries, Kibbutz Beit Haemek, Israel) in an incubator (5% CO_2 at 37°C) for 1 hour before media samples were collected for corticosterone assessment. To

our surprise, when the perineal cavity of the adrenalectomized rats was wide open, we found a pink-red tissue surrounded by numerous blood vessels in proximity to the site of the original glands. These adrenal-like tissues varied in specific location and shape, sometimes exhibiting few adjacent foci (Fig. 1A).

To test the functionality of these potentially regenerated adrenal-like tissues, all remaining adrenalectomized rats were shaved and subjected to standard ultrasonography (see the following text) to detect any adrenal-like tissue (Fig. 1B). Next, five sham and nine adrenalectomized rats that exhibited such suspected tissue were exposed to stress. Thereafter, blood was collected, the rats were euthanized, and the adrenal-like tissues were excised and cut into 1-mm pieces and placed within standard medium in an incubator for 1 hour (as described previously) before media samples were collected for corticosterone and aldosterone assessment. To histologically examine the regenerated adrenal-like tissue, naive adrenal glands and regenerated adrenal tissues were either fixed in 4% formaldehyde or flash frozen. Thereafter, the glands were embedded in paraffin, and sectioned at 20-μm slices for hematoxylin and eosin or 10-μm slices for oil red O staining (as described later).

Experiment 3: the effects of age and of the extent of margins removed with the gland on the regeneration of adrenocortical tissue

To eliminate any concerns that some microscopic fragments or single cells of the adrenal gland may have been left in

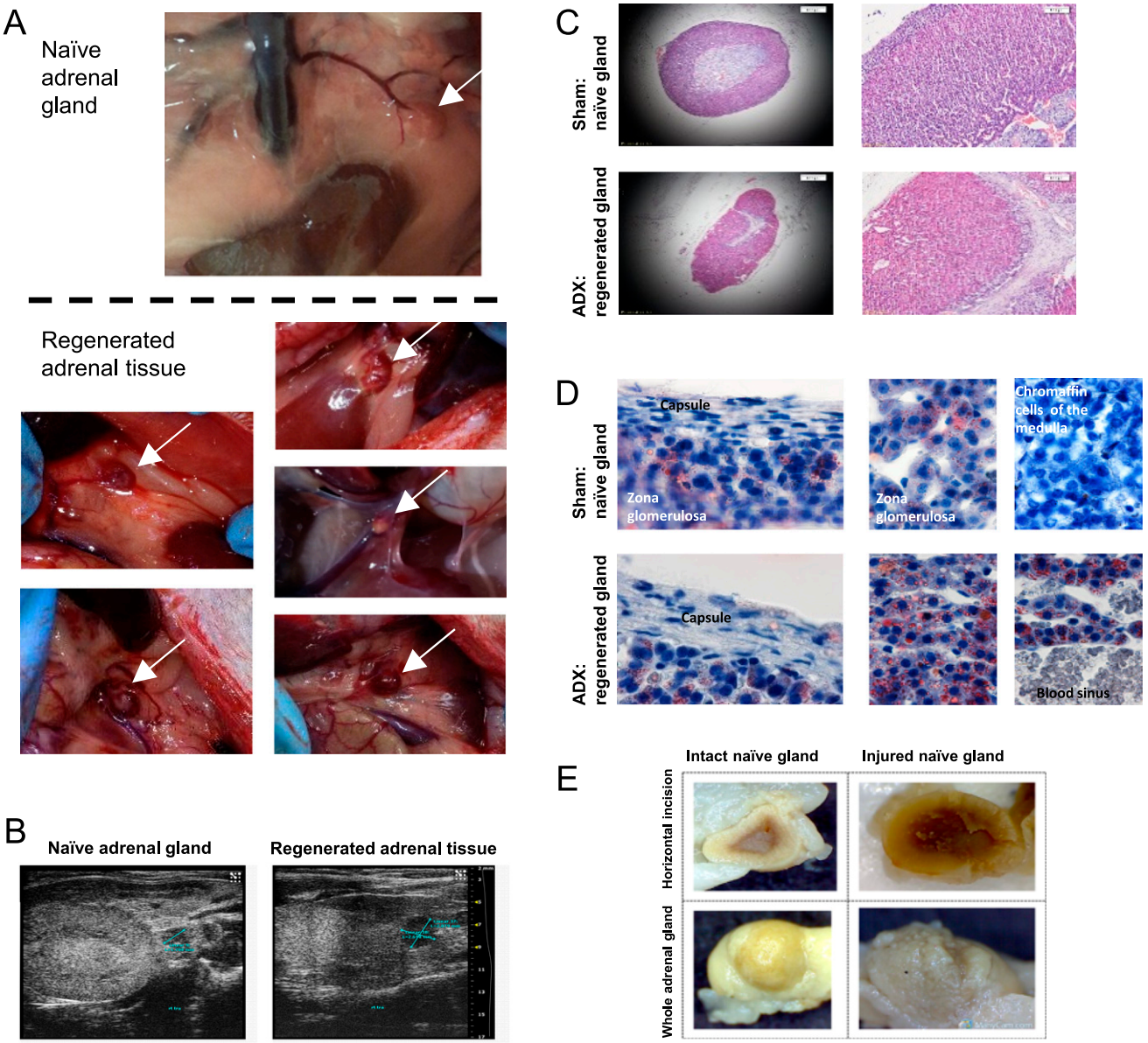

Figure 1. Representative images. (A) A naive adrenal gland (top) and regenerated adrenal tissue (bottom) are indicated with white arrows. (B) Ultrasonography images of a naive adrenal gland (left) and regenerated adrenal tissue (right) are shown. (C) Hematoxylin and eosin staining of a naive adrenal gland (top) and regenerated adrenal tissue (bottom). The ruler scale is as follows: top and bottom left, 500μm; top right, 50μm; and bottom right, 25μm. (D) Oil red O staining of a naive adrenal gland (top) and regenerated adrenal tissue (bottom) are shown. (E) Intact (left) and injured (right) naive adrenal glands were collected during ADX in male F344 rats. The horizontal incision is presented at the top, and the whole gland is shown at the bottom.

the abdominal cavity after ADX, this study tested whether the extent of margins removed with the gland determined the likelihood of adrenal regeneration. In addition, the impact of age on adrenal regeneration was examined, as all previous studies were conducted in 8-week-old rats. Stress levels of corticosterone were assessed 2, 12, and 30 weeks after the ADX/sham operation, 2 hours after light onset in each time point. This study was conducted in (1) 2-month-old female F344 rats, (2) 2-month-old male F344 rats, and (3) 6-month-old male F344 rats. ADX was performed in an extensive manner, removing a substantial amount of adipose tissue surrounding the adrenal glands. Eighty-nine male and female F344 rats underwent either extensive ADX or a sham operation. In the extensive ADX procedure, a special effort was made to remove the adrenal glands with all surrounding adipose tissue without touching or inflicting damage to the original gland (in the standard ADX procedure, the glands are removed with less surrounding adipose tissue without contacting or damaging them). Blood samples were collected 2 hours after light onset after 15 minutes of restraint stress at 2, 12, and 30 weeks after surgery.

Experiment 4: the effect of chronic stress on adrenocortical regeneration

Given the role of ACTH in the development and maintenance of the adrenal cortex (26), we sought to test whether activation of the HPA axis facilitated adrenal regeneration. Seventy-five male and female F344 rats underwent extensive ADX (as in experiment 3: *The effects of age and of the extent of margins removed with the gland on the regeneration of adrenocortical tissue*) and were randomly divided into three subgroups: (1) ADX-corticosterone (ADX-CORT), (2) ADX-Control, and (3) ADX-Stress.

Two stress paradigms were used: (1) twice a week, animals were placed in a 20 × 40 cm cage with 1-cm deep water for 60 minutes, and (2) twice a week, the home cage with its bedding was replaced by a new clean cage [instead of once a week—known to increase stress levels (27)]. After surgery, ADX groups received a high dose of corticosterone (30 mg/L; twice the standard dose) in their drinking saline (0.9% NaCl). Six days after surgery, all ADX groups were weaned from corticosterone replacement except for the ADX-CORT group, which continued to drink corticosterone replacement for 8 weeks after surgery and thereafter was weaned from corticosterone replacement and the animals allocated to either the stress or the control group for the rest of the study duration. While the ADX-CORT group was under corticosterone replacement, corticosterone was withdrawn from the group 14 hours prior to blood withdrawal and was restored immediately after. At 10 days and 6, 12, 18, and 32 weeks, blood was collected after 15 minutes of restraint stress. Overall, 20% of adrenalectomized animals reached the end of the experiment (32 weeks), whereas the rest lost >20% of body weight or became sick and were euthanized.

ADX and animal maintenance

Rats were anesthetized with 2.5% isoflurane, and a 4-cm midline abdominal incision was performed. We used this midline approach to enable the removal of the gland with its surrounding fat tissue while ascertaining no contact with the gland itself when removed. The gland extraction was conducted in three steps: (1) gently lifting the fat tissue 2 to 3 mm from the gland with forceps, which elevated the gland and further exposed it, (2) inserting blunt forceps below the gland to protect it from potential damage, and (3) using a scalpel to cut the fat tissue below the forceps and around the gland. The extracted glands were first inspected outside the animals to verify complete removal (as described later). The abdomen was then sutured using a 0.2-mm monofilament nylon string. To facilitate the animals' recovery, corticosterone was administered subcutaneously (s.c.) after surgery (3 mg/kg; twice, 3 hours apart). Thereafter, adrenalectomized animals received saline (0.9% NaCl) supplemented with corticosterone (15 mg/L) as a replacement for their drinking water. This replacement regimen was maintained for 3 weeks in the first experiment (ADX-CORT reestablishment, described previously), and for 1 week in all other experiments. Thereafter, adrenalectomized animals received saline alone as a replacement for their drinking water. Control animals underwent a sham operation, in which a 4-cm midline abdominal incision was performed and sutured, without the removal of the adrenal glands. All operated animals were administered etodolac during surgery (12.5 mg/kg s.c.) to alleviate pain.

Inspection of the excised glands

To minimize the chances for incomplete removal of the adrenal glands, all glands were removed without direct contact, as described previously. When the surgeon had doubts about whether a gland was removed completely or was injured as a result of the surgical procedure, the animal was excluded from the study. Immediately after excision, all glands were carefully inspected visually for completeness outside the animal. After fixation in 4% formaldehyde, the excised glands were microscopically inspected to identify potential incompleteness of the external capsule (all animals in the extensive ADX experiments), and some glands were also histologically examined for completeness (on the basis of other outcomes in the same animal).

Histology

Adrenal tissues were either fixed in 4% formaldehyde or flash frozen for histopathological examination. Before histology, glands were inspected through a light microscope to identify potential incompleteness of the external capsule or any irregularities. Thereafter, the tissues were either embedded in paraffin and sectioned as 20-μm slices for hematoxylin and eosin or 10-μm slices for oil red O staining, as described elsewhere (28). A board-certified toxicological pathologist who was blinded to the treatment performed the histopathological evaluation.

Baseline and poststress blood withdrawal and plasma collection

At different time intervals after ADX/sham operations (*e.g.*, 2, 6, and 12 weeks in experiment 2: *Time dependency of corticosterone restoration following ADX*), blood was drawn four times from each animal as follows: 2 hours after light onset, immediately before and immediately after 15 minutes of restraint stress, and a week later, 2 hours after dark onset, immediately before and after stress. The order of light/dark blood collections was counterbalanced in each study so that for half of the animals in each group, blood was first withdrawn during the dark period and then, a week later, during the light period; the opposite procedure was used for the other half. In experiment 3 (*The effects of age and of the extent of margins removed with the*

gland on the regeneration of adrenocortical tissue), blood was drawn only during the light phase (see previous description), and in experiment 4 (*The effect of chronic stress on adrenocortical regeneration*), blood was withdrawn only after 15 minutes of restraint stress (see earlier description). For blood withdrawal, animals were anesthetized with isoflurane, and 0.5 mL of blood was drawn from the tail end within <3 minutes of approaching the animal cage when testing for baseline levels and/or after 15 minutes of restraint stress. The blood was collected in 1.5-mL Eppendorf tubes containing EDTA solution (0.9 mg EDTA in 50 μL of phosphate-buffered saline). Blood was then centrifuged for 20 minutes at 930*g*, 4°C, and plasma was collected and stored at −20°C until assayed for CORT, aldosterone, or epinephrine levels.

Restraint stress

Animals were placed in a standard restrainer for 15 minutes. The transparent restrainer tube (28 cm long) limited the ability of the animal to turn around while enabling some movement and free breathing.

Assessment of corticosterone levels

Plasma corticosterone levels and *in vitro* secretion of corticosterone were measured using radioimmunoassay (RIA) (ImmuChem Double Antibody Corticosterone 125I RIA kit; MP Biomedicals, Orangeburg, NY), per manufacturer's instructions. The intra-assay coefficient of variability was <5%, as reported by the manufacturer and as evident in our assay.

Assessment of epinephrine levels

Plasma epinephrine levels were measured using an epinephrine enzyme-linked immunosorbent assay kit (Labor Diagnostika Nord, Nordhorn, Germany) according to the manufacturer's protocol. The manufacturer reports a correlation of $r = 0.99$ with high-performance liquid chromatography measured levels. The intra-assay coefficient of variability was <11%, as reported by the manufacturer and as evident in our assay.

Assessment of aldosterone levels

Plasma aldosterone levels and *in vitro* secretion of aldosterone were measured using an aldosterone enzyme-linked immunosorbent assay kit (Cloud-Clone Corp., Katy, TX) according to the manufacturer's protocol. The intra-assay coefficient of variability was <10%, as also reported by the manufacturer.

Animal treatment in cases of weight loss and exclusion from the study

Adrenalectomized animals often do not regain their presurgical weight, or they continuously lose weight after surgery. In the latter case, animals were treated with 3 mg/kg of corticosterone (Sigma-Aldrich, Rehovot, Israel) (a single or repeated injections s.c.) and 15% glucose in their drinking saline. Animals who lost 20% of their body weight were euthanized.

Ultrasonography imaging

Animals were anesthetized and had the abdomen shaved to reduce imaging artifacts in the ultrasonography examination. A sound-conducting gel was applied, and an ultrasonography examination was performed by a blinded board-certified ultrasound technician. The animals were examined in both right and left oblique positions to assess both adrenal gland locations. The ultrasound technician evaluated the glands for existence, location, and number of foci by multiple transversal and longitudinal scans. At the end of the experiment, animals were dissected and adrenal glands were exposed to confirm ultrasonography findings.

Statistical analyses

Depending on experimental design, we conducted two-, three-, or four-way factorial analysis of variance (ANOVA) or an unpaired one-tailed *t* test, with a predetermined significance level of 0.05. Repeated-measures ANOVA was conducted when studying within-animal time-dependent changes. Provided significant group differences were found, *post hoc* Fisher protected least significant difference contrasts were performed to compare pairwise comparisons on the basis of *a priori* hypotheses.

Results

Experiment 1: 10 weeks after ADX, rats exhibited significant corticosterone plasma levels, including circadian rhythms and stress responses

Sham animals exhibited circadian corticosterone levels and significant stress responses at both light and dark phases, as expected (Fig. 2). Surprisingly, adrenalectomized animals also exhibited a significant corticosterone stress response during the light phase (from 87 to 246 ng/mL) ($P < 0.0001$). Furthermore, during the dark phase, baseline levels of adrenalectomized animals were significantly higher than during the light phase (222 vs 87 ng/mL), but stress did not significantly increase corticosterone levels (*i.e.*, 222 vs 254 ng/mL).

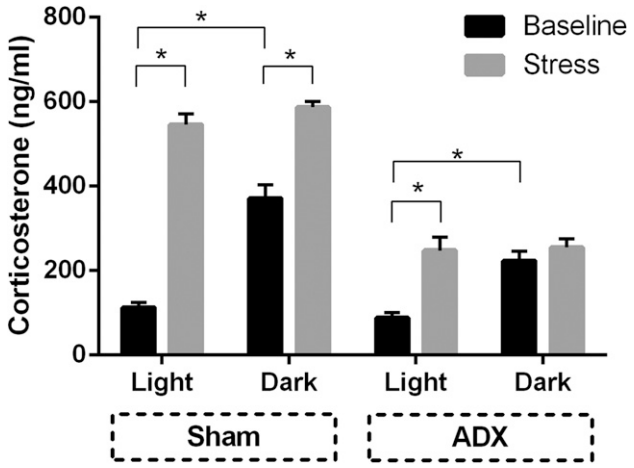

Figure 2. Three months after ADX (n = 16) and sham operation (n = 20), rats exhibited significant corticosterone plasma levels, including circadian rhythms and stress responses (15 minutes of restraint stress). Corticosterone replacement was discontinued 3 weeks after ADX. Data are presented as mean ± standard error of the mean. *$P < 0.05$.

Importantly, under all conditions, corticosterone levels in adrenalectomized animal were higher than the near-zero levels expected in the absence of adrenal glands, reaching ~50% of their respective levels in sham operated animals. Thus, we suspected that our adrenalectomized animals developed an alternative source for corticosterone production and sought to elucidate this phenomenon in the experiments described previously.

Experiment 2A: the elevation of systemic corticosterone levels after ADX was time dependent

As expected, the three groups showed significantly different overall corticosterone levels (F344 females > F344 males > Lewis males) ($P < 0.0001$). The same pattern of effects (described later) were evident within each of the three sex/strains, but because of differences in the absolute levels and variance between these groups, we conducted a separate four-way ANOVA for each sex/strain to assess the impact of ADX, light/dark phase, stress exposure, and time interval after ADX/sham operation. No sex differences were found among the F344 rats.

As seen in Figs. 3–5, sham animals in all groups exhibited normal physiological corticosterone levels at 2, 6, and 12 weeks after the operation. In contrast, 2 weeks after operation, adrenalectomized animals showed low corticosterone levels (mean, 9 to 30 ng/mL, depending on the group), which did not increase in the dark phase or after stress. However, at the 6- and 12-week time points, adrenalectomized animals in all groups exhibited significant circadian alteration of corticosterone, increasing corticosterone levels from 70 ng/mL in the light phase to 200 ng/mL in the dark phase ($P < 0.0003$). Furthermore, in the light phase, adrenalectomized animals showed two- to fourfold stress-induced increases in corticosterone levels ($P < 0.0001$). This stress response at 6 and 12 weeks, which was not evident at 2 weeks, reinforced our suspicion that an alternative source of corticosterone was developing over time.

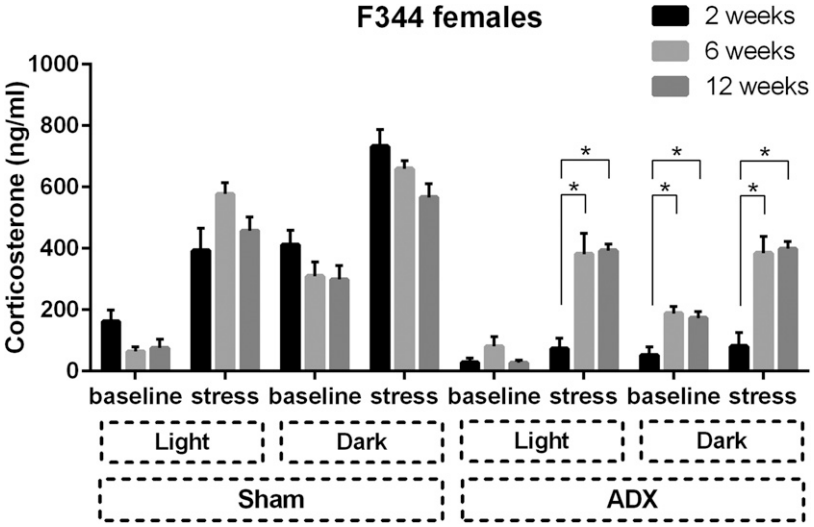

Figure 3. Adrenalectomized female F344 rats (ADX, n = 10; sham, n = 7) exhibited low corticosterone levels 2 weeks after surgery that did not increase during the dark phase or after stress. Six and 12 weeks after ADX, rats exhibited significant increases in corticosterone levels, including circadian rhythms and stress responses (15 minutes of restraint stress). Corticosterone replacement was discontinued 1 week after ADX. Data are presented as mean ± standard error of the mean. *$P < 0.05$.

Experiment 2B: the search for the origin of corticosterone in adrenalectomized rats and the discovery of regenerated functional adrenocortical-like tissue

An RIA confirmed that the media sampled from tubes containing adrenal-like tissues contained ample amounts of corticosterone, similar to the amount secreted by intact adrenal glands and >100-fold greater than that of any other organ tested (Fig. 6A). These findings suggest that the regenerated tissue contained cellular machinery for corticosterone synthesis and secretion. Furthermore, the regenerated tissue secreted physiological levels of aldosterone (Fig. 6B), which was also detected in the plasma of these rats, although at lower concentrations than in the sham rats ($P < 0.05$) (Fig. 6C). Secretion of epinephrine was undetected both *in vitro* and *in vivo* while observed in naive animals (data not shown). The detection of corticosterone and aldosterone in the adrenal-like tissue suggests that the adrenocortical zona fasciculata and zona glomerulosa were regenerated in these adrenalectomized rats. In contrast, the inability to detect epinephrine in the regenerated tissue or in the animals suggests the absence of the functional medulla or chromaffin cells in the regenerated tissue.

In Fig. 1C, the naive adrenal gland is exhibited, containing all cortex layers, capsule, and inner medulla. In contrast, regenerated tissue from an adrenalectomized rat displays a different morphology; zona fasciculata and zona glomerulosa are observed, but organized structure is lacking. Furthermore, we found no evidence for the medulla or chromaffin cells (Fig. 1C and 1D). The capsule morphology in both ADX-regenerated tissue and naive glands is very similar.

Experiment 3: the effects of age and of the extent of margins removed with the gland on the regeneration of adrenocortical tissue

Thirty weeks after ADX, the number of animals that survived was too low for statistical analysis. However, eight adrenalectomized animals exhibited significant corticosterone levels after stress (increasing from 50 to 300 ng/mL); four of them were from the 6-month-old male group, two

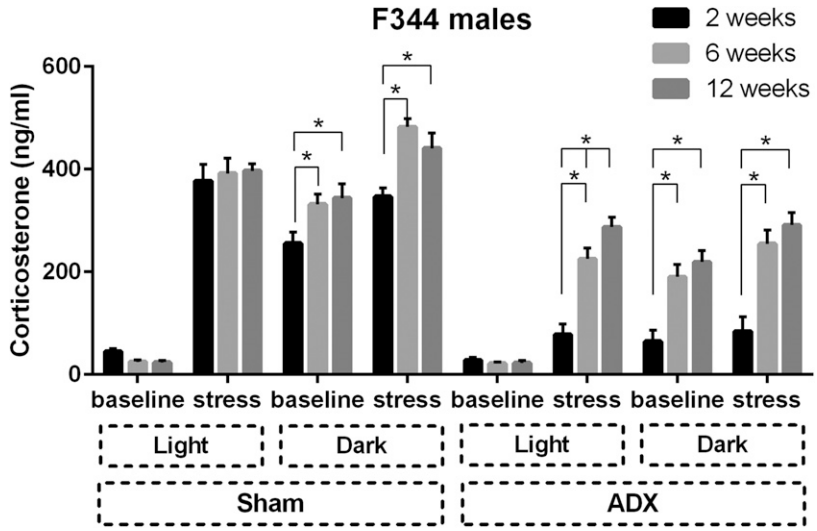

Figure 4. Adrenalectomized male F344 rats (ADX, n = 13; sham, n = 12) exhibited low corticosterone levels 2 weeks after surgery that did not increase during the dark phase or after stress. Six and 12 weeks after ADX, rats exhibited significant increases in corticosterone levels, including circadian rhythms and stress responses (15 minutes of restraint stress). Corticosterone replacement was discontinued 1 week after ADX. Data are presented as mean ± standard error of the mean. $*P < 0.05$.

were from the 2-month-old male group, and two were from the 2-month-old female group (age groups from the beginning of the experiment). In five of the eight animals that exhibited significant corticosterone levels, we were able to locate regenerated adrenal tissue. Interestingly, regenerated adrenal tissue was also found in two animals in which corticosterone levels were below detection levels (Table 1). Next, the original naive adrenal glands that were excised in the ADX procedure were inspected under a binocular microscope for capsule or cortex imperfections or damage that may have occurred during their removal (Fig. 1E). Of the seven rats in which regenerated adrenal tissue was found, five had no signs of noticeable damage in the excised adrenals; in the adrenal glands of the other two rats, we observed a minor injury in the form of a slight tear in the capsule.

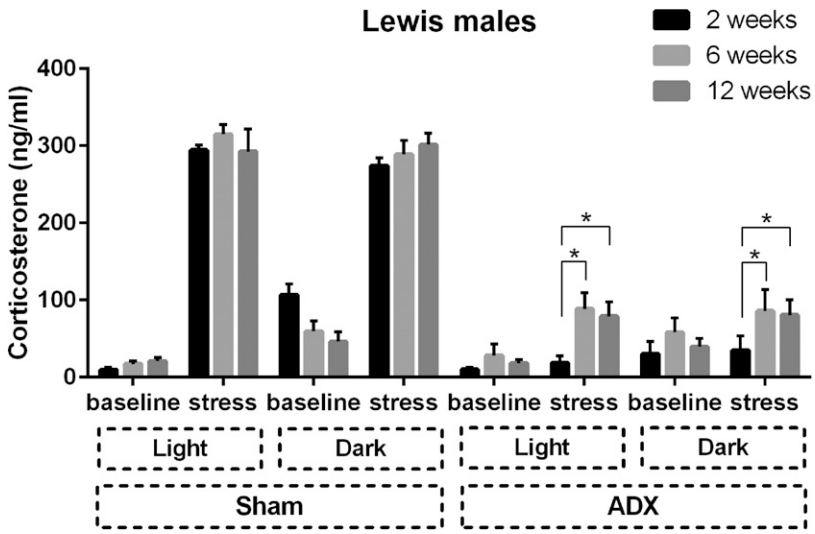

Figure 5. Adrenalectomized male Lewis rats (ADX, n = 11; sham, n = 7) exhibited low corticosterone levels 2 weeks after surgery that did not increase during the dark phase or after stress. Six and 12 weeks after ADX, rats exhibited significant increases in corticosterone levels, including circadian rhythms and stress responses (15 minutes of restraint stress). Corticosterone replacement was discontinued 1 week after ADX. Data are presented as mean ± standard error of the mean. $*P < 0.05$.

Experiment 4: chronic stress accelerated adrenocortical regeneration

By the end of the experiment, the number of animals that survived was too low for statistical analysis. However, 12 adrenalectomized animals exhibited significant corticosterone levels after stress. Of these 12 rats, we found regenerated adrenal tissue in six rats. Furthermore, of these 12 rats, nine had no signs of any damage in the excised adrenals, and the adrenal glands of the other three were found to have a minor tear in their capsule (Table 1). Interestingly, upon examination of the excised glands from animals that did not exhibit detectable corticosterone levels, 14 rats had injured their excised gland (capsule or cortex). In any case of an injured gland, it was unclear whether damage occurred *in vivo* during the ADX or when removing the fat tissue for the examination.

Because the number of animals was not sufficient for statistical analysis at 32 weeks after ADX, a two-way ANOVA was conducted at 18 weeks (both sexes together). A main effect of treatment was found ($P < 0.0025$) (Fig. 7). Fisher protected least significant difference for preplanned comparisons indicated significant higher corticosterone levels in the ADX-Stress group than in both the ADX-CORT and the ADX-Control groups, both at 12 weeks ($P < 0.019$ and $P < 0.041$, respectively) and at 18 weeks ($P < 0.009$ and $P < 0.039$, respectively).

Discussion

The current study was conducted given occasional observations of elevated corticosterone levels in adrenalectomized rats stressed >3 weeks after ADX. Although extra-adrenal sources for glucocorticoid production are known (*e.g.*, skin, thymus, gonads),

they are believed to be insufficient to induce significant systemic levels or stress responses (17, 18, 22, 23). Our findings demonstrated a time-dependent reestablishment of corticosterone levels after bilateral ADX and minimal corticosterone replacement in a significant portion of these animals and on the basis of development of functional tissue of adrenal cortex without evidence of adrenal medulla tissue or systemic epinephrine. In the standard ADX procedure (experiments 1 and 2), the adrenal glands were removed with minimal surrounding adipose tissue without contacting or damaging the gland. In the extensive ADX procedure (experiments 3 and 4), a special effort was made to remove the adrenal glands with all surrounding adipose tissue, which may have also contained microscopic glandlike tissue or a few adrenocortical cells. Whereas ~80% of the rats undergoing the standard ADX procedure demonstrated significant corticosterone levels and survived until the end of the study, only ~20% of the rats in the extensive ADX procedure were not euthanized and demonstrated significant corticosterone levels. In most of these 20%, no damage was evident in the original excised glands, dissociating between a potential microscopically noticeable injury to the gland and adrenal-like tissue regrowth.

Our study suffers from several limitations. We did not measure ACTH levels or perform analysis of messenger RNA, protein, or the activity of steroidogenic enzymes in the regenerated tissue. These may help elucidate the nature of the regenerated tissue and the mechanism underlying its regrowth. In addition, the origin of the regenerated tissue in our adrenalectomized rats remains unknown. It could have originated in adrenal rest tumors similar to those seen in congenital adrenal hyperplasia, as described in animal models of steroidogenic factor 1 manipulation (29). Alternatively, it could have originated from preexisting microscopic accessory/ectopic adrenal foci, as further discussed next. Nonetheless, the fundamental finding of a regenerated tissue that is capable of secreting adrenocortical hormones after successful bilateral ADX remains.

Only a few published case reports have described similar phenomena in humans (15, 16, 30). Most surprising is a case report of an 11-year-old German boy undergoing total bilateral ADX for Cushing syndrome and treated with steroid supplementation. Three years after the operation, cortisol levels were still undetected. However, 5 years after the operation, his baseline cortisol value increased to normal levels; 4 years later, normal adrenocortical responses were evident, and steroid replacement was terminated. Adrenal cortex scintigraphy revealed bilateral orthotopic normal-sized glands with normal activity. Twelve years after the operation, the patient was healthy and active (16).

Of note, any damage to the excised glands could potentially affect the development of adrenal tissue. In the extensive ADX experiments, from a total of 136 adrenalectomized rats, 110 exhibited intact adrenal glands after their excision, whereas 26 (~1 of 5) rats exhibited microscopically damaged glands (Fig. 5E), which may have occurred during or after gland excision. Of the 136 adrenalectomized rats, we were able to find regenerated adrenal tissues in 13 rats, 4 of which were in rats whose excised adrenal glands were found to exhibit microscopic damage and 9 of which were in rats whose excised adrenal glands were found intact. These results suggest that each adrenalectomized rat had at least an 8% chance of developing regenerated adrenal tissue when the original adrenal glands were found intact after excision. The chances rose to 16% in animals with damaged original glands. Notably, in 22 of the 26 rats (84%) whose excised glands were found to be damaged, no regenerated tissue was found and no detectable corticosterone levels were observed. These findings indicate a double dissociation between the development of regenerated tissue and microscopic damage to the excised gland.

In addition, in some cases we clearly observed significant corticosterone levels in an adrenalectomized animal but were unable to find regenerated adrenal tissue. For example, from 12 rats that exhibited significant corticosterone levels in the ADX-stress experiment, we were able to find regenerated adrenal tissues in only six. Three more rats in the age experiment exhibited significant corticosterone levels, but we were unable to detect any regenerated adrenal tissue. We suggest that in these animals, regenerated adrenal tissue developed somewhere in the intraperitoneal cavity, and we did not succeed in locating it. In abdominal surgeries such as ADX, the internal organs often adhere to one other, which constitutes an obstacle in finding regenerated tissue.

Overall, all eight scenarios have occurred: animals exhibited microscopically intact/damaged excised glands, we were/were not able to find regenerated adrenal tissue, and rats exhibited/did not exhibit significant corticosterone levels.

On the basis of these findings, we suggest four nonexclusive hypotheses to explain the development of regenerated adrenal tissue in adrenalectomized rats. (1) During ADX, a microscopic portion of adrenal cortex remained in the abdominal cavity. These few cells served as precursors for regenerated adrenal cortical tissue. As alluded to in the following text, this scenario may underlie only a small portion of the observed regenerated cortical tissue. (2) Preexisting microscopic accessory/ectopic adrenal foci were present, most likely in the abdominal cavity proximal to the original gland, and proliferated after ADX. (3) Extra adrenal glucocorticoid production occurred in other organs that ramped up

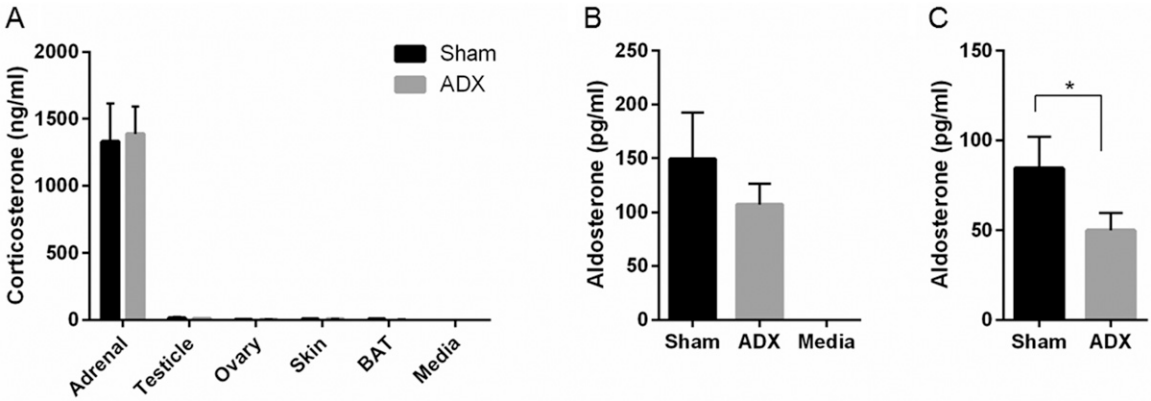

Figure 6. (A) Adrenal-like tissues excised from adrenalectomized rats (n = 9) and incubated in media for 1 hour secreted amounts of corticosterone similar to those of the intact adrenal glands from sham rats (n = 5) and >100-fold greater than that of any other organ tested (n = 2 per group). (B) Adrenal-like tissues excised from adrenalectomized rats (n = 9) and incubated in media for 1 hour secreted amounts of aldosterone similar to those of the intact adrenal glands from sham rats (n = 5). Media control tubes, n = 2. (C) Adrenalectomized rats (n = 9) exhibited substantial aldosterone plasma levels; however, they were lower than those of sham rats (n = 5). Data are presented as mean ± standard error of the mean. $*P < 0.05$.

production to compensate for the lack of adrenal glands. (4) Adrenalectomized animals regenerated an adrenal-like tissue through yet unknown mechanisms, such as circulating adrenocortical precursor cells.

Several internal organs and glands have the ability to perform "reparative regeneration" after injury, including the thymus, thyroid gland, intestine, lungs, heart, liver, kidney and bladder, skin, pancreas, and bone. However, most organs initiate the regeneration process with a large cell mass, typically with 80% or more of the original organ (31). In contrast, in adrenalectomized rats, the few remaining cells may be sufficient to initiate a regeneration process of the cortex. Although we have conducted this study in a manner that minimized the potential for such a scenario (including extensive ADX) and the glands were verified histologically for completeness, adrenal-cortex damage may have occurred as a result of tearing or cutting of the blood vessels and the splanchnic nerves that penetrate the gland. It would be challenging to accurately determine whether a few adrenal cells were shed through the blood circulation during the excision procedure and whether adrenocortical cells had been embedded in proximal blood vessels or were extravasated to fat tissue before adrenal removal. If indeed such phenomena occurred, clinical and experimental practices of complete bilateral ADX may similarly result in disseminated adrenocortical cells. Of note, the findings indicated that ~20% of the excised glands had some level of injury that was potentially surgery related, but regenerated adrenal tissue was found mainly in animals whose excised glands were intact. Furthermore, most rats whose original glands were found to be injured did not develop regenerated adrenal tissue (84%, or 22 of 26), further weakening the association between the observed injury to the excised gland and the development of the regenerated tissue.

A second hypothesis that could explain the development of regenerated adrenal tissue in our studies is that preexisting accessory adrenal foci were present and

Table 1. Number of Regenerated Adrenal Glands Found in Adrenalectomized Rats in the Age and ADX-Stress Experiments (Extensive ADX Experiments)

	Age Experiment		Stress Experiment	
	Regenerated Glands	No Regenerated Glands Found	Regenerated Glands	No Regenerated Glands Found
Corticosterone	5	3	6	6
No corticosterone	2	51	0	53

Many animals did not survive the extensive ADX, in which a substantial amount of adipose tissue surrounding the adrenal glands was removed with the gland. However, in the age experiment, in 7 of 61 adrenalectomized rats, regenerated adrenal tissues were detected; 5 of these were from animals whose adrenal glands were found to be intact, and 2 were from impaired adrenalectomized animals. Eight of the 61 adrenalectomized animals exhibited an increase in corticosterone levels after stress exposure; five of these increased levels were from animals in which regenerated adrenal tissue was detected, three were from animals in which no adrenal tissue was found. In the stress experiment, in 6 of 65 adrenalectomized rats, regenerated adrenal tissues were detected; 4 of these were from animals whose adrenal glands were found to be intact, and 2 were from impaired adrenalectomized animals. Twelve of the 65 adrenalectomized animals exhibited an increase in corticosterone levels after stress exposure; 6 of these increased levels were from animals in which regenerated adrenal tissue was detected, and 6 were from animals in which no adrenal tissue was found.

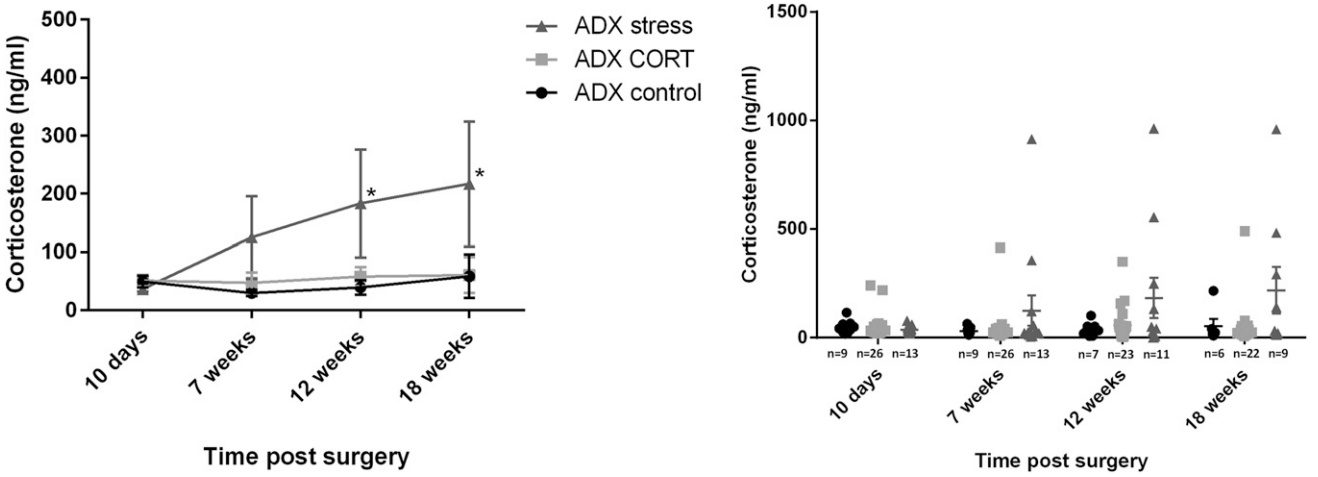

Figure 7. Adrenalectomized male F344 rats exposed to chronic stress reestablished their corticosterone levels 12 weeks after extensive ADX, whereas adrenalectomized rats that were not exposed to stress and adrenalectomized rats that were maintained on high levels of corticosterone did not reestablish corticosterone levels by 18 weeks. Corticosterone replacement was discontinued 1 week after ADX for the ADX-Stress and ADX-Control groups and 8 weeks after ADX for the ADX-CORT group. Left panel: summarized data. Right panel: individual data points. Data are presented as mean ± standard error of the mean. $*P < 0.05$, significantly different from ADX-control (no treatment) at the same time point.

proliferated after ADX. Ample clinical and animal studies reported the existence of ectopic adrenal glands (additional macroscopic adrenal-like tissues located elsewhere in the body), typically in the abdominal cavity (32–44). In patients without adrenal disease, additional adrenal-like glands were found in the celiac region in 30% of autopsies (39). Additional studies reported ectopic adrenal tissues in proximity to the kidney (43) or genital structures (35, 41, 45), adherent to the base of the appendix, and within the inguinal canal (33, 43). Anderson and Ross (33) suggested that accessory adrenal tissue exists in ~50% of neonates and children but markedly regresses by adulthood. Of note, most examples of ectopic adrenal tissue are found incidentally during surgery or autopsy (33, 35, 42). The existence of ectopic adrenal tissues suggests that normal embryological development of adrenal glands involves dissemination of progenitor adrenal cells, of which few progress to fully developed glands with an innervated medulla, whereas others may remain dormant. Compensatory functional hypertrophy of ectopic adrenal-like tissue after destruction or removal of normal adrenal glands has also been suggested (35, 40). A possible driving mechanism could be high ACTH levels after ADX (3, 31, 46–48), which have been shown to cause marked thickening and vacuolation of the cortical tissue in both normal and accessory adrenal glands (40), as may have occurred herein in the ADX-stress experiment.

Hummel (32) studied the occurrence of accessory adrenocortical nodules in nine different mice strains and found them in between 38% and 60% of mice, depending on the strain, some exhibiting multiple nodules. Furthermore, in all of the studied strains higher percentages of accessory adrenal nodules were found on the left side, with a 4:1 ratio between the left and right sides. This observation corresponds well with the current findings, in which most regenerated adrenal glands were found on the left side. Notably, accessory adrenals are also found in dogs, moles, armadillos, squirrels, lagomorphs, humans, and nonhuman primates (49) and are typically found in the perirenal adipose tissue. Parker and Valerio (34) wrote that "accessory adrenocortical tissue can partially, and perhaps entirely, replace adrenocortical function in ADX animals, thus complicating studies of adrenocortical function." This may well be the case in the current study.

A third hypothesis for elevated corticosterone levels after ADX is extra-adrenal glucocorticoid production that ramps up production to compensate for the lack of adrenal glands. In the past 30 years, a large number of studies have demonstrated that corticosterone and aldosterone can also be synthesized *de novo* in extra-adrenal locations, including the gonads, liver, heart, and skin (17–22). These organs express *CYP11B1* long after ADX or adrenal enucleation. One cannot exclude the possibility that after ADX other organs might increase their transcription of the enzymes involved in corticosterone synthesis and gain the synthesis capacity to impact systemic levels as a compensatory reaction. However, excluding the adrenal glands, the ability of these organs to synthesize corticosterone is limited and does not significantly impact systemic corticosterone levels (17, 18, 22, 23). In addition, after the ADX-time dependency experiment, we tested the main candidate organs for pronounced extra-adrenal secretion of corticosterone and aldosterone (gonads, heart, BAT, and thymus) and found no evidence of substantial production of these hormones.

Finally, one cannot exclude the possibility that the regenerated adrenal glands in the current study may

reflect regeneration from non−original gland sources, namely, neo-regeneration. Such neo-regeneration is different from all cases reported in the literature that are based on residuals from the original adrenal glands. To the best of our knowledge, there are no reports on this phenomenon in rats. To date, the ability to regenerate whole organs or body parts once the original organ has been fully excised occurs only in a few organisms. Little is known about this phenomenon, and most of what is known is learned from salamanders, who have the remarkable ability to regenerate complex structures such as limbs, tails, and the spinal cord, along with some sections of the heart and brain (50, 51).

Evidence from the current study also suggests that corticosterone synthesis increases with time after ADX, individual differences are prevalent, and environmental or internal stressors affect this process. Prominent among these factors, as evident in the ADX-stress experiment, are environmental and psychological stressors, such as cage replacement and restraint stress, and physiological stressors, such as immune activation or imbalanced sodium levels.

The current findings have ramifications for basic science, the methodology of ADX, and clinical practice. At the methodological level, long-term studies employing ADX animals should consider not only ongoing replacement of baseline corticosterone levels (*e.g.*, using a sustained-release pellet), but also additional supplementation in circumstances characterized by higher levels (*e.g.*, active periods, through adding corticosterone to drinking fluid) and minimization of stress-induced HPA responses. Shortening the duration from ADX to experimentation may reduce the risk for unwanted corticosterone responses, and it is imperative to verify lack of corticosterone response to stress. It should also be noted that adrenalectomized animals may quickly develop compensatory mechanisms in cases of low endogenous or replaced corticosterone and aldosterone levels, which may enable small changes in corticosterone levels that induce the same consequences as greater changes in naive animals. Potential clinical ramifications may be markedly supportive for patients, although further preclinical studies are necessary before implementation.

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