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Development, Maintenance, and Function of the Adrenal Gland in Early Postnatal Proopiomelanocortin-Null Mutant Mice

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Adult mouse mutants homozygous for an engineered proopiomelanocortin (POMC)-null allele lack macroscopically distinct adrenal glands and circulating adrenal hormones. To understand the basis for this adrenal defect, we compared the development of adrenal primordia in POMC-null mice and littermate controls. POMC-null mutant mice are born with adrenal glands that are morphologically indistinguishable from those of their wild-type littermates. However, in mutants adrenal cells fail to proliferate postnatally and adrenals atrophy until they have disappeared macroscopically in the adult. While present, mutant adrenals are differentiated as evidenced by the presence of enzymes for the final steps in the synthesis of corticosterone, aldosterone, and catecholamines. However, in contrast to adrenals of wild-type littermates, adrenals of POMC-null mutants do not produce corticosterone, not even in response to acute stimulation with exogenous ACTH. They do produce aldosterone; however, it is produced at reduced levels correlating with adrenal size. Transplantation of POMC-null mutant adrenals to adrenalectomized wild-type littermates results in adrenals with normal morphology and production of both corticosterone and aldosterone. These findings demonstrate that POMC peptides are not required for prenatal adrenal development and that POMC peptides in addition to ACTH are required for postnatal proliferation and maintenance of adrenal structures capable of producing both glucocorticoids and mineralocorticoids. (*Endocrinology* 146: 2555–2562, 2005)

THE ADRENAL GLAND regulates a number of essential physiological functions in the adult organism through production of steroids and catecholamines. Maintenance of adrenal structure and function are regulated through integration of extra- and intracellular signals. Central to this regulation is the pituitary hormone ACTH, which is derived from the proopiomelanocortin (POMC) polypeptide precursor (1–3). ACTH is the principal hormone stimulating adrenal glucocorticoid biosynthesis and secretion (4, 5). It is a central component of the stress response as part of the hypothalamus-pituitary-adrenal axis. Briefly, CRH secreted by the hypothalamus binds to pituitary corticotrophs expressing CRH receptor 1. This stimulates the secretion of ACTH by corticotrophs; ACTH in turn binds to the adrenal expressing melanocortin receptor 2 (Mc2r). This interaction stimulates glucocorticoid secretion by the adrenal gland. Serum glucocorticoid levels regulate CRH levels through glucocorticoid receptors in the hypothalamus. Interruption of this circuit at different sites, either surgically, pharmacologically, or genetically, has validated this model (6–12).

Whereas these results show the centrality of ACTH in regulating the hypothalamus-pituitary-adrenal axis, other data suggest that POMC-derived peptides, other than ACTH, play roles in adrenal development, function, and maintenance. Specifically, cleavage of the N-terminal POMC1–74 would generate shorter peptides with mitogenic (POMC1–28, POMC1–49, and/or POMC1–52; Refs. 13–18) and ACTH-potentiating (Lys-γ-MSH; Refs. 19–23) properties. Furthermore, there are experimental data indicating that α-MSH augments adrenal steroidogenesis (24, 25) and stimulates function of the aldosterone-producing zona glomerulosa (25–27).

We previously generated a mouse model that completely lacks all POMC-derived peptides by targeted deletion of exon 3 containing the POMC coding region (28). Mouse mutants homozygous for the engineered POMC-null allele are born at one quarter of the expected frequency; those surviving to adulthood show obesity, pigmentation defects, and adrenal insufficiency (28). Recently, the same findings were reported for a POMC-null mouse mutant model generated by replacing the initiator ATG with a stop codon and the majority of exon 3 with a targeting cassette (29, 30).

With respect to the adrenal insufficiency, we previously showed that homozygous POMC-null mutants at 6 months of age lacked morphologically distinct adrenal glands and circulating adrenal hormones (28). To understand the basis of this defect, we have analyzed adrenal development, maintenance and function in early postnatal POMC-null mutant mice. Furthermore, we assessed the potential for structural and functional rescue of mutant adrenals through transplantation studies.

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Abbreviations: H&E, Hematoxylin and eosin; Mc2r, melanocortin receptor 2; PCNA, proliferating cell nuclear antigen; POMC, proopiomelanocortin; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

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Materials and Methods

Animals

POMC wild-type, heterozygous, and mutant mice in the 129SvEv background (28) were housed under a 12-h light, 12-h dark cycle, with a standard laboratory diet (PMI5053; Purina Mills, Richmond, IN), provided *ad libitum* unless otherwise stated. Mice were genotyped by PCR analysis of tail DNA (28). All procedures described below follow National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

Serum

Blood was collected into microfuge tubes from tail veins or through retroorbital phlebotomy using heparinized capillary tubes. The blood was allowed to clot at room temperature for 15–20 min and then put on ice. Samples were spun at 4 C, 3500 rpm, for 15 min, and serum was transferred to new microfuge tubes. Serum was flash-frozen in dry ice-ethanol and stored at −80 C until analyzed.

RIA assays

Levels of corticosterone and aldosterone were determined in serum samples using RIA kits according to the manufacturer's specifications (Corticosterone RIA, ICN Biomedicals Inc., Irvine, CA; Coat-A-Count Aldosterone RIA, Diagnostic Products Corp., Los Angeles, CA). The limits of detection for these RIAs are 25 ng/ml for corticosterone and 25 pg/ml for aldosterone, respectively.

Histology

Immediately after mice were killed, tissues were carefully removed and fixed in 10% formalin in PBS. They were then dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks. A series of 5-μm-thick sections were cut and stained with hematoxylin and eosin (H&E) or used for immunohistochemistry.

Immunohistochemistry

Immunohistochemical analyses were carried out on formalin-fixed paraffin-embedded tissue sections. Rabbit polyclonal antiserum against tyrosine hydroxylase and mouse monoclonal antibody against rat 11-β-hydroxylase were obtained from Chemicon International, Inc. (Temecula, CA). Immunoreactivity was visualized using the Vectastain ABC-AP kit and the Vector M.O.M. (Peroxidase) Kit, both from Vector Laboratories, Inc. (Burlingame, CA), for tyrosine hydroxylase and 11-β-hydroxylase, respectively.

Proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) analysis

PCNA was detected in paraffin sections by staining according to the manufacturer's recommendations (PCNA staining kit; Zymed, Inc., San Francisco, CA). For detecting fragmented DNA through TUNEL, paraffin sections were treated and stained following the manufacturer's protocols (NeuroTACS II *in situ* apoptosis kit; Trevigen, Inc., Gaithersburg, MD).

Northern blot analysis

Adrenals were collected and immediately frozen on dry ice. RNA was prepared using the TRIzol method (Invitrogen, Carlsbad, CA) followed by column purification (RNeasy Mini Kit; QIAGEN, Valencia, CA). Seven micrograms of total RNA were used for formaldehyde gel electrophoresis. Gels were blotted onto nylon membranes (BrightStar Plus; Ambion, Austin, TX) and hybridized with ^{32}P-labeled oligonucleotides using NorthernMax buffers (Ambion). The following antisense oligonucleotides were used: ACTH receptor, 5′-GCCAACAGAAGATGAAGACTCC; 11-β-hydroxylase, 5′-CCATCTGCACATCCTCTTTCTCTT; aldosterone synthase, 5′-CATTTGCAGCTTGCTGCCCCTA; tyrosine hydroxylase, 5′-TGGCACCTGTGGGTGGTACCCTAT; cyclophilin, 5′-CAAAACGCTCCATGGCTTCCACAA. Autoradiographs were quantified by densitometry using the AlphaImager2200 (Alpha Innotech Corporation, San Leonardo, CA). The signal for the constitutively expressed cyclophilin gene served as the control for the amount of RNA loaded. The hybridization signals for the sets of P7 and of P35 samples were normalized according to the cyclophilin control for each sample. These normalized data were then graphed as a percentage of wild-type signal.

Supplementation of ACTH

For acute stimulation, ACTH (1–24), synthesized by Invitrogen was used. Mice were injected with ACTH (1 μg/0.1 ml PBS/0.5% BSA per mouse) sc between the shoulder blades. Control animals received carrier (PBS/0.5% BSA) only. Wild-type animals received 0.4 mg dexamethasone 2 h before ACTH or carrier injection (31). Tail blood was collected for corticosterone RIA 1 h after ACTH stimulation.

Transplantation studies

The adrenal transplantation technique described for rats was used (32–34). All manipulations were carried out using a stereomicroscope (Nikon SMZ-U; Nikon, Melville, NY). For transplantation of postnatal d 9 adrenals into age-matched littermates adrenal glands were removed from donor animals and cleaned of connective tissue but were otherwise left intact. Adrenal grafts were placed on gauze soaked in isotonic saline at room temperature until transplanted; transplantation was completed within several minutes of graft preparation. Recipient littermates were bilaterally adrenalectomized under Metofane (Schering-Plough, Kenilworth, NJ) inhalation anesthesia through a dorsal midline skin incision and lateral retroperitoneal incisions. The right kidney was exteriorized through the retroperitoneal incision, and adrenals were placed under the kidney capsule through a slit in the adrenal capsule made with a fine scalpel blade, one adrenal per recipient. After careful repositioning of the kidney and closure of peritoneal and skin wounds by suturing, the mice were returned to their home cage. Nursing of pups after surgery by the dam was improved when all pups of a litter were surgically manipulated, either undergoing adrenalectomy and transplantation or sham surgery, *i.e.* incisions and suturing only. Transplant recipients and sham-manipulated littermates grew up indistinguishably.

At 3 months of age, mice were killed. Careful dissection confirmed that the adrenalectomized mice had none of their own adrenal tissue left; the adrenal transplant was visible in the mid-portion of the right kidney.

Statistical analysis

Data were analyzed by one-way ANOVA. Significance was accepted at $P < 0.05$ unless indicated otherwise. Results are expressed as the mean ± SEM.

Results

Adrenal gland structures develop prenatally in the absence of POMC peptides

Adult POMC-null mutants lack discernable adrenal gland structures (28). To determine how adrenal glands develop in POMC-null mutant mice, we dissected pups at birth and at weekly intervals postnatally. Adrenal glands in homozygous POMC-null mutant pups were indistinguishable in size and histological appearance from those of heterozygous and wild-type littermates at birth (Fig. 1A). Notably, this was also the case for adrenals from newborn homozygous POMC-null mutant pups born to homozygous POMC-null mutant dams (Fig. 1B); in this case POMC peptides are lacking in both fetus and dam. Together, these results suggest, but do not prove, that POMC-derived peptides are not required for prenatal development of adrenal structures.

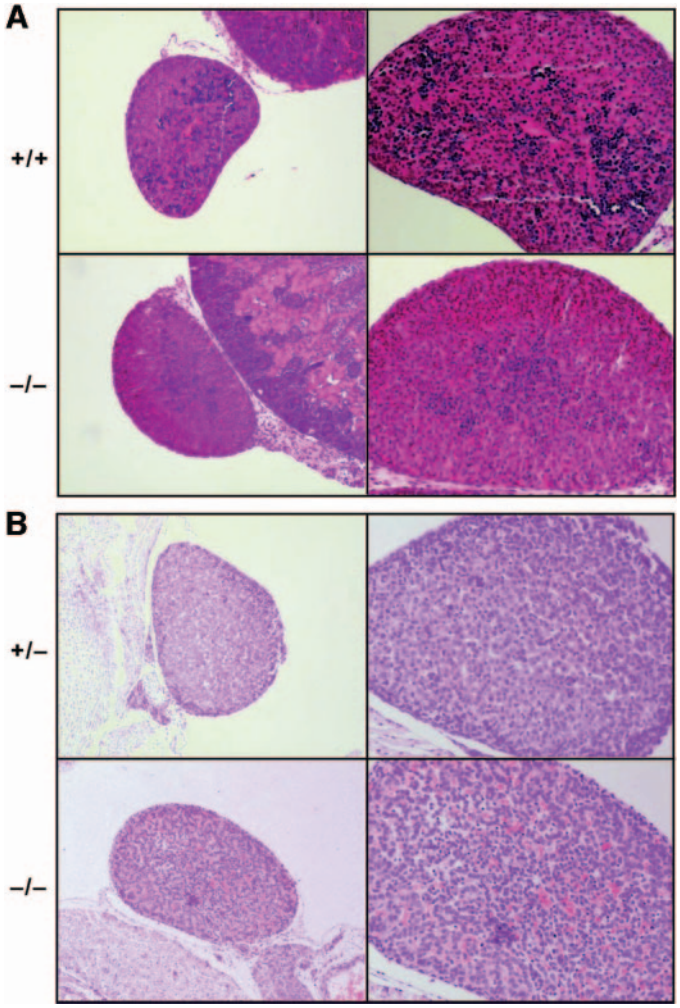

FIG. 1. Structure of neonatal wild-type, heterozygous, and POMC-null mutant adrenals. H&E-stained paraffin sections of neonatal adrenals are shown at ×10 and ×20 magnification from (A) wild-type (+/+) and mutant (−/−) littermates born to heterozygous dams and (B) heterozygous (+/−) and mutant (−/−) littermates born to homozygous POMC-null mutant dams.

Adrenal gland structures are not maintained in POMC-null mutant mice

To find out what happens to the adrenal glands between the early postnatal days and the adult stage, we collected adrenals at weekly intervals. Visually adrenal sizes in POMC-null mutants are comparable to those of their wild-type littermates for the first 7–14 d postnatally, whereas by 28–35 d the wild-type adrenal is severalfold larger than the mutant adrenal (Fig. 2, A and B). Quantification of adrenal weights (Fig. 2C) shows that mutant adrenals weigh slightly, but significantly, less than wild-type adrenals already at 1 wk of age (wild-type 0.48 ± 0.02 mg *vs.* mutant 0.30 ± 0.02 mg, $P < 0.00005$). By 5 wk of age wild-type adrenals have over 6-fold the weight of mutant adrenals (wild-type 2.50 ± 0.17 mg *vs.* mutant 0.40 ± 0.06 mg, $P < 0.000005$); the weight of mutant adrenals did not change significantly between 1 and 5 wk. By the time the POMC-null mutants are several months of age, adrenal remnants can rarely be found macroscopically in the fat tissue surrounding the kidneys. This lack of adrenal maintenance affects all layers of the adrenal equally, *i.e.* medulla and cortex, and within the cortex it affects the different zones equally. This is evident in adrenal sections stained with H&E (Fig. 2B). Thus, adrenals in POMC-null mutant mice regress proportionally, *i.e.* the different adrenal layers regress roughly equally. These results reveal an essential role for POMC peptides in maintenance of the whole organ.

Lack of maintenance of adrenals is due to failure of cell proliferation

Regression of adrenals in POMC-null mutants could be due to decreased proliferation, increased cell death, or both. Wild-type adrenals from postnatal d 14 pups displayed high numbers of proliferating cells in all layers of the adrenal. Adrenals from POMC-null mutant mice had considerably fewer proliferating cells, and these were mainly restricted to the outermost layer of the adrenal cortex (Fig. 3A). Staining for apoptotic cells in postnatal adrenals between 1 and 5 wk revealed only a few individual apoptotic cells in wild-type adrenals; these cells were mainly found in a small band between cortex and medulla. In mutant adrenals, a comparable section had one apoptotic cell or none at all (Fig. 3B). Postnatally, adrenals undergo vigorous proliferation. Our results show that this postnatal proliferation does not happen in the absence of POMC peptides. The lack of proliferation accounts at least to a large part for the difference in adrenal mass between wild-type and mutant adrenal by 5 wk of age.

Adrenal gland structures in young POMC-null mice are not functional with respect to corticosterone release

To test whether the adrenal gland structures present in young POMC-null mutant mice are functional, we assessed serum levels of corticosterone and aldosterone in 1-wk- and 1-month-old POMC-null mutant and wild-type controls (Fig. 4, A and B). No corticosterone was detected in POMC-null mutants at 1 wk or 1 month of age (Fig. 4A), nor at any later time point (sera of mice up to 9 months of age were analyzed; data not shown). Serum levels of aldosterone in POMC-null mutants were not significantly different in wild-type *vs.* mutant mice at 1 wk of age (wild-type *vs.* mutant, 357 ± 145 *vs.* 89 ± 5 pg/ml; not significant) but were significantly decreased in POMC-null mutants at 1 month of age (wild-type *vs.* mutant, 793 ± 103 *vs.* 203 ± 39 pg/ml; $P < 0.0001$) (Fig. 4B). At the later age, adrenals in POMC-null mutants are considerably smaller than in wild-type littermates but are still present. Aldosterone measurements are an indicator for the presence of adrenal structures: with increasing age fewer mutant mice have measurable levels of aldosterone, reflecting fewer POMC-null mutants with adrenal structures left (nine of 10 mice at 3 months *vs.* zero of 10 mice at 9 months; data not shown).

We next tested whether we could induce corticosterone release after acutely stimulating the mice with ACTH, especially at 1 wk of age when adrenal structures in POMC-null mutants are morphologically indistinguishable from those of wild-type littermates. Whereas the wild-type mice respond to the challenge with ACTH by releasing corticosterone at increasing levels with increasing age, the POMC-null mutant

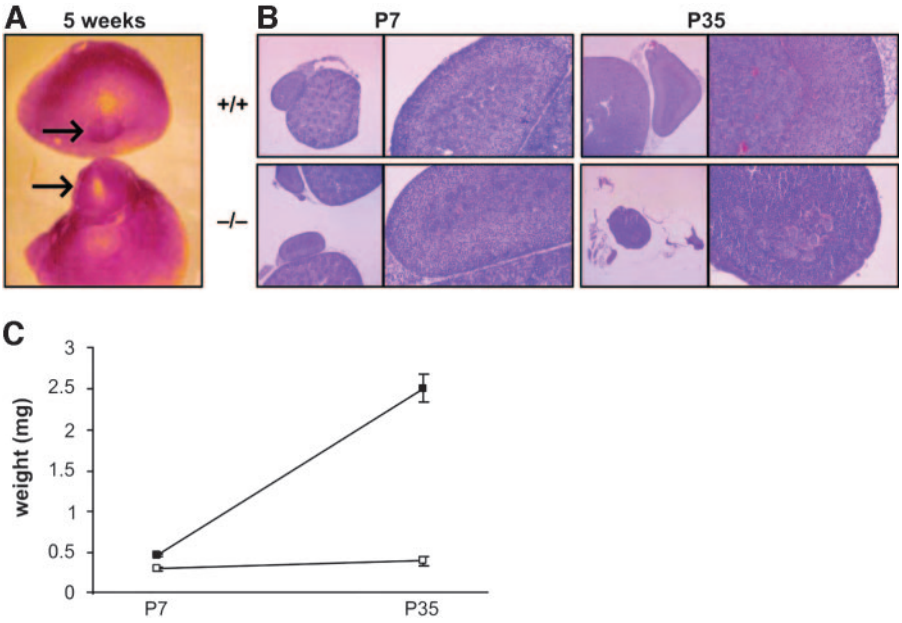

FIG. 2. Adrenal maintenance *vs.* regression in wild-type *vs.* POMC-null mutant mice. A, Stereomicroscope view on the top of the kidney with the adrenal gland attached (*arrow* pointing at adrenal) from 5-wk-old POMC-null mutant (upper kidney plus adrenal) and wild-type (lower kidney plus adrenal) littermates. B, H&E-stained paraffin sections of adrenals from wild-type (+/+) and POMC-null mutant (−/−) littermates at postnatal d 7 (P7) and 35 (P35), ×4 and ×10 magnifications. C, Weights of adrenals collected from 1- and 5-wk-old mice (n = 5–8) after fixation in formalin.

mice did not have any detectable levels of corticosterone in their serum at any age (Fig. 4C). Corticosterone levels in control animals receiving carrier only were below detection (data not shown). These results indicate that in the absence of POMC peptides there is no release of corticosterone, neither at basal levels nor after acute stimulation with amounts of ACTH eliciting a robust response in the presence of POMC peptides, *i.e.* in wild-type controls.

Adrenal gland structures in young POMC-null mice are differentiated

A possible explanation for the results above is that adrenals in POMC-null mutants are morphologically developed but at the molecular level lack essential components required for steroidogenesis. Therefore, we tested the POMC-null mutant adrenals for key components of steroidogenesis, including the ACTH receptor (Mc2r), and the enzymes required for the last steps in the synthesis of corticosterone (11-β-hydroxylase), aldosterone (aldosterone synthase), and catecholamines (tyrosine hydroxylase) (Fig. 5). Results from Northern blots, *in situ* hybridizations, and immunohistochemistry experiments showed that POMC-null mutant adrenals express transcripts and the proteins of those genes. Quantitation of Northern blot autoradiographs showed that POMC-null mutant adrenals contain transcripts of these components on a per cell basis comparable to those of wild-type littermates with the exception of Mc2r, which is expressed at decreased levels in POMC-null mutant adrenals compared with wild-type adrenals (75 *vs.* 100%; Fig. 5B).

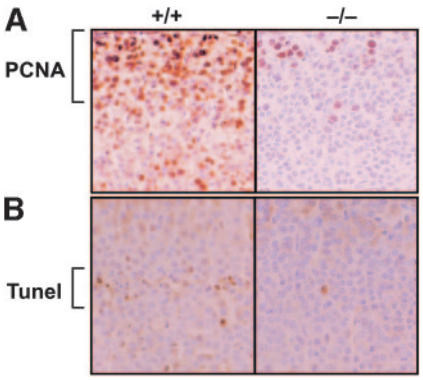

FIG. 3. Cell proliferation and death in adrenals from wild-type and POMC-null mutant mice. Staining of paraffin sections of adrenals from wild-type (+/+) and POMC-null mutant (−/−) littermates with antibodies against PCNA for detection of proliferating cells at postnatal d 14 (A) and for detection of apoptotic cells by TUNEL reaction at postnatal d 35 (B); ×20 magnification. *Dark cells* are positive; *brackets* highlight stained cells in wild-type sections.

Transplantation of POMC-null mutant adrenals to wild-type recipients rescues adrenal structure and function

During the early postnatal period adrenal glands in POMC-null mutant mice are morphologically indistinguishable from, and are as differentiated as, those of their wild-type littermates. To investigate whether placing of POMC-null mutant adrenals into an environment providing all POMC peptides, starting at early postnatal stages, would rescue structure and function of POMC-null mutant adrenals, we carried out transplantation studies. At postnatal d 9, adrenals from POMC-null mutant pups were transplanted into adrenalectomized wild-type littermates, one adrenal per recipient; and vice versa, adrenalectomized POMC mutant pups received adrenals from wild-type littermates. To test for functionality of our transplanted structures, we assayed the serum of the transplant recipients 2 months after transplantation for the presence of corticosterone and aldosterone. The mutant adrenals transplanted into the wild-type recipients turned out to be fully functional, producing expected levels of corticosterone and aldosterone (266 ± 31 mg/ml and 899 ± 75 pg/ml, respectively). In contrast, the wild-type adrenals in the POMC mutant hosts did not produce corticosterone at all and produced reduced levels of aldosterone (194 ± 48 pg/ml; Fig. 6A), replicating the results found in

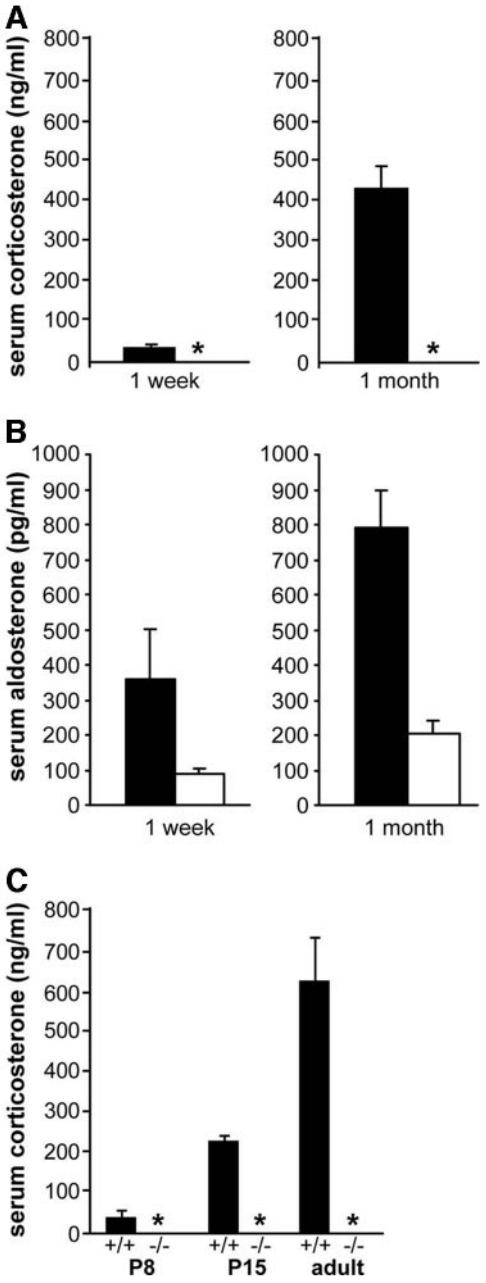

FIG. 4. Function of postnatal wild-type and POMC-null mutant adrenals. A and B, Blood was collected from wild-type (+/+; *black bars*) and POMC-null mutant (−/−; *white bars*) littermates at 1 wk and at 1 month of age, and adrenal steroids were determined by RIA. Values represent the mean ± SEM for three (1 wk; A and B), nine (1 month; A), and eight (1 month; B) mice per group. *, Not detectable. C, Mice, wild-type (+/+) and POMC-null mutant (−/−) littermates, ages postnatal d 8 and 15 (P8, P15) and 2 months (adult), were injected with 1 μg synthetic ACTH (wild-type mice had gotten 0.4 mg dexamethasone 2 h earlier), and tail blood was collected for corticosterone RIA 1 h later. Values represent the mean ± SEM for five mice per group. *, Not detectable.

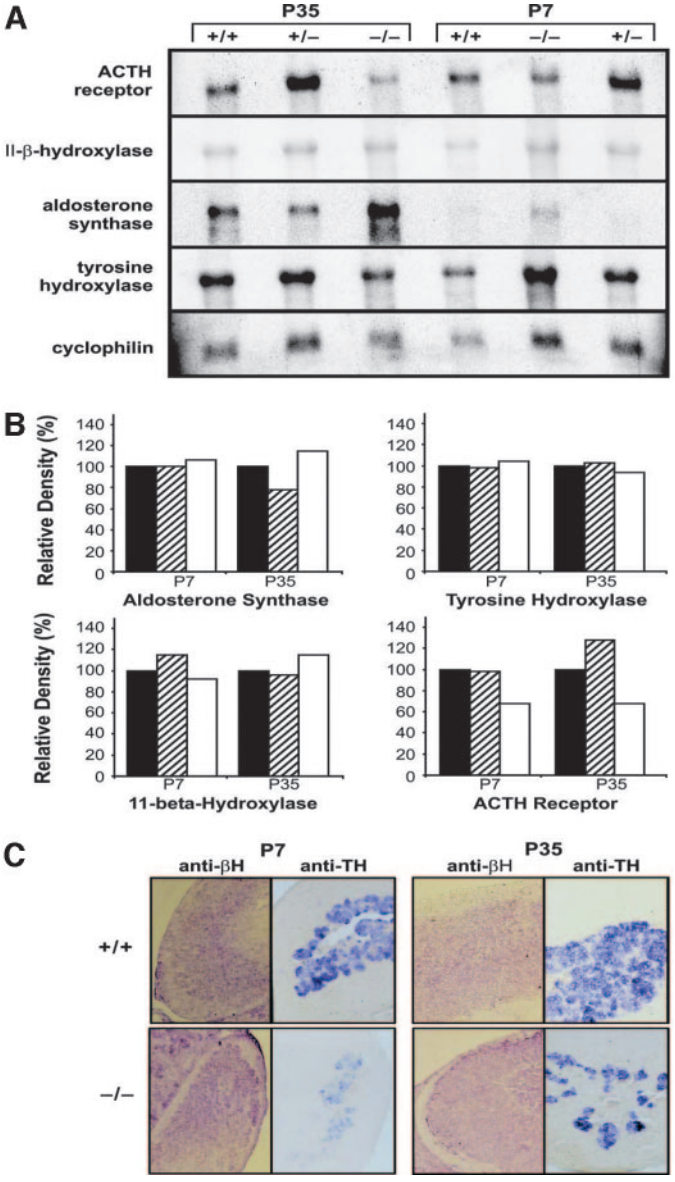

FIG. 5. Differentiation of postnatal wild-type and POMC-null mutant adrenals. A, Northern blot analyses with radioactively labeled probes as indicated; equal amounts of RNA were loaded; cyclophilin is the control. Mice were 7 d (P7) or 5 wk old (P35) wild-type (+/+), heterozygous (+/−), and POMC homozygous-null mutant (−/−) littermates. B, Densitometry graphs of the autoradiographs in A; wild-type, *black bars*; heterozygotes, *striped bars*; mutant, *white bars*. Hybridization signals for each probe are normalized to the cyclophilin signals and presented as percentage of wild-type signal. C, Immunohistochemical staining of paraffin sections of adrenals from wild-type (+/+) and POMC-null mutant (−/−) littermates with antibodies against 11-β-hydroxylase (anti-βH; positive cells are *red*) and tyrosine hydroxylase (anti-TH; positive cells are *blue*) at postnatal d 7 (P7) and 35 (P35), ×20 magnification.

POMC-null mutants. At 3 months of age, mice were killed; H&E sections of adrenals from both a wild-type recipient carrying a mutant adrenal and a mutant recipient carrying a wild-type adrenal are shown in Fig. 6B. The adrenal transplanted from a POMC-null mutant pup into an adrenalectomized wild-type littermate has developed into the typical adrenal structure with a layered cortex and a central medulla. The adrenal transplanted from a wild-type pup into an adrenalectomized mutant mouse is considerably smaller and less organized. Thus adrenals from POMC-null mutant mice retain the potential to differentiate into, and to be maintained as, functional adrenal glands, demonstrating that prenatal

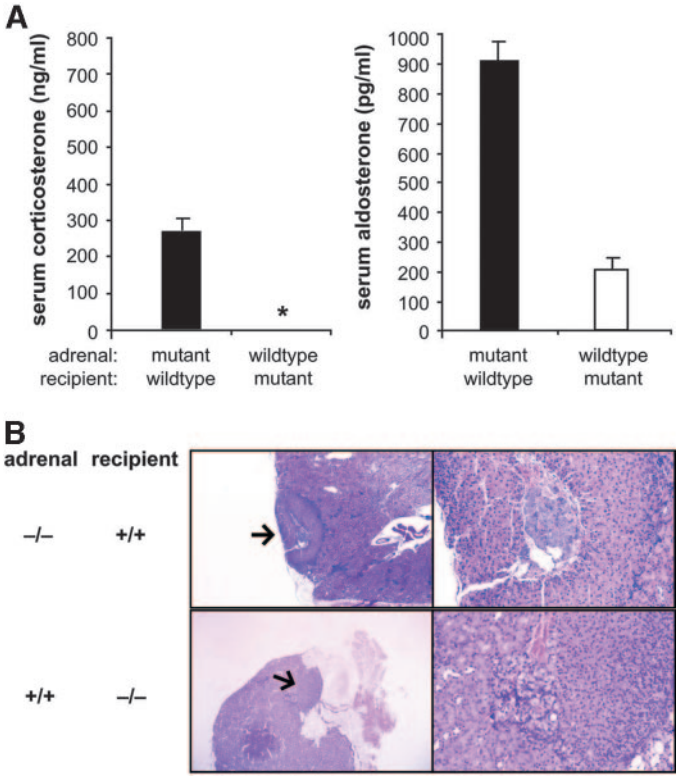

FIG. 6. Transplantation of POMC-null mutant adrenals into adrenalectomized wild-type littermate recipients and vice versa. A, RIA analysis of sera for corticosterone and aldosterone from adrenalectomized wild-type recipients carrying mutant adrenal transplants and from mutant recipients carrying wild-type adrenal transplants 2 months after transplantation. Values represent the mean ± SEM for three to five mice per group. *, Not detectable. B, Example of mutant adrenal transplanted into wild-type recipient (adrenal, −/−; recipient, +/+; *upper panel*) and of wild-type adrenal transplanted into mutant recipient (adrenal, +/+; recipient, −/−; *lower panel*). *Arrows* point at adrenal transplants surrounded by kidney tissue. H&E-stained paraffin sections, ×4 and ×10 magnifications.

development of adrenal glands is not affected by absence of POMC peptides. However, this potential can only be realized in an environment providing POMC peptides.

Discussion

Adrenals in homozygous POMC-null mutant pups were indistinguishable in size and histological appearance from those of heterozygous and wild-type littermates at birth. These results demonstrate that POMC-derived peptides are not required for prenatal development of adrenal structures in the mouse. This conclusion is confirmed through transplantation experiments, where POMC-null mutant adrenals are fully functional upon early postnatal transplantation into adrenalectomized wild-type recipients. This is in contrast to conclusions drawn from results in other experimental systems reported in the literature. Experiments with hypophysectomized fetal sheep (35, 36) or pigs (37) showed significant perinatal inhibition of growth of the adrenal cortical zone, particularly that of the zona fasciculata, but normal adrenal medullar development, as judged by volume and number of cells. Glucocorticoid treatment of pregnant rats resulting in decreased availability of ACTH in the fetus led to considerable atrophic changes in adrenocortical cells (38) as well as in adrenal medulla (39) in offspring. The differences in these results to those in POMC-null mutant mice may reflect species differences and/or the reaction of the organism to a sudden change in hormones during development compared with absence of hormones throughout development. As our results show, prenatal development of the adrenal in mouse to a morphologically normal organ takes place in the continuous absence of all POMC-derived peptides. And absence of POMC peptides during development does not lead to aberrant consequences *per se*, because transplantation of 1-wk-old mutant adrenals to an environment containing POMC peptides results in functioning adrenals.

Although not required for prenatal development, POMC peptides are essential for postnatal maintenance of the adrenal structures in that in their absence adrenals fail to proliferate postnatally and atrophy slowly. Lack of proliferation rather than apoptosis seems to account for the failure in adrenal growth. This is different from the effects seen in hypophysectomized rats, in which adrenocortical cell decrease results from apoptosis (40–42). A possible explanation is that acute withdrawal of pituitary POMC peptides has a different effect than their continuous absence.

Although early postnatal adrenal structures in POMC-null mutant mice are differentiated, as evidenced by the presence of enzymes required in adrenal steroid synthesis, these structures do not produce corticosterone. Furthermore, they produce aldosterone at reduced levels. In this regard, the POMC-null mutant mouse model is different from patients with POMC-null mutations, who are reported to have no defects of mineralocorticoid function (43). This might reflect a species difference, but the basis remains to be elucidated. It is also different from other mouse mutant models with primarily low levels of ACTH, such as mice lacking CRH (6) or its receptor (7, 8) or mice lacking differentiated pituitary corticotrophs (Tpit-null mouse; Ref. 10). In these mouse models, the zona fasciculata atrophies, whereas the other adrenal layers remain relatively unchanged compared with wild-type adrenals, resulting in hypocorticosteronemia with normal levels of aldosterone. The major difference between these models and the POMC-null mutant is the complete absence of all POMC peptides, at all times and from all sources, in the latter.

The adrenal gland has the capacity to produce corticosteroids after transplantation into adrenalectomized recipients (32–34). We used a cross-transplantation paradigm to show that adrenals developed in POMC-null mutant mice are capable of being maintained and producing corticosteroids upon transplantation into an environment providing all POMC peptides, whereas wild-type adrenals transplanted into POMC-null mutant recipients cease producing corticosteroids and atrophy. In previously reported experiments, about half the corticosterone levels were found in transplanted animals *vs.* nonmanipulated or unilaterally adrenalectomized animals (32–34, 44). We found this to be the case in our transplantation experiments of POMC-null mutant adrenals into adrenalectomized wild-type recipients as well; aldosterone levels in transplant recipients were in the normal range. These experiments demonstrate that 1) POMC peptides are not required for prenatal development of the ad-

renal glands, and 2) presence of all POMC peptides allows maintenance of an adrenal structure capable of producing both gluco- and mineralocorticoids.

The POMC-null mutant mouse model provides the opportunity to test which of the POMC peptides, individually or in combination, rescues structure and/or function of the adrenals. Two approaches have been explored so far: reconstitution of POMC-null mutants with synthetic ACTH alone (Refs. 30 and 45, this report, and our unpublished data) and exposure of POMC-null mutant adrenals to all POMC peptides by transplantation into wild-type hosts (this report). There is no corticosterone release in POMC-null mutant mice upon acute stimulation with exogenous ACTH. At 1 wk of age, when adrenals from POMC-null mutant mice are morphologically indistinguishable from those of wild-type mice, acute stimulation with 1 μg ACTH results in robust stimulation of corticosterone release 1 h later in dexamethasone-suppressed wild-type littermates but does not stimulate any corticosterone release in POMC-null mutant mice. Whereas acute stimulation with higher doses of ACTH has not been tested, the failure of POMC-null mutant adrenals to produce corticosterone in response to ACTH, as their wild-type littermates do, suggests that the presence of other POMC peptides is required to permit ACTH to elicit corticosterone production. Reconstitution of POMC-null mutants for several weeks with low supraphysiological doses of ACTH (1 μg per mouse per day), *i.e.* doses that do not cause increase of adrenal weight and of corticosterone production (Cushing symptoms) in wild-type controls, does not rescue adrenal structure nor function (Ref. 45 and our own unpublished data). However, reconstitution for 10 d with doses of ACTH (30 μg per mouse per day) that cause Cushing symptoms in wild-type controls leads to increase in adrenal weight and production of corticosterone in POMC-null mutants to levels comparable to those of unstimulated wild-type mice (30). Coll *et al.* (30) showed that the increase in adrenal weight in their ACTH reconstitution experiments is due to hypertrophy of existing cells rather than proliferation of new cells; however, we identified a lack of early postnatal cell proliferation in POMC-null mutant adrenals. Interestingly, ACTH alone does not stimulate the production of aldosterone (30). In contrast, transplantation of mutant adrenals to an environment providing all POMC peptides restores both corticosterone and aldosterone production.

Interpretation of these different results has to take into consideration the concentration of plasma ACTH in the context of absence or presence of other POMC peptides. Normal ACTH levels in mice are in the 100 pg/ml range (46, 47), with peak secretion occurring before the onset of the dark cycle (48). Providing mice daily with 1 μg ACTH alone, *i.e.* achieving a maximum concentration of approximately 30 ng/ml, does not rescue adrenal structure or function. Providing mice daily with 30 μg of ACTH, *i.e.* achieving a maximum concentration of approximately 1 μg/ml (10,000-fold increase over basal levels), results in increase of adrenal weight through hypertrophy of adrenal cells and stimulates corticosterone production but does not stimulate aldosterone production. Plasma levels of ACTH in adrenal transplant recipients are expected to be lower than in either of the pharmacological reconstitution experiments. Wilkinson *et al.* (32) determined the time course of changes in the concentration of plasma ACTH in rats after transplantation of adrenals to bilaterally adrenalectomized recipients. Whereas plasma ACTH was elevated at 1 wk, by 3–5 wk after transplantation the concentration of plasma ACTH was not different from that in intact rats. In mice, adrenalectomy without adrenal transplants or corticosterone replacement leads to a 10- to 20-fold increase (1–2 ng/ml) over basal levels in serum ACTH 3 h after adrenalectomy (49); elevated levels can still be measured 1 wk after adrenalectomy (46, 50), but ACTH levels have returned to basal levels without corticosterone replacement by 4 wk after adrenalectomy (47). Because we transplanted into our adrenalectomized wild-type mice a mutant adrenal eventually producing corticosterone and thus providing feedback to pituitary ACTH release, levels of 2 ng/ml ACTH are unlikely to even have been reached; however, this remains to be determined. Thus, it is unlikely that the rescue of adrenal structure and function in the transplantation experiments is due to the effects of ACTH alone.

Together, these results support the notion that POMC peptides in addition to ACTH are required 1) to permit physiological amounts of ACTH to elicit corticosterone production, 2) to effect increase of adrenal weight through proliferation of adrenal cells, and 3) to restore aldosterone production.

There are conflicting data in the literature over the last 3 decades concerning the role of different POMC-derived peptides in different aspects of adrenal growth, maintenance, and function. Further analyses of the POMC-null mutant model, especially through genetic complementation experiments, offers the opportunity to experimentally resolve these issues.

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