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The Effects of Proopiomelanocortin Deficiency on Murine Adrenal Development and Responsiveness to Adrenocorticotropin

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The mature adrenal cortex is dependent upon proopiomelanocortin (POMC)-derived peptides for the maintenance of its size, structure, and endocrine function. Recent studies in mice genetically deficient in POMC have suggested that early exposure to POMC-derived peptides might also be necessary for the development of a functionally competent adrenal. We examined adrenal morphology and function in an independent line of mice lacking all POMC-derived peptides (*Pomc*$^{-/-}$). Adrenal glands were found in all mice, although the glands of *Pomc*$^{-/-}$ mice had markedly reduced weight compared with control animals (0.5 ± 0.1 *vs.* 2.1 ± 0.1 mg, respectively; $P < 0.05$) and had disrupted cortical architecture. In *Pomc*$^{-/-}$ mice, plasma corticosterone was undetectable, and plasma aldosterone was significantly reduced compared with wild-type mice (498 ± 88 *vs.* 1845 ± 168 nmol/liter, respectively; $P < 0.001$). Heterozygous mice (*Pomc*$^{+/-}$) had smaller adrenal glands with significantly lower levels of corticosterone both basally and in response to CRH and ACTH than wild-type mice, indicating that two functional copies of the *Pomc* gene are necessary to support the fully normal function of the hypothalamic-pituitary-adrenal axis. Three-month-old *Pomc*$^{-/-}$ mice were treated for 10 d with a highly specific ACTH analog. This treatment restored adrenal weight, cortical morphology, and plasma corticosterone to the levels seen in wild-type littermates. In conclusion, murine adrenal glands can develop without exposure to endogenous POMC-derived peptides during fetal and neonatal life. Although such glands are atrophic and hypofunctional, exposure to ACTH alone can restore their size, morphology, and corticosterone secretion. (*Endocrinology* 145: 4721–4727, 2004)

ONCE FULLY DEVELOPED, the glucocorticoid-producing elements of the adrenal cortex are clearly dependent upon proopiomelanocortin (POMC) peptides produced by pituitary corticotrophs to maintain normal structure and function (1). Thus, hypophysectomy or down-regulation of corticotroph activity secondary to exogenous glucocorticoid both result in adrenal atrophy (1, 2). Of the POMC-derived peptides, ACTH is the classic adrenocorticotropic hormone and is the most important pituitary-derived peptide controlling steroidogenesis in the adult adrenal.

Additionally, there is evidence that POMC-derived peptides may play a role in the control of adrenal development, with exposure to these peptides during fetal and neonatal life being a potential requirement for the development of a functionally competent adrenal cortex. Yaswen *et al.* (3) reported that *Pomc*-null mice have no discernible adrenals macroscopically, only rudimentary adrenal glands microscopically, and undetectable circulating levels of both glucocorticoid and mineralocorticoid. Congenital deficiency of POMC in humans also results in hypocortisolemia and a markedly disrupted adrenal cortex (4, 5). A crucial role for ACTH in the development of the adrenal is highlighted by the anatomical findings in humans affected by familial glucocorticoid deficiency (6). Familial glucocorticoid deficiency is caused by loss of function mutations in the melanocortin 2 receptor, the endogenous ACTH receptor, resulting in adrenal unresponsiveness to ACTH and severe glucocorticoid deficiency (6). Affected adrenal glands are atrophic and have a disordered zona glomerulosa with no evidence of fasciculata or reticularis cells within the adrenal cortex.

However, evidence that POMC-derived peptides other than ACTH may be involved in the control of adrenal growth and development is also accumulating (7–11). In particular, some studies have proposed that a potent adrenal mitogenic peptide resides within the N-terminal 16-kDa fragment of POMC (N-POMC) (7–11). This large peptide is itself nonmitogenic and would require postsecretional cleavage to release the smaller active fragment. The recent description of an adrenal serine protease that can specifically cleave rat N-POMC to generate a small peptide has given considerable weight to this hypothesis (12).

We have examined the adrenal phenotype in an independent mouse model of POMC deficiency (*Pomc*$^{-/-}$) lacking all POMC-derived peptides. In addition, we have used mice heterozygous for this null mutation in the *Pomc* allele to determine whether *Pomc* haploinsufficiency (*Pomc*$^{+/-}$) results in adrenal hypofunction. Finally, we have treated these mice with a specific ACTH analog to determine whether it is capable of acting alone to restore adrenal structure and function.

Abbreviations: HPA, Hypothalamic-pituitary-adrenal; hpf, high-power field; PCNA, proliferative cell nuclear antigen; POMC, proopiomelanocortin.

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Materials and Methods

Animal studies

POMC-deficient mice were generated on a 129/SvEv background as previously described (13). $Pomc^{-/-}$ mice used in this study were offspring of either heterozygous matings or female $Pomc^{+/-}$ mice/male $Pomc^{-/-}$ matings. As we have previously reported (13), heterozygous matings resulted in $Pomc^{-/-}$ mice being born at a frequency of 8%, rather than the 25% expected for a recessive disorder, indicating that POMC deficiency is associated with substantial prenatal lethality. Genotypes were determined by PCR of DNA from tail tissue as previously described (13). Mice were maintained under controlled temperature (22 C) and light (12 h light from 0700–1900 h) and had *ad libitum* access to water and standard chow (4.5% fat chow; Special Diet Services, Witham, UK). Animals were individually caged and handled daily for 1 wk before peptide administration and/or blood collection, unless otherwise stated. Trunk blood was collected within 1 min of initial handling in all experiments. All animal protocols used in these studies were approved by the United Kingdom Home Office.

Unstimulated hormone levels

Blood for corticosterone was collected at the indicated clock time. All sampling done in dark cycle was collected using a dark room red light to minimize stress to the mice. Blood for aldosterone was collected between 1600 and 1700 h.

CRH test

Two-month-old male wild-type and $Pomc^{+/-}$ mice were subcutaneously injected at 0900 h with either 1 μg of CRH (Sigma-Aldrich, Poole, UK) or saline. Blood was collected after 30 min.

ACTH test

Two-month-old male wild-type and $Pomc^{+/-}$ mice were ip injected at 0900 h with either $ACTH_{1-24}$ (Sigma-Aldrich) at a dose of 10 mg/kg or saline. Blood was collected after 60 min.

Depot ACTH administration

Three-month-old male wild-type and $Pomc^{-/-}$ mice were sc injected once daily at 1800 h for 10 d with either 30 μg of Depot Synacthen (Alliance Pharmaceuticals, Wiltshire, UK) or saline (sham injection). Blood was collected at 0900 h on the day after the last injection.

Histology and immunohistochemistry

Adrenal glands were rapidly dissected, cleaned of fat, and weighed. Glands that were to be used for histology were left uncleaned and attached to kidney and placed in 4% paraformaldehyde overnight at 4 C. Tissues were dehydrated and embedded in paraffin, and 7-μm sections were cut and stained with hematoxylin and eosin using standard protocols.

For proliferative cell nuclear antigen (PCNA) immunohistochemistry, paraffin-embedded sections were rehydrated, boiled in 10 nM sodium citrate (pH 6.0) for 5 min, blocked with 0.3% hydrogen peroxide in methanol for 10 min, and incubated at 4 C overnight with a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:100 in a blocking buffer. Bound antibody was detected using the Immunocruz Kit (Santa Cruz Biotechnology) according to the manufacturer's protocol. Sections were counterstained with hematoxylin.

To assess response to ACTH, hematoxylin and eosin-stained adrenal glands were examined under standard light microscopy using ×400 magnification. Cell nuclei within the cortical region of three independent sections from three different animals per group were counted.

Hormone assays

Plasma corticosterone and aldosterone were determined using commercially available kits according to the manufacturers' protocols (corticosterone, OCTEIA kit; Immunodiagnostic Systems Limited, Tyne and Wear, UK; and aldosterone, ^{125}I RIA kit; Diagnostics Products Corp., Los Angeles, CA).

Statistics

All data reported are mean ± SEM unless otherwise reported. Analysis was by paired or unpaired Student's *t* test using Prism software (GraphPad Software Inc., San Diego, CA). Diurnal variation was analyzed by two-way ANOVA. Results were considered statistically significant at $P < 0.05$.

Results

$Pomc^{-/-}$ mice have small adrenal glands with disordered cortical architecture

Three-month-old $Pomc^{-/-}$ mice all had macroscopically detectable adrenal glands, but they were significantly smaller than those found in wild-type littermates (mean weight of gland, 0.5 ± 0.1 *vs.* 2.1 ± 0.1 mg, respectively; $P < 0.001$; Fig. 1A). Microscopically, they had a distinct cortex and medulla, although the normal cortical architecture was lost with no clearly distinguishable zona fasciculata or glomerulosa (Fig. 1F). On hematoxylin and eosin staining, the adrenal medulla was clearly present in $Pomc^{-/-}$ mice, although reduced in size compared with wild-type mice. $Pomc^{+/-}$ mice also had smaller glands than wild-type animals (1.8 ± 0.1 *vs.* 2.1 ± 0.1 mg, respectively; $P < 0.05$; Fig. 1A), but the gross histological appearance was identical to that of wild-type mice.

$Pomc^{-/-}$ mice have undetectable corticosterone and $Pomc^{+/-}$ mice have significantly reduced corticosterone production compared with wild-type littermates

Homozygous mutant mice had undetectable plasma corticosterone levels, even when measured at the diurnal peak just before the onset of the dark cycle. In addition, compared with wild-type mice, heterozygous mice had a marked reduction in diurnal corticosterone secretion, with significantly lower levels of glucocorticoid at both 1300 h (74 ± 8 *vs.* 154 ± 27 ng/ml, $Pomc^{+/-}$ *vs.* wild-type; $P < 0.05$) and 1900 h (101 ± 17 *vs.* 200 ± 41 ng/ml, $Pomc^{+/-}$ *vs.* wild-type; $P < 0.01$; Fig. 2A). Circulating mineralocorticoid levels (Fig 2B) were also affected by POMC insufficiency; both homozygous mutant (498 ± 88 nmol/liter) and heterozygote (990 ± 121 nmol/liter) mice had significantly lower levels of aldosterone than wild-type (1845 ± 168 nmol/liter) mice ($P < 0.001$ for $Pomc^{-/-}$ *vs.* wild-type, and $P < 0.01$ for $Pomc^{+/-}$ *vs.* wild-type).

POMC haploinsufficiency results in abnormal responses to dynamic tests of the hypothalamic-pituitary-adrenal (HPA) axis

Given the reduction in basal corticosterone seen in $Pomc^{+/-}$ mice, we undertook dynamic testing of the HPA axis in these animals and compared the corticosterone responses with those of wild-type littermates. The CRH stimulation test caused a significantly higher rise in corticosterone in wild-type compared with $Pomc^{+/-}$ mice (795 ± 97 *vs.* 516 ± 64 ng/ml, respectively; $P < 0.05$; Fig. 3A). In addition, the ACTH stimulation test caused corticosterone to rise to

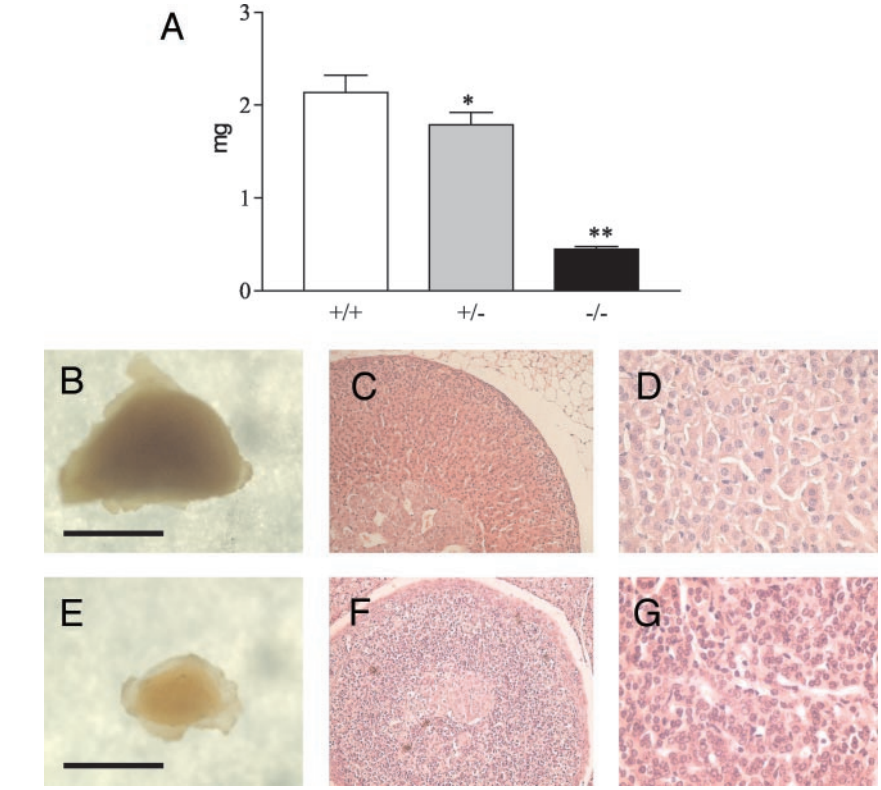

FIG. 1. A, Mean size of adrenal gland in 3-month-old male mice (*, $P < 0.05$; **, $P < 0.01$). Appearance of wild-type (B–D) and mutant (E–G) adrenal gland. Macroscopic (B and E; magnification, ×20; *scale bar*, 1 mm) and microscopic hematoxylin and eosin staining (C and F: magnification, ×200; and D and G: magnification, ×400; *scale bar*, 1 mm).

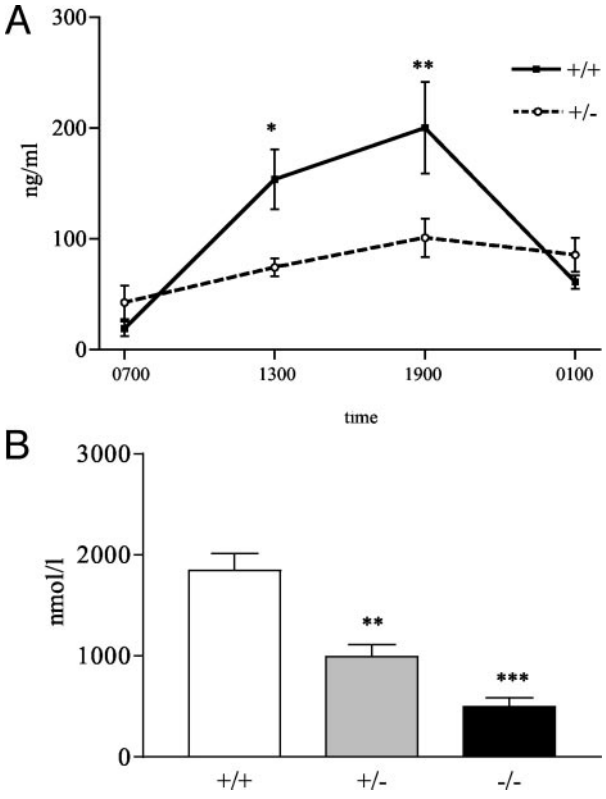

FIG. 2. A, Diurnal variation in wild-type and heterozygous 2-month-old male mice. Each point represents data from six mice. Plasma corticosterone in $Pomc^{-/-}$ was below detectable limit of assay. B, Basal plasma aldosterone in wild-type, heterozygous, and mutant mice (+/+, n = 7; +/−, n = 7; and −/−, n = 6; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

297 ± 55 ng/ml in wild-type mice compared with only 156 ± 21 ng/ml in $Pomc^{+/-}$ mice ($P < 0.05$; Fig. 3B).

Treatment with ACTH restores adrenal size and cortical architecture in $Pomc^{-/-}$ mice

To determine whether ACTH alone is capable of restoring normal size, structure, and function to an adrenal gland deprived of endogenous POMC-related peptides from early life, we administered a depot ACTH analog once daily for 10 d to 3-month-old $Pomc^{-/-}$ mice and compared these mice with wild-type animals given sham injections for 10 d. After 10 d of depot ACTH treatment, the adrenal glands of $Pomc^{-/-}$ mice showed an approximately 5-fold increase in mass, achieving an adrenal weight similar to that of wild-type sham-treated animals (2.3 ± 0.3 *vs.* 1.9 ± 0.3 mg, respectively; P = not significant; Fig. 4A). Administration of 10 d of Depot Synacthen to wild-type mice resulted in a further increase in adrenal size (3.3 ± 0.3 *vs.* 1.9 ± 0.3 mg, ACTH *vs.* sham, $P < 0.05$; Fig. 4A).

Microscopically, the normal cortical architecture of ACTH-treated $Pomc^{-/-}$ mice was restored with clear zonation into glomerulosa and fasciculata (Fig. 4C). To gain some insights into whether the effect of ACTH was predominantly on cell proliferation (hyperplasia) or cell growth (hypertrophy), we measured the number of cells per high-power field (hpf) and used immunohistochemistry to assess qualitative changes in staining for the proliferation marker PCNA. ACTH treatment of both wild-type and $Pomc^{-/-}$ mice resulted in a significant reduction of cells per hpf compared with sham-injected animals (for wild-type mice: 136 ± 8 *vs.* 57 ± 2 cells/hpf, sham *vs.* ACTH; $P < 0.05$: for $Pomc^{-/-}$ mice: 256 ± 32 *vs.* 83 ± 4 cells/hpf, sham *vs.* ACTH; $P < 0.05$; Fig.

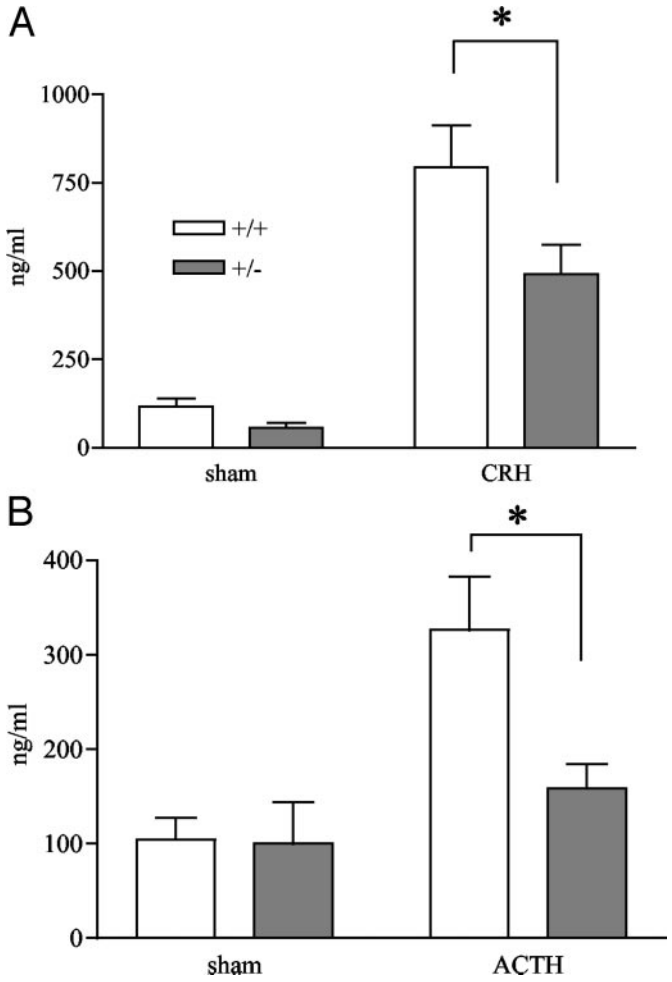

FIG. 3. Corticosterone response after CRH (A) or ACTH (B) in wild-type (*clear bar*) or heterozygous (*dark bar*) 2-month-old male mice. Each *bar* represents data from six mice. *, $P < 0.05$.

5A). Immunohistochemical staining for the proliferation marker PCNA did not change in adrenal sections from both wild-type and mutant animals after ACTH (Fig. 5, B–E). The results of both of these measurements would provide support for the notion that the adrenal growth seen with ACTH is more likely to be a hypertrophic, rather than a hyperplastic, response.

Treatment with ACTH restores corticosterone levels in Pomc$^{-/-}$ mice

In wild-type mice, ACTH treatment resulted in a mean plasma corticosterone level over 6 times higher than that measured in sham-injected animals (526 ± 193 *vs.* 81 ± 26 ng/ml, respectively; $P < 0.05$; Fig. 6). Plasma corticosterone levels in ACTH-treated *Pomc*$^{-/-}$ mice matched sham-treated wild-type mice (100 ± 36 *vs.* 81 ± 26 ng/ml, respectively; P = not significant), whereas corticosterone remained undetectable in sham-injected *Pomc*$^{-/-}$ mice. Interestingly, plasma aldosterone levels did not increase significantly in ACTH-treated mice (data not shown).

Discussion

In contrast to what has been previously reported (3), we find that 129/SvEv mice lacking all endogenous POMC-derived peptides develop macroscopically identifiable adrenal glands. *Pomc*$^{-/-}$ mice have undetectable circulating corticosterone but, again in contrast to previous models of congenital POMC deficiency, have detectable plasma levels of aldosterone. We show that *Pomc* haploinsufficiency impacts significantly on both diurnal corticosterone production and the response of the HPA to dynamic testing. Additionally, the administration of a highly specific ACTH analog for 10 d is capable of producing an adrenal gland that is indistinguishable from a wild-type gland in terms of size, histological morphology, and glucocorticoid production.

The adrenal phenotype in the strain of *Pomc*-null mice

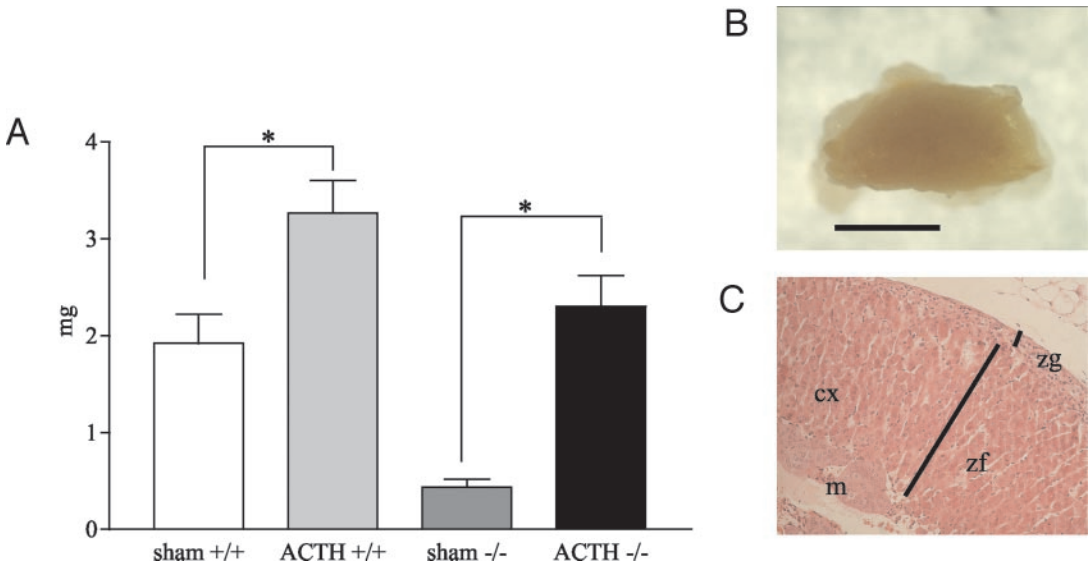

FIG. 4. Adrenal response to ACTH. A, Weight of single adrenal gland after sham or ACTH treatment in wild-type and mutant mice (n = 4 in each group; *, $P < 0.05$). Macroscopic (B; magnification, ×20) and microscopic (C; magnification, ×200) appearance of adrenal gland from *Pomc*$^{-/-}$ mouse after 10 d of depot ACTH. *Scale bar*, 1 mm. cx, Cortex; m, medulla; zf, zona fasciculata; zg, zona glomerulosa.

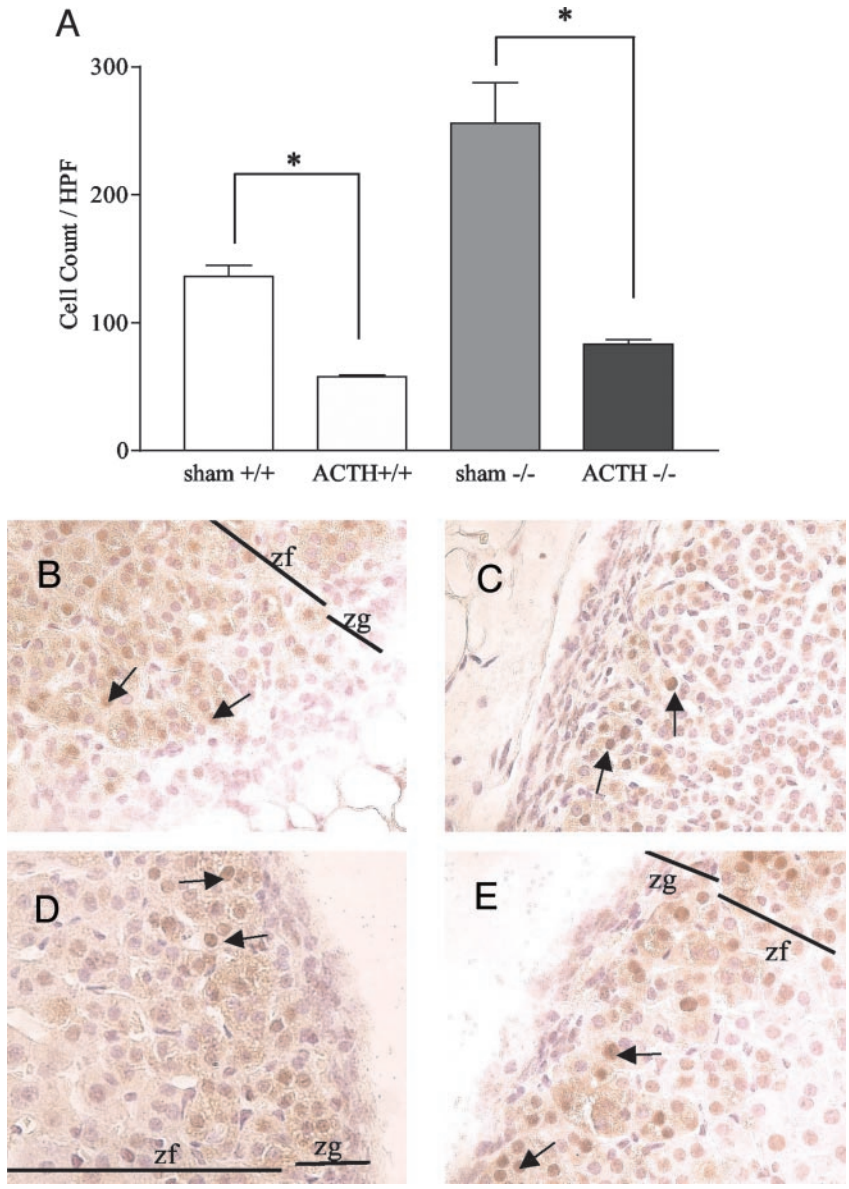

FIG. 5. A, Cell count of hpf within zona fasciculata of adrenals from wild-type and mutant mice after sham and ACTH treatment. *, $P < 0.05$. Immunohistochemistry for proliferative marker PCNA in wild-type (B and D) and knockout (C and E) mice after sham injection (B and C) or ACTH (D and E). *Arrows* indicate positively stained nuclei. zg, Zona glomerulosa; zf, zona fasciculata.

described here differs significantly from previous reports of congenital POMC deficiency in mice (3, 14). All the homozygous mutant mice we examined had visible adrenal glands, which, despite being markedly small and highly dysmorphic, clearly had both cortex and medulla. The mice reported by Yaswen *et al.* (3) had no macroscopically visible adrenal tissue, and the rudimentary gland that was seen microscopically had no clearly discernible cortical and medullary zones. One relatively trivial potential reason for the differences between the two studies is the fact that these mice develop progressive and severe obesity, and the large amount of abdominal fat can make adrenals hard to locate. Our study used younger mice than Yaswen *et al.* (3 *vs.* 6 months, respectively) that would have been less obese, and this may have made localization easier. Indeed, since their first report in 1999, Hochgeschwender *et al.* (15) have subsequently reported the presence of adrenal glands in these mice, with reduced size and altered cellular composition at pre- and early postnatal stages.

The absence of corticosterone in our *Pomc*-null mice is consistent with the findings of Yaswen *et al.* (3) and is not unexpected given the central role of ACTH and the melanocortin 2 receptor in glucocorticoid biosynthesis. However, the presence of detectable circulating aldosterone contrasts with the previous knockout mouse model, which reported undetectable aldosterone, a finding that might be thought to support the concept of a failure of adrenal development in mice congenitally deficient in POMC because it is known that the major regulators of aldosterone production from the mature adrenal are not POMC derived. Humans affected by congenital POMC deficiency are hypocortisolemic but have normal aldosterone levels (5), which is indicative of a normally functioning zona glomerulosa. Further, postmortem studies in such subjects have revealed structurally intact zona glomerulosa and adrenal medulla but an absence of zona fasciculata and reticularis (5). Thus, the presence in our mice of discernible adrenal tissue with a disordered cortex but a clear cortical/medullary demarcation and detectable

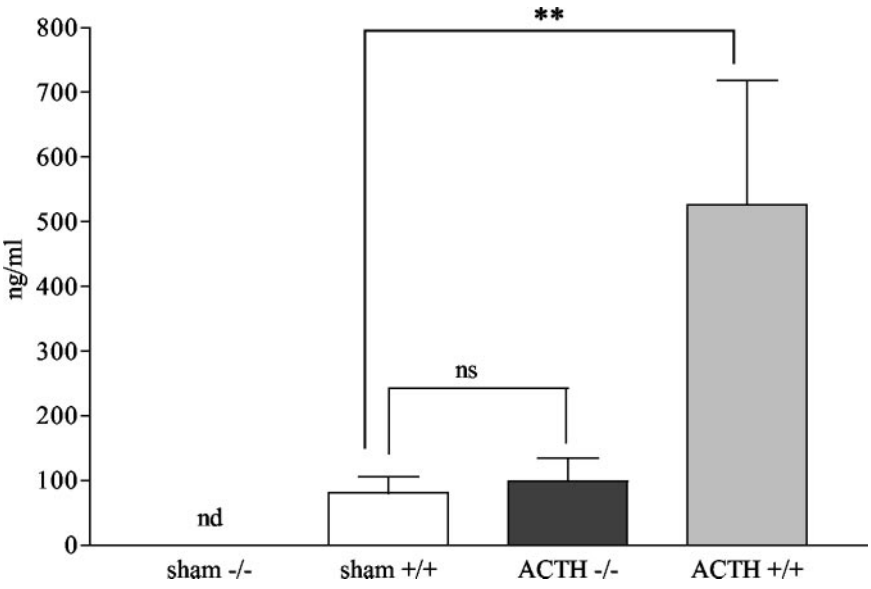

FIG. 6. Plasma corticosterone after ACTH treatment in $Pomc^{-/-}$ mice. Plasma corticosterone in sham-treated $Pomc^{-/-}$ mice was below the detection limit of assay (n = 4 in each group; **, $P < 0.01$). nd, Not detectable; ns, not significant.

mineralocorticoid suggest that the structural and functional sequelae of congenital POMC deficiency seen in our knockout model resemble much more closely those seen in humans. Why the two POMC knockout models differ in terms of aldosterone levels is not entirely clear but may relate to issues such as assay type, timing of samples, and age of mice at time of study.

Of note, we have demonstrated that *Pomc* haploinsufficiency significantly reduces corticosterone production throughout the diurnal cycle, indicating that for fully normal function, the HPA axis requires two full copies of the *Pomc* gene. In addition, we have demonstrated that *Pomc* heterozygosity significantly blunts the response of the HPA axis to both CRH and ACTH. Yaswen *et al.* (3) also reported that heterozygous mice have a reduced corticosterone level, although it is uncertain at what time of day these samples were taken.

An important question that has not previously been answered with confidence is the extent to which there is a developmental period during which the creation of a functionally competent adrenal cortex is dependent on exposure to POMC-derived peptides. A second related question is whether or not ACTH alone is capable of promoting the growth and development of an adrenal gland or whether there might be an additional requirement for other peptides derived from POMC. Smart and Low (14) have recently back-crossed the *Pomc* mutant allele from the 129/SvEv strain used by Yaswen *et al.* onto a C57BL/6 background. These mice had undetectable corticosterone but did have identifiable, severely hypoplastic adrenals, indicative of the effect genetic background can have on phenotype. However, when these mice were treated with 1 μg of $ACTH_{1-24}$ twice daily by ip injection for 2 wk, there was no increase in corticosterone production. This result led these authors to conclude that the development of a functionally competent adrenal cortex might be dependent on exposure to POMC-derived peptides in fetal and or neonatal life. In contrast to Smart and Low, the administration of a highly selective ACTH analog (Depot Synacthen) to our $Pomc^{-/-}$ mice for 10 d resulted in the development of an adrenal gland that was indistinguishable from wild-type gland in terms of size, morphology, and glucocorticoid production. This difference may be because the ACTH in our study was administered at a higher dose and in the form of a sc depot preparation.

Histological and immunohistochemical analysis show this response as being primarily hypertrophy rather than hyperplasia, which is indicative of a role for ACTH in adrenocortical differentiation of cells already present in the *Pomc*-null adrenal. However, at present, it is unknown whether more prolonged treatment could also result in cell proliferation.

Nevertheless, our data show that in mice that lack all endogenous POMC-derived peptides throughout uterine and postnatal life, ACTH alone can transform previously dysmorphic, hypofunctional adrenals into glands capable of synthesizing corticosterone at levels close to those of wild-type mice. The finding that plasma aldosterone levels were detectable but reduced in *Pomc*-null animals but that these levels did not increase after ACTH administration is intriguing and requires further exploration.

Finally, recent studies by Bicknell *et al.* (12) strongly suggest that adrenal growth, at least under certain circumstances, may require not only ACTH but also a peptide or peptides derived from the N terminus of POMC, whose bioactivity is liberated by the actions of an adrenal-specific protease. This phenomenon was demonstrated to be particularly involved in situations such as the compensatory growth of the remaining adrenal in response to unilateral adrenalectomy. Although our studies were not designed to test the hypothesis that compensatory adrenal growth might require N-terminal POMC-derived peptides, it is notable that we were able to restore structure and function to the adrenal gland in the absence of any POMC-derived peptide other than a highly specific ACTH analog. The POMC-deficient model will be very useful in the future to more directly test hypotheses regarding the requirement for other peptide elements of the *Pomc* gene in adrenal structure, growth, and function.

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