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Central Dysregulation of the Hypothalamic-Pituitary-Adrenal Axis in Neuron-Specific Proopiomelanocortin-Deficient Mice

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Proopiomelanocortin (POMC) is synthesized predominantly in pituitary corticotrophs, melanotrophs, and arcuate hypothalamic neurons. Corticotroph-derived ACTH mediates basal and stress-induced glucocorticoid secretion, but it is uncertain whether POMC peptides produced in the brain also regulate the hypothalamic-pituitary-adrenal axis. To address this question, we generated neuron-specific POMC-deficient mice by transgenic (*Tg*) replacement of pituitary POMC in a global *Pomc*$^{-/-}$ background. Selective restoration of pituitary POMC prevented the adrenal insufficiency and neonatal mortality characteristic of *Pomc*$^{-/-}$ mice. However, adult *Pomc*$^{-/-}$*Tg*/+ mice expressing the pituitary-specific transgene exhibited adrenal cortical hypertrophy, elevated basal plasma corticosterone, elevated basal but attenuated stress-induced ACTH secretion, and inappropriately elevated CRH expression in the hypothalamic paraventricular nucleus. In addition, *Pomc*$^{-/-}$*Tg*/+, *Pomc*$^{+/-}$*Tg*/+, and *Pomc*$^{+/-}$ mice, which all displayed varying degrees of elevated CRH, frequently developed melanotroph adenomas after 1 yr of age, whereas *Pomc*$^{-/-}$ mice, with maximal CRH expression and glucocorticoid disinhibition, developed corticotroph and melanotroph adenomas. These results indicate that neuronal POMC peptides are necessary to regulate CRH within physiological limits and that a chronic reduction or absence of hypothalamic POMC leads to trophic stimulation of pituitary cells directly or indirectly through elevated CRH levels.

THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) axis mediates the core neuroendocrine response to stress (1). Within the hypothalamus, the paraventricular nucleus (PVH) is the nodal point for integration of environmental and interoceptive triggers of stress. A subpopulation of parvicellular, hypophyseotropic PVH neurons releases CRH from terminals in the median eminence whereby CRH reaches its target pituitary cells via a portal venous plexus. CRH activates CRH-R1 receptors located on both anterior lobe corticotrophs and intermediate lobe melanotrophs to induce proopiomelanocortin (*Pomc*) gene expression and hormone secretion (2–4). ACTH, derived from the posttranslational processing of proopiomelanocortin (POMC) in pituitary corticotrophs, is the principal mediator of basal and stress-induced glucocorticoid secretion from the adrenal cortex (5). Circulating glucocorticoids provide a negative feedback signal to the limbic-HPA axis at multiple levels in the brain and also directly on corticotrophs, but not melanotrophs, due to the differential cell-specific expression of glucocorticoid receptors (3, 4, 6).

In addition to its expression in the pituitary, the *Pomc* gene is highly expressed in a subpopulation of neurons in the arcuate nucleus of the hypothalamus (ArcN) that extend axonal projections to multiple subcortical nuclei (7, 8). POMC processing in ArcN and in a second smaller population of POMC neurons located in the nucleus tractus solitarius yields a family of melanocortin peptides together with the potent opioid β-endorphin (9). POMC peptides function physiologically within the central nervous system to modulate appetite, reward, and autonomic outflow (10–13). Neuronal targets implicated in the anorectic and catabolic effects of melanocortins include PVH CRH neurons, which are densely innervated by ArcN POMC terminals (14). A large percentage of these neurons express melanocortin-4 receptors and respond directly to melanocortin stimulation accompanied by increased plasma ACTH, corticosterone, and decreased food intake (14–17). Opioid peptides including β-endorphin can directly modulate the function of CRH neurons in the PVH with predominantly inhibitory actions by μ opioid receptor activation and facilitatory actions by κ opioid receptor activation and thereby alter HPA activity (18, 19). Furthermore, published data conflict with each other concerning whether glucocorticoids have long-loop feedback effects on the activity of POMC neurons in ArcN and expression of the *Pomc* gene analogous to their clearly established inhibitory control of pituitary corticotrophs (20, 21).

There is growing evidence for complex reciprocal interactions between nutritional state and HPA axis activity and among the humoral and neural factors that regulate both homeostatic systems (22, 23). POMC expression, processing, and peptide signaling may have coevolved in pituitary cells and the ontogenetically related medial-basal hypothalamic neurons (24) to coordinate energy balance and stress respon-

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Abbreviations: ArcN, Arcuate nucleus; EGFP, enhanced green fluorescent protein; HPA, hypothalamic-pituitary-adrenal; POMC, proopiomelanocortin; PRL, prolactin; PVH, paraventricular nucleus hypothalamus.

siveness. A total absence of POMC peptides secondary to null *Pomc* gene mutations produces a syndrome of adrenal insufficiency and early-onset obesity in both humans and mice (25–27). However, because of the dual nature of POMC expression in pituitary and brain, it has not been possible to fully distinguish the contribution of POMC peptides from each site to the varied components of the syndrome occurring in $Pomc^{-/-}$ mice.

Therefore, to differentiate among the unique functions and potential redundancies of POMC produced in the pituitary and brain, we have restored POMC expression specifically to the pituitary of $Pomc^{-/-}$ mice using a compound transgenic strategy. Here, we characterize the function of the HPA axis and report the unanticipated consequences of sustained HPA overactivity and pituitary adenoma induction secondary to central CRH drive in neuronal-specific POMC-deficient mice.

Materials and Methods

Care and use of animals

All mice for these studies were maintained under controlled temperature and photoperiod (14 h light, 10 h dark; lights on at 0500 h) with food and water provided *ad libitum*. All experimental procedures were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and followed established guidelines of the Public Health Service.

Generation and breeding of mice

*pHalEx2** transgenic mice were generated by microinjection of a genomic *Tg* construct that selectively expresses POMC in pituitary cells, but not neurons, with normal hormonal regulation and posttranslational processing (28–30). These transgenic mice were crossbred to the $Pomc^{-/-}$ strain on a mixed hybrid B6;D2;129X1;129S6 genetic background as described fully in a previous report (28). The resulting compound mutant mice segregated into six possible distinct genotypes: $Pomc^{+/+}$, $Pomc^{+/-}$, or $Pomc^{-/-}$ at the endogenous *Pomc* locus in combination with either the hemizygous transgene (*Tg*/+) or wild-type chromosomal integration site (+/+). For one experiment, mice were further bred to homozygosity for the *Tg* allele to produce a cohort consisting of $Pomc^{+/+}Tg/Tg$ and $Pomc^{-/-}Tg/Tg$ genotypes. Additionally, small numbers of a second compound mutant strain were produced by crossing $Pomc^{+/-}$ breeders to an existing inbred B6 transgenic strain that expresses enhanced green fluorescent protein [enhanced green fluorescent protein (EGFP)] (Clontech Laboratories, Inc., Mountain View, CA) under the control of pituitary and neuronal *Pomc* promoter/enhancer elements (31). A third strain of mutant mice, official designation B6;129S2-$Pomc1^{tm1Low}$/J (Induced Mutant Resource, The Jackson Laboratories), that expresses a truncated POMC prohormone resulting in a phenotype of selective β-endorphin deficiency was produced in our laboratory (32) and maintained on a congenic B6 genetic background as described previously (12).

Genotyping

Genotyping of compound *Pomc* mutant mice was performed as described in detail previously (28). An additional PCR detected the *Pomc-EGFP* transgene 220 bp product using primer pair 5′-TATATCATGGCCGACAAGCA-3′ and 5′-GAACTCCAGCAGGACCATGT-3′.

Blood and tissue collection

Mice were singly housed for at least 2 d before blood sampling to obtain basal, nonstressed hormone levels. Cages were transported individually to an adjacent room between 0700 and 1000 and the mice were decapitated without anesthesia within 30 sec of cage handling. Trunk blood was collected into tubes containing EDTA and plasma was immediately separated by centrifugation and frozen. Brains were rapidly removed, frozen in isopentane, and stored at −80 C for later sectioning. Pituitaries were weighed and stored in buffered 10% formalin or submersed in a cryomold containing optimal cutting temperature compound and frozen on dry ice. Adrenal glands were dissected free of surrounding fat, weighed as a pair, and stored in 10% formalin. For acute stress studies, mice were individually constrained for 20 min inside cylindrical tubes and then immediately decapitated for trunk blood. Additional mice were anesthetized with 2% Avertin, perfused via the left ventricle with 4% buffered paraformaldehyde, and their brains removed and postfixed for 16 h.

Histology and immunohistochemistry

Histology was performed on 5-μm paraffin sections of adrenal or pituitary glands stained with hematoxylin and eosin or by the Gordon-Sweet silver method for reticulin fibers. Pituitary hormone immunohistochemistry was performed on either 20-μm frozen sections or 5-μm paraffin sections. Antigen retrieval was performed on the latter sections after deparaffinization and rehydration by treatment of slides with boiling 10 mM trisodium citrate buffer, pH 6.0, for 30 min. After three rinses in 10 mM 140 mM NaCl, 20 mM K_2HPO_4, pH 7.4 (KPBS), pituitary sections were incubated with 2% normal goat serum and 0.3% Triton X-100 in KPBS containing one of the following primary antisera (A. Parlow, National Hormone and Peptide Program) at the indicated dilutions: antihuman GH (1:2000), antirat prolactin (PRL) (1:25), antirat FSH (1:6000), antirat TSH (1:500), and antirat ACTH (1:5000) at 4 C overnight. Sections were then rinsed three times in KPBS and sequentially incubated with 20 μg/ml biotinylated goat-antirabbit IgG (Vector Laboratories, Burlingame, CA) for 2 h at 25 C followed by incubation with an avidin-biotin horseradish peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories) for 1 h at 25 C. Reaction product was revealed with 1 mg/ml diaminobenzidine containing 0.03% H_2O_2 yielding a brown precipitate. CRH immunohistochemistry was performed on free-floating 50-μm vibratome brain sections fixed in 4% paraformaldehyde with a rabbit anti-CRH antiserum (1:4000; Peninsula Laboratories, San Carlos, CA) in KPBS buffer, 2% normal goat serum, and 0.3% Triton X-100 at 4 C for 48 h followed by the steps outlined previously.

In situ hybridization

Initial screening for *pHalEx2** transgene expression used a radiolabeled 23-bp antisense oligonucleotide corresponding to the exon 2 insertion and was performed as described previously (30). Definitive *in situ* hybridization studies used single-stranded 700 nucleotide [^{35}S]-radiolabeled cRNA riboprobes generated by T3/T7 polymerases from a mouse *Pomc* template containing exon 3 sequences subcloned into pGEM-7 (Promega, Madison, WI). The antisense probe hybridized equally to endogenous and *pHalEx2** transgenic *Pomc* mRNA. The *Pomc* sense probe was used as a control and produced no specific signal on pituitary or brain sections. *Crh* sense and antisense [^{35}S]-radiolabeled cRNA probes were generated from a 578-bp *PstI-PstI* fragment contained in exon 2 of the mouse *Crh* gene (a gift from A. Seasholtz, University of Michigan) subcloned into pBluescript II (Stratagene, La Jolla, CA). Probes were separated from unincorporated nucleotides over NICK columns (Sephadex G-50 DNA Grade; Amersham Pharmacia Biotech, Piscataway, NJ) and applied to slides containing serial 16-μm coronal sections at 55–60 C for 24 h in hybridization buffer at a concentration of 5×10^6 cpm/ml as described previously (33). After hybridization, slides were washed four times with 150 mM NaCl, 15 mM Na citrate, pH 7.0 (SSC), digested with RNAseA (30 min), rinsed again in SSC containing DTT, dehydrated through a series of ethanol concentrations, vacuum dried, and analyzed by autoradiography (Biomax MR; Kodak, Rochester, NY) followed by phosphoimaging (Molecular Dynamics, Eugene, OR). Digital images were analyzed using IP-Lab Gel and NIH Image. Hybridization signal was quantified in selected regions of interest with constant area size from each section. Background signal was quantified from sections not containing any visible specific hybridization signal and then subtracted from total signal in each section. *Pomc* mRNA signal in the ArcN and pituitary was averaged from the three sections of each tissue with the highest signal per mouse (n = 3–4 mice per genotype). *Crh* mRNA was analyzed similarly by averaging the three most robustly labeled PVH sections per mouse (n = 3–6 mice per genotype).

Hormone assays

Plasma hormones were measured using a two-site ACTH immunoradiometric assay (Nichols Diagnostic Institute, San Juan Capistrano, CA) and a double antibody corticosterone RIA (MP Biomedicals, Solon, OH) according to the manufacturers' instructions. Maximal sensitivity was achieved by adjusting the plasma dilutions.

Statistics

All data presented are the mean ± SE of mean unless stated otherwise. Data were analyzed by multifactor analyses of variance appropriate for the design of each experiment with genotype and sex as independent variables using StatView version 5.0.1 for Macintosh Power PC (SAS Institute Inc., Cary, NC). One-factor analyses of variance were used to follow-up significant main effects and *post hoc* pairwise comparisons between groups were performed by the Tukey/Kramer test with a criterion for significant differences set at $P < 0.05$ unless stated otherwise.

Results

Transgenic rescue of pituitary POMC in Pomc$^{-/-}$ mice

In situ hybridization of tissue sections with a riboprobe that hybridized to exon 3 sequences common to both the wild-type *Pomc*$^{+}$ and *pHalEx2* Tg* alleles, but absent from the mutant *Pomc*$^{-}$ allele, confirmed that the transgene selectively rescued POMC expression in intermediate and anterior lobes of the pituitary but not the ArcN (Fig. 1). The spatial distribution of *Pomc* mRNA transcribed from the *pHalEx2* Tg* allele in the anterior lobes of *Pomc*$^{-/-}$*Tg*/+ mice matched that of *Pomc*$^{+/+}$ mice and the expression patterns of previously reported transgene constructions that contained analogous *Pomc* promoter regions (29, 30, 34). Quantification of total pituitary *Pomc* mRNA hybridization signals revealed no significant difference between *Pomc*$^{-/-}$*Tg*/+ and *Pomc*$^{+/+}$ mice (Table 1). Furthermore, there was no significant reduction or increase in *Pomc* mRNA in *Pomc*$^{+/-}$ *vs. Pomc*$^{+/+}$ or *Tg*/+ *vs.* +/+ mice, respectively. Similarly, quantification of *Pomc* mRNA in the ArcN revealed no differences among *Pomc*$^{+/+}$, *Pomc*$^{+/+}$*Tg*/+, *Pomc*$^{+/-}$, and *Pomc*$^{+/-}$*Tg*/+ mice (Table 1). Immunohistochemistry and RIAs confirmed the restored production of β-endorphin, melanocyte-stimulating hormone, and ACTH in the pituitary but not hypothalamus of *Pomc*$^{-/-}$*Tg*/+ mice at physiological levels (28).

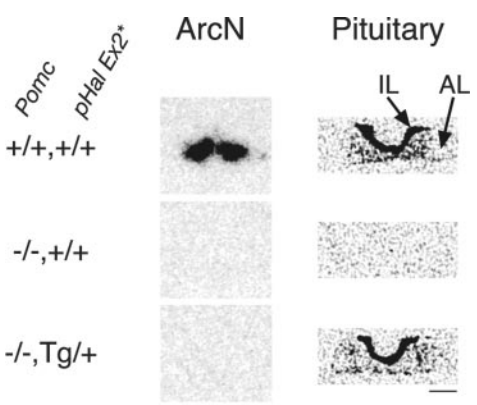

FIG. 1. Transgenic rescue of pituitary POMC expression in *Pomc*$^{-/-}$*Tg*/+ mice. *Pomc* mRNA *in situ* hybridization was performed on coronal sections of ArcN and pituitary from 6-month-old male mice of the indicated compound genotypes and representative autoradiograms are shown. IL, Intermediate lobe; AL, anterior lobe. *Scale bars*, 200 μm for ArcN and 300 μm for pituitary sections.

Perinatal lethality and adrenal insufficiency characteristic of Pomc$^{-/-}$ mice were reversed in Pomc$^{-/-}$Tg/+ mice

Breeder pairs of compound *Pomc*$^{+/-}$*Tg*/+ mice produced the expected Mendelian ratio of genotypes in their offspring with the exception of *Pomc*$^{-/-}$ mice. Compared with the ~60% frequency of perinatal deaths in *Pomc*$^{-/-}$ pups, consistent with previous reports (27, 35), there was no excess mortality observed in *Pomc*$^{-/-}$*Tg*/+ mice. *Pomc*$^{-/-}$ pups that did not die on postnatal d 1 were indistinguishable by visual observation from their littermates, regardless of genotype, until after weaning and they then survived into adulthood without any specific treatment.

Adult *Pomc*$^{-/-}$ mice of both sexes had small but easily identifiable adrenal glands by gross inspection of their suprarenal fat (Fig. 2A). Microscopic sections revealed a clearly distinguishable adrenal medulla and cortex, but the cortical zona fasciculata was decreased in height and the characteristic columnar cell arrangement was replaced by disorganized, densely packed cells with scant cytoplasm compared with *Pomc*$^{+/+}$ siblings (Fig. 2B). Pituitary POMC replacement in *Pomc*$^{-/-}$*Tg*/+ mice resulted in enlarged adrenal glands primarily due to hypertrophy of the zona fasciculata as indicated by an increased volume of eosinophilic cytoplasm per cell and decreased numbers of cells per high-power field (Fig. 2B). A marked sexual dimorphism was apparent in the *Pomc*$^{+/+}$, *Pomc*$^{+/+}$*Tg*/+, *Pomc*$^{+/-}$, and *Pomc*$^{+/-}$*Tg*/+ genotypes characterized by nearly a 2-fold greater weight of the female adrenals ($F_{1,145} = 170$, $P < 0.0001$, main effect of sex) (Fig. 2C). The atrophic adrenals of male and female *Pomc*$^{-/-}$ mice had similarly low weights, and although pituitary POMC restoration resulted in adrenal gland enlargement in both sexes, the percentage increase compared with their respective *Pomc*$^{+/+}$ controls was substantially greater in males (~100%) than females (~25%).

Plasma corticosterone was measured previously in unstressed 10- to 15-wk-old *Pomc*$^{+/+}$*Tg*/+ and *Pomc*$^{-/-}$*Tg*/+ mice at intervals of 3 h from 0800 to 2000 h (28). *Pomc*$^{+/+}$*Tg*/+ mice exhibited the expected diurnal rhythm of corticosterone secretion with a nadir early in the light phase and a peak before the onset of darkness. Female *Pomc*$^{+/+}$*Tg*/+ mice had higher peak corticosterone values than males, paralleling their sexually dimorphic adrenal size. Although female *Pomc*$^{-/-}$*Tg*/+ mice retained a normal diurnal pattern of corticosterone secretion with normal basal and only modestly increased peak values, male *Pomc*$^{-/-}$*Tg*/+ mice exhibited a strikingly altered pattern of corticosterone secretion with exaggerated nadir and peak levels. In the current study, basal morning corticosterone and ACTH levels were measured in 9- to 11-month-old mice of all six genotypes (Table 1). Body weights of these mice were very similar to our previous report (28). *Pomc*$^{-/-}$ mice had no detectable level of either hormone. Excluding this genotype from the analysis of variance analyses, there was a significant main effect of genotype in male mice for both basal corticosterone ($F_{4,23} = 9.0$, $P < 0.0001$) and ACTH ($F_{4,23} = 5.7$, $P < 0.005$). *Pomc*$^{-/-}$*Tg*/+ mice had significantly increased basal levels of corticosterone

TABLE 1. POMC mRNA levels in the pituitary and ArcN, basal hormone levels, and body weight in unstressed mice

Genotype	POMC mRNA (% of $Pomc^{+/+}$)		Basal hormone levels (EDTA plasma)				Body weight (g)	
			Corticosterone (ng/ml)		ACTH (pg/ml)			
	Pituitary male	ArcN male	Male	Female	Male	Female	Male	Female
$Pomc^{+/+}$	100 ± 12	100 ± 3	20 ± 2	43 ± 21	17 ± 7	23 ± 4	42 ± 2	34 ± 1
$Pomc^{+/+}Tg/+$	63 ± 9	128 ± 45	12 ± 2	66 ± 32	8 ± 6	23 ± 9	43 ± 3	36 ± 2
$Pomc^{+/-}$	74 ± 11	85 ± 15	12 ± 4	22 ± 11	8 ± 4	14 ± 7	47 ± 1	39 ± 2
$Pomc^{+/-}Tg/+$	108 ± 17	91 ± 9	16 ± 7	8 ± 2	18 ± 10	22 ± 7	48 ± 2	45 ± 3
$Pomc^{-/-}$	<1	<2	ND	ND	ND	ND	58 ± 1	54 ± 5
$Pomc^{-/-}Tg/+$	76 ± 6	<2	84 ± 21[a]	36 ± 7	50 ± 9[b]	37 ± 11	74 ± 4[a]	71 ± 5[a]
$Pomc^{+/+}Tg/Tg$			6 ± 2	16 ± 3			40 ± 2	30 ± 2
$Pomc^{-/-}Tg/Tg$			37 ± 11[c]	49 ± 11[c]			73 ± 2[d]	74 ± 2[d]

Data in the table are the mean ± standard error of mean from mice of the indicated genotypes and sex. Mice in the first cohort consisted of six different genotypes and were age 9–11 months (n = 3–4 mice per group for mRNA; n = 4–8 mice per group for hormones and body weight, except n = 3 for male $Pomc^{-/-}$ mice). Mice in the second cohort were homozygous for the *pHalEx2** transgene (*Tg/Tg*) and age 6 months (n = 8–12 mice per group). POMC mRNA was quantified by *in situ* hybridization using a Molecular Dynamics phosphoimager and NIH Image as described in *Materials and Methods* and normalized to mean values for the $Pomc^{+/+}$ genotype set to 100%. ND, Not detectable at maximal sensitivity for the assays.

[a] $P < 0.05$ *vs.* all other genotypes (Tukey/Kramer pairwise *post hoc* comparisons).

[b] $P < 0.05$ *vs.* all other genotypes except $Pomc^{+/+}$ (Tukey/Kramer pairwise *post hoc* comparisons).

[c] $P < 0.05$ *vs.* $Pomc^{+/+}Tg/Tg$ (Student's *t* test).

[d] $P < 0.0001$ *vs.* $Pomc^{+/+}Tg/Tg$ (Student's *t* test).

compared with all other genotypes. The corresponding increase in ACTH in male $Pomc^{-/-}Tg/+$ mice was also significantly different from all other genotypes except $Pomc^{+/+}$ mice by the Tukey/Kramer post hoc test; however, a planned pairwise comparison with Fisher's projected least significant difference test gave a $P < 0.01$. Increased basal corticosterone was further confirmed in neural-specific POMC-deficient males and also detected in mutant females by analysis of an independent cohort of 6-month-old mice consisting of lean $Pomc^{+/+}Tg/Tg$ and obese $Pomc^{-/-}Tg/Tg$ genotypes (Table 1). Notably, homozygosity of the *Tg* allele on the wild-type $Pomc^{+/+}$ background had no effect on basal corticosterone levels, body weight, or adrenal weight (adrenal data not shown).

Altered stress activation of the HPA axis and hypothalamic CRH expression in $Pomc^{-/-}Tg/+$ mice

Mice of all genotypes except $Pomc^{-/-}$ responded briskly to an acute 20-min restraint stress with elevated plasma ACTH (Fig. 3A) and corticosterone (Fig. 3B) relative to their respective nonstressed levels (Table 1 and Fig. 3, *insets*). However, there was a highly significant main effect of genotype for stress-induced ACTH by a two-factor analysis of variance excluding the $Pomc^{-/-}$ group ($F_{4,59} = 12.6$, $P < 0.0001$). There was also a significant effect of sex ($F_{1,59} = 6.2$, $P < 0.05$) due to higher ACTH levels in males but no interaction between the two factors ($F_{4,59} = 2.0$, $P = 0.11$). The magnitude of the ACTH stress response was significantly blunted by 50–75% in both male and female $Pomc^{-/-}Tg/+$ mice compared with $Pomc^{+/+}$ mice or the other responding groups (Fig. 3A).

Corresponding analyses of the corticosterone response to restraint stress in the same mice (Fig. 3B) revealed a highly significant main effect of sex ($F_{1,59} = 66.9$, $P < 0.0001$) due to higher plasma corticosterone levels in females. There was also a main effect of genotype ($F_{4,59} = 2.9$, $P < 0.05$) but no interaction between the two factors ($F_{4,59} = 1.6$, $P = 0.19$). Other than the absence of detectable corticosterone after stress in the $Pomc^{-/-}$ mice, *post hoc* pairwise comparisons among the other groups did not reach statistical significance for either sex. In a separate experiment, pair-feeding of $Pomc^{-/-}$Tg/+ mice to the daily food intake of $Pomc^{+/+}$ mice also resulted in amplified corticosterone responses in $Pomc^{-/-}$Tg/+ females compared with males (data not shown), indicating that the sexual dimorphism in the response to psychogenic or metabolic stressors was maintained in the neural selective POMC-deficient mice.

Steady-state CRH mRNA levels in the PVH determined by quantitative *in situ* hybridization were more than 6-fold higher in $Pomc^{-/-}$ mice, which lack all negative glucocorticoid feedback to the central nervous system than $Pomc^{+/+}$ mice ($F_{5,13} = 35.5$, $P < 0.0001$; main effect of genotype) (Fig. 4, A and C). Although restoration of pituitary POMC expression in $Pomc^{-/-}$Tg/+ mice significantly reduced CRH mRNA levels relative to $Pomc^{-/-}$ mice, these levels still remained 2-fold higher than in the PVH of $Pomc^{+/+}$ mice and inappropriately elevated relative to the increased circulating corticosterone (Fig. 4C). $Pomc^{+/-}$ mice had hypothalamic CRH mRNA levels that were increased more modestly, but still significantly, by ~50% over that of $Pomc^{+/+}$ mice, suggesting a gene dosage effect for neuronal POMC on CRH levels ($F_{1,11} = 10.8$, $P < 0.01$, data collapsed across *pHalEx2** *Tg* genotype because there was no main effect of *Tg* nor a significant interaction of *Tg* with *Pomc* genotype in an initial two-factor analysis of variance limited to the four genotypes, excluding the $Pomc^{-/-}$ and $Pomc^{-/-}$Tg/+ groups). In contrast to the expression pattern in the PVH, CRH mRNA was suppressed in the central nucleus of the amygdala (CeA) of $Pomc^{-/-}$ mice and restored to normal levels in the $Pomc^{-/-}Tg/+$ mice (Fig. 4D). Similar levels of expression were observed in the CeA of all other groups.

Immunohistochemical analysis of CRH expression in the PVH paralleled the results of CRH mRNA *in situ* hybridization (Fig. 4B). As expected, without colchicine pretreatment to inhibit axonal transport of peptides, there was almost no

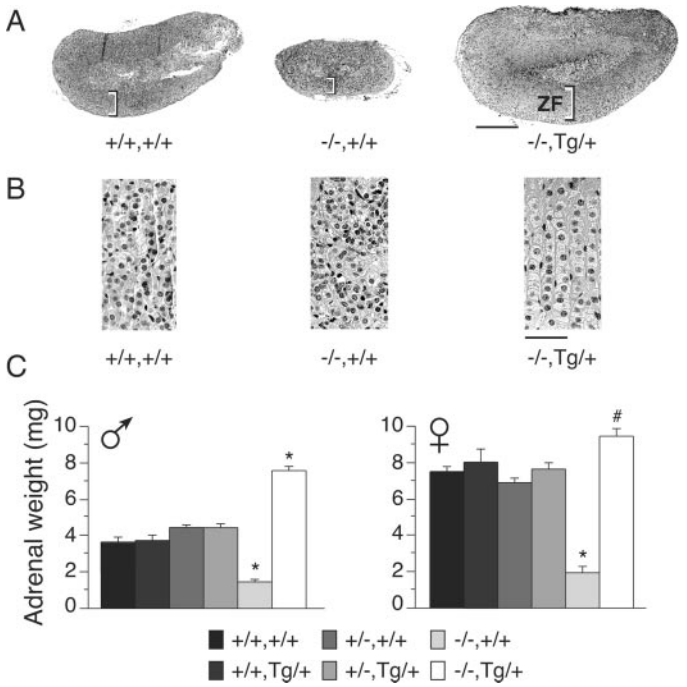

FIG. 2. Adrenal gland morphology, adrenal cortical organization, and adrenal weights in mutant mice. A, Five-micrometer sections of 6-month-old male adrenals stained with hematoxylin and eosin showing relative sizes of hemisected glands. ZF, Zona fasciculata. *Scale bar*, 400 μm. B, Higher magnification of the adrenal cortex showing the histology of the zona fasciculata. *Scale bar*, 100 μm. C, Wet weights of paired adrenal glands obtained from male and female mice of all six genotypes, age 10 $\pm$ 0.2 months (range, 4–17 months) and excluding mice with pituitary tumors. Genotypes were equivalently distributed across the age range and there was not a significant correlation of adrenal weight with age ($r^2 = 0.014$; $F_{1,155} = 2.2$, $P = 0.14$). There were significant main effects of genotype for males ($F_{5,86} = 53.8$, $P < 0.0001$) and females ($F_{5,60} = 69.1$, $P < 0.0001$). *, $P < 0.05$ compared with all other groups; and #, $P < 0.05$ compared with $Pomc^{+/+}$and $Pomc^{+/-}$ groups (Tukey/Kramer pairwise *post hoc* analyses).

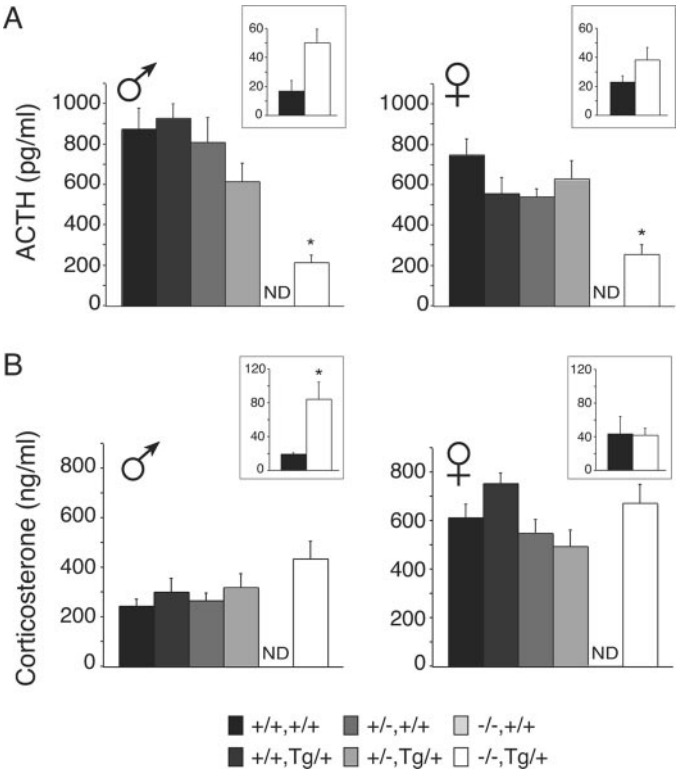

FIG. 3. Activation of the HPA axis after 20 min of acute restraint stress. Plasma ACTH (A) and corticosterone (B) levels after stress compared with levels in basal conditions (*insets*). Stress data were obtained from male and female mice of all six genotypes, age 11 $\pm$ 0.5 months (range, 4–20 months) and excluding mice with pituitary tumors. Genotypes were equivalently distributed across the age range and there was not a significant correlation of ACTH levels with age ($r^2 = 0.049$; $F_{1,66} = 3.4$, $P = 0.07$). Neither hormone was detectable in $Pomc^{-/-}$ mice (ND) and therefore these groups were not included in the analyses of variance. Despite a significant blunting of ACTH responses in both male and female $Pomc^{-/-}Tg/+$ mice (*, $P < 0.05$ compared with all other genotypes of the same sex, Tukey/Kramer *post hoc* pairwise comparisons), there was not a corresponding attenuation in corticosterone levels.

immunoreactive CRH detected in cell bodies of $Pomc^{+/+}$ mice, but positive neuronal processes were present. In contrast, CRH-immunoreactive soma and processes were abundant and strongly labeled in the PVH of $Pomc^{-/-}$ mice. Although not quantified, there also appeared to be moderately more cell body staining of CRH neurons in both $Pomc^{+/-}$ and $Pomc^{-/-}Tg/+$ compared with $Pomc^{+/+}$ mice in parallel with the levels of CRH mRNA expression.

We next tested whether a selective absence of β-endorphin in mice expressing normal levels of melanocortin peptides would recapitulate the elevated levels of CRH expression observed in the PVH of $Pomc^{-/-}Tg/+$ mice that lack all central POMC peptides. Quantitative mRNA *in situ* hybridization for CRH was performed in C57BL/6 congenic β-endorphin$^{+/+}$ and β-endorphin$^{-/-}$ male mice, which have previously been demonstrated to have normal adrenal gland size, plasma ACTH, and corticosterone levels (32). Steady-state levels of CRH mRNA were identical between these two groups of mice (Fig. 4E).

$Pomc^{+/-}$ and $Pomc^{-/-}$ mice developed pituitary adenomas

Male and female mice, either heterozygous or homozygous for the *Pomc* null allele, exhibited a high incidence of pituitary glands weighing more than 2 SD above the mean of $Pomc^{+/+}$ mice after age 12 months (Fig. 5). Expression of the *pHalEx2*Tg* did not appear to alter either the latency to pituitary tumorigenesis or the distribution of tumor weights in compound mutant $Pomc^{+/-}Tg/+$ or $Pomc^{-/-}Tg/+$ mice. In most cases, visual examination of tumors that weighed <10 mg with a stereodissecting microscope revealed enlargement of the intermediate lobe. In addition to or in place of intermediate lobe tumors, pituitaries from $Pomc^{-/-}$ mice frequently contained one or more translucent anterior lobe nodules suggesting the presence of microscopic anterior lobe tumors even when the pituitary weights were still within normal limits.

Histological analyses were performed to further characterize the pituitary tumors (Fig. 6). Immunostaining revealed the presence of normal corticotrophs, thyrotrophs, lactotrophs, and somatotrophs in the anterior lobe and melanotrophs in the intermediate lobe of $Pomc^{+/+}$ mice (Fig. 6A, *top row*) and $Pomc^{+/+}Tg/+$ mice (data not shown). The pituitary of a 12-month-old $Pomc^{-/-}$ mouse had no immunopositive staining for ACTH but contained immunonegative anterior lobe nodules surrounded by cells staining for TSH,

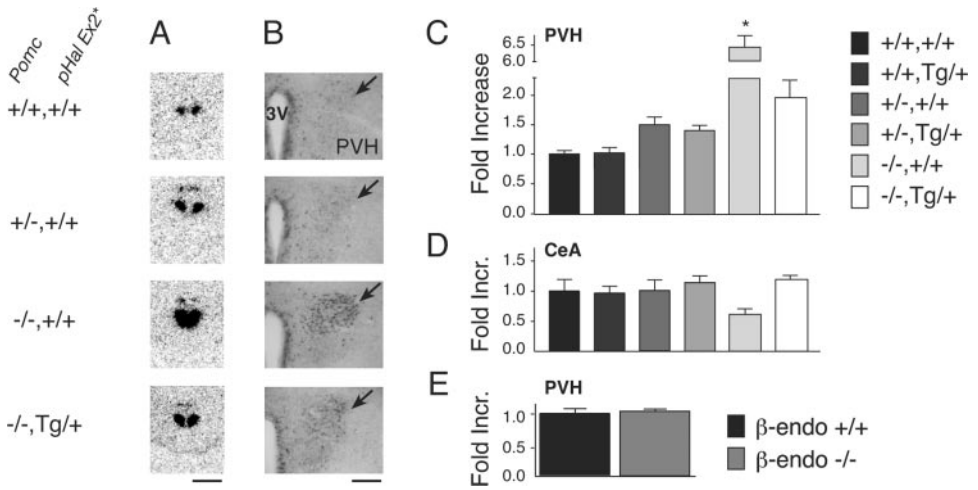

FIG. 4. CRH expression in the hypothalamus and amygdala of male mice. CRH mRNA was analyzed by *in situ* hybridization (A) and CRH peptide by immunocytochemistry (B) on coronal brain sections of 9-month-old *Pomc* mutant mice. PVH, Paraventricular nucleus of the hypothalamus; 3V, third ventricle. *Scale bars*, 1 mm (A) and 200 μm (B). C, CRH mRNA levels in PVH were quantified using a phosphoimager and the data expressed relative to a normalized value of 1.0 for $Pomc^{+/+}$ controls. (*, $P < 0.05$ compared with all other genotypes, Tukey/Kramer *post hoc* pairwise comparisons.) D, CRH mRNA levels in the central nucleus of the amygdala (CeA) were quantified from the same sections as those containing the PVH and the data expressed relative to a normalized value of 1.0 for $Pomc^{+/+}$ controls ($F_{5,13} = 2.5$, $P = 0.09$). E, CRH mRNA levels in PVH were quantified from congenic C57BL/6 male β-endorphin$^{+/+}$ and β-endorphin$^{-/-}$ mice and the data expressed relative to a normalized value of 1.0 for β-endorphin$^{+/+}$ controls ($F_{1,9} = 0.11$, $P = 0.75$).

PRL, and GH (Fig. 6A, *middle row*) and FSH (not shown). A similar pattern of immunostaining was observed in another 10-month-old $Pomc^{-/-}$ mouse whose pituitary gland was still within the normal weight range (data not shown). In contrast, ACTH immunostaining of pituitary glands from $Pomc^{+/-}$ mice often revealed tumors that clearly originated in the intermediate lobe such as the example shown (Fig. 6A, *bottom row*). The anterior lobe of this $Pomc^{+/-}$ mouse had a normal distribution of corticotrophs and other cell types.

We observed POMC-positive tumors of widely varying size that appeared to arise exclusively from the intermediate lobe not only in $Pomc^{+/-}$ mice, but also in $Pomc^{+/-}Tg/+$ and $Pomc^{-/-}Tg/+$ mice. Figure 6B shows sections of two representative abnormal pituitaries weighing 4.2 mg (*top row*, *left panel*) and 31 mg (all other panels) from $Pomc^{-/-}Tg/+$ mice. Both glands contained normal anterior lobe tissue with a mixture of typical acidophilic and basophilic cell types. Immunostaining for GH and PRL was positive only in cells of the anterior lobe, whereas immunostaining for ACTH revealed scattered corticotrophs in the anterior lobes and continuous sheets of melanotrophs in the intermediate lobe tumor (Fig. 6B, *lower row*). Large intermediate lobe tumors displaced and compressed underlying anterior lobe tissue and overlying hypothalamus but did not appear to invade the contiguous structures.

Pituitary tumors originating in both the intermediate and anterior lobes were observed in $Pomc^{-/-}$ mice. Figure 6C shows two such representative pituitary tumors weighing 8.2 mg and 11 mg. The first pituitary contained a nodular intermediate lobe composed only of basophilic cells (Fig. 6C, *upper row*). Multiple mitotic figures were present per high-power field. A Gordon-Sweet silver stain revealed areas of enlarged acini and disrupted reticulin fibers consistent with hyperplasia, contiguous with frankly adenomatous tissue characterized by a total loss of all extracellular reticulin matrix. The second pituitary (Fig. 6C, *lower row*) was composed of apparently normal intermediate lobe tissue overlying an enlarged anterior lobe, which contained only a thin margin of mixed acidophilic and basophilic cells. These marginal cells were immunopositive for GH and PRL and were adjacent to the large immunonegative mass of tumor cells. The tumor had the histopathological features of an adenoma, including loss of the reticulin network, enlarged pleomorphic nuclei with numerous mitotic figures, peliosis, and hematoid depositions.

To further confirm the presumptive melanotroph and corticotroph cell type of origin in the nonfunctioning pituitary tumors of $Pomc^{-/-}$ mice, we crossed the $Pomc^{-/-}$ genotype to transgenic mice expressing EGFP, specifically in POMC cells under control of *Pomc* gene regulatory elements (31). A relatively normal-sized pituitary gland from one of these compound mutant mice showed a medial anterior lobe nodule composed mostly of EGFP-positive corticotrophs as well as individual EGFP-positive corticotroph cells in the lateral wings of the anterior lobe and a distinct intermediate lobe of normal size composed of EGFP-positive melanotrophs (Fig. 6D). A macroadenoma that was several times larger than a normal pituitary gland was harvested from a second compound mutant $Pomc^{-/-}PomcEGFP\text{-}Tg$ mouse. This tumor had no grossly identifiable intermediate or anterior lobe structures but was composed of serpentine cords and sheets of predominantly EGFP-positive cells surrounding cystic spaces (Fig. 6E).

Discussion

In the present study, the goal was to establish a model system to formally test the hypothesis that loss of central nervous system and pituitary-derived POMC peptides contribute uniquely to the adrenal insufficiency associated with

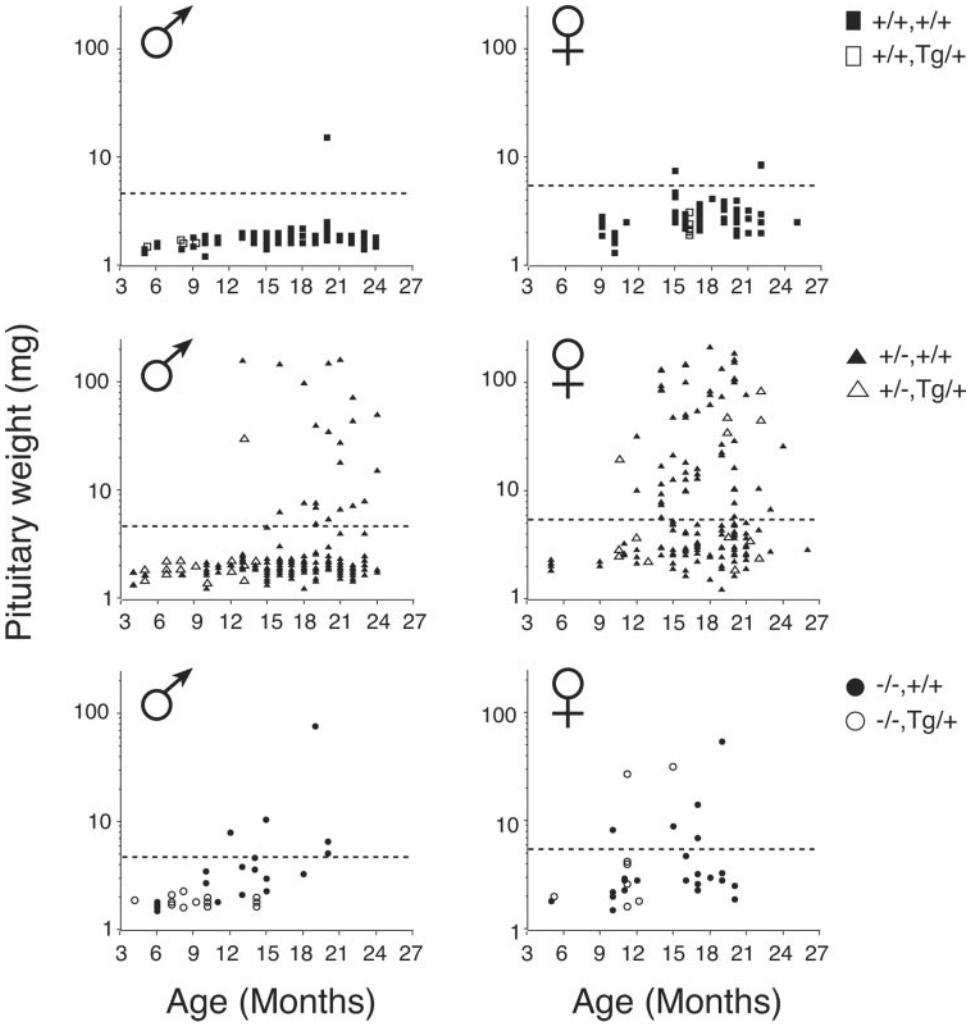

FIG. 5. Distribution of pituitary weights in male and female compound *Pomc* mutant mice. The scatterplots show the wet weights of pituitary glands (logarithmic y-axis) obtained from 4- to 26-month-old mice of the indicated genotypes. *Dotted lines* indicate 2 SD values above the mean weights of male or female pituitary glands from $Pomc^{+/+}$ mice. Most of the mice showed no obvious signs of pituitary disease and were killed for other purposes or at the indicated ages to obtain a snapshot of tumor development over time. Mice with tumor weights greater than 50 mg were more likely to have developed a head tilt, bulging cranium, arched back, or rapid weight loss and were killed when those signs were apparent.

null mutations of the *Pomc* gene. Two general strategies have been used to differentiate and attribute specific consequences of gene-inactivating mutations to the spatial location of gene expression in distinct cell types, tissues, or organs. The first strategy depends on spatially restricted gene inactivation, most commonly involving some form of conditional, somatic cell-based intrachromosomal recombination event (36). A major difficulty that can limit the use of this approach is the achievement of complete gene inactivation in the specific location of interest. In the current studies, we used the alternative genetic approach to create a *de facto* central nervous system-specific inactivation of POMC function by the transgenic rescue of *Pomc* gene expression to pituitary cells in mice with a preexisting targeted null mutation of *Pomc* affecting all sites of expression. Previous studies from our laboratory have identified regulatory elements in the proximal mouse *Pomc* promoter that confer high-fidelity *in vivo* transgene expression specifically to pituitary corticotrophs and melanotrophs with no detectable expression in either the ArcN or nucleus tractus solitarius (30, 37). In addition to their spatial selectivity, these promoter elements have also faithfully recapitulated the developmental onset, diurnal rhythm, and stimulatory/inhibitory regulation of *Pomc* expression by adrenalectomy, dexamethasone, metyrapone blockade of glucocorticoid biosynthesis, hypertonic saline, and dopaminergic agonist/antagonist treatment in the anterior and intermediate pituitary lobes (29, 34, 38).

Rescue of adrenal function

POMC peptides are known to play a critical role in the development, maturation, and function of the adrenal glands (25, 39, 40). Our data confirm that the pituitary is a sufficient source of POMC and that neuronal POMC expression is not necessary for synthesis and secretion of corticosterone from the adrenal cortex. In addition to ACTH, which stimulates adrenal cortical cells by activation of melanocortin-2 receptors, a peptide derived from the aminoterminal half of POMC by extracellular endoproteolytic cleavage has been reported to be mitogenic for adrenal cortical cells *in vitro* (40, 41). The *pHalEx2** transgene encodes all POMC peptides and therefore cannot differentiate between the possible contributions of ACTH and the aminoterminal peptide to the observed restoration of both adrenal gland morphology and function; however, two recent studies by Coll *et al.* (25, 42) involving the injection of an ACTH analog (1–24 ACTH, synacthen) or the short peptide derived from the aminoterminal region of POMC (1–28 POMC) to $Pomc^{-/-}$ mice strongly suggest that

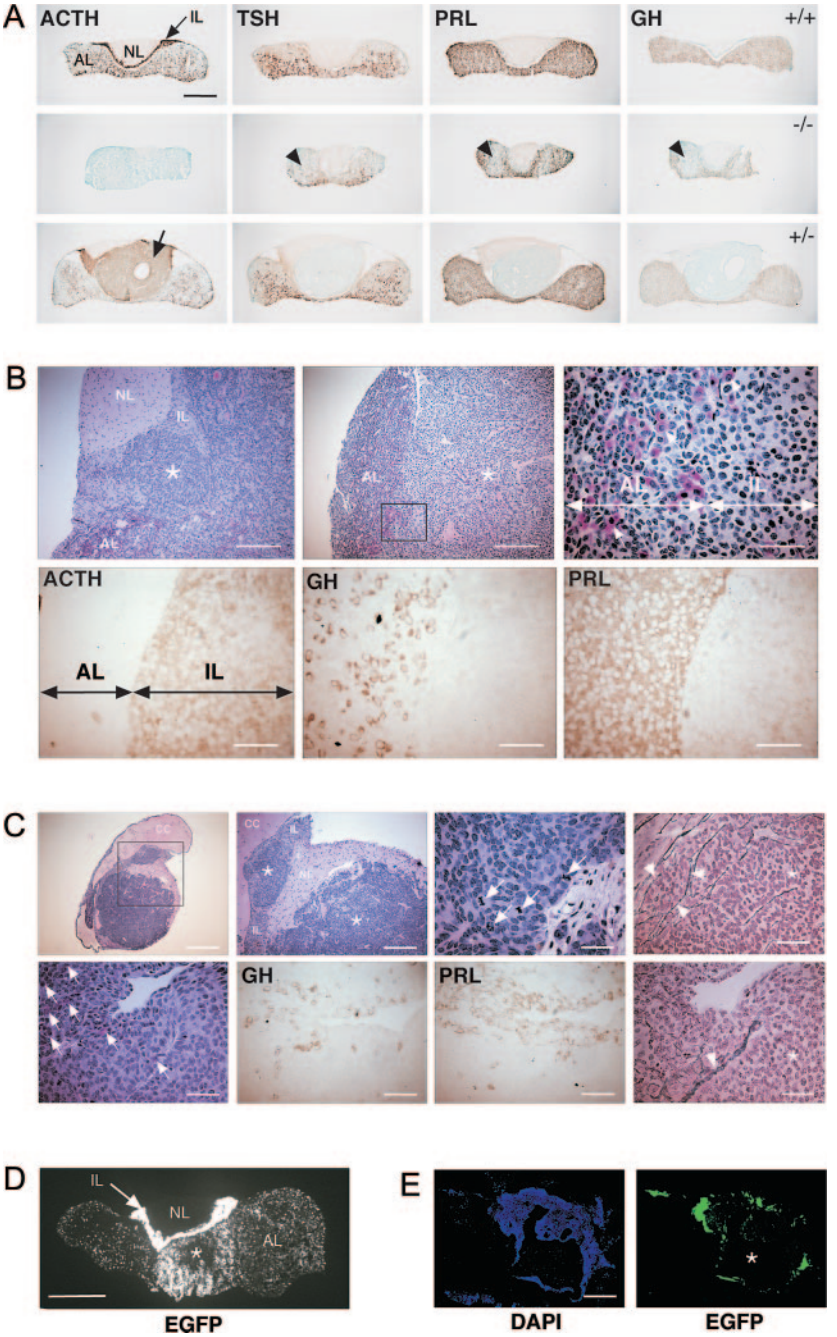

FIG. 6. Histological analysis of pituitary tumors in *Pomc* mutant mice. A, Immunohistochemical localization of ACTH, TSH, PRL, and GH in coronal pituitary sections from 11- to 12-month-old male mice. Arrows indicate a normal intermediate lobe (IL) (*top row*) and a melanotroph IL tumor (*bottom row*) in $Pomc^{+/+}$ and $Pomc^{+/-}$ mice, respectively. Arrowheads indicate a large anterior lobe (AL) nodule that does not express any pituitary hormone in a $Pomc^{-/-}$ mouse (*middle row*). NL, Neural lobe. *Scale bar*, 300 μm. B, Early (*top left*) and late-stage (*top middle*) IL tumors (*) from 11- and 15-month-old female $Pomc^{-/-}$ *Tg*/+ mice, respectively. The remaining panels are higher-magnification photomicrographs of the area indicated by the square. Hematoxylin and eosin staining (*top right*) shows the boundary between normal AL, which contains numerous pink acidophilic cells (*arrows*), and adenomatous IL. Melanotrophs in the IL tumor are uniformly positive for ACTH immunoreactivity but negative for GH and PRL (*bottom row*). *Scale bars* represent either 120 μm (*first two panels*) or 30 μm. C, (*Top row*) IL pituitary tumor in a 10-month-old female $Pomc^{-/-}$ mouse. Tumor nodules (*) are adjacent to areas of relatively normal melanotrophs and a colloidal cyst (cc). Numerous mitotic figures are present (*arrows*). Gordon-Sweet silver stain reveals areas of hyperplasia with enlarged acini (*arrowheads*) adjacent to frank adenoma (*) (*top right*). *Bottom row*, AL pituitary tumor in a 15-month-old male $Pomc^{-/-}$ mouse. Hematoxylin and eosin staining reveals residual normal AL containing pink acidophils (*arrows*) adjacent to basophilic adenoma. GH and PRL immunoreactive cells are only present in the normal AL tissue. Reticulin fibers are condensed at the border between normal AL and adenoma (arrowhead) and disrupted or completely absent in the adenoma (*). *Scale bars*, 300 μm (*panel I*), 120 μm (*panel II*), 20 μm (*panel III*), and 30 μm (*panel IV* and *bottom row*). D, EGFP-positive, presumptive corticotroph tumor (*) in the AL of a compound mutant $Pomc^{-/-}$*PomcEGFP-Tg* mouse. *Scale bar*, 300 μm. E, Portion of a large pituitary tumor with no grossly identifiable structure from an 18-month-old $Pomc^{-/-}$ *PomcEGFP-Tg* mouse. Tumor cells are variably positive for EGFP fluorescence and surround numerous cyst-like cavities (*). *Scale bar*, 120 μm.

ACTH is the critical product secreted by pituitary corticotrophs. This latter conclusion is further supported by our observation that another pituitary-specific transgene *pHal** that encodes the amino-terminal POMC peptide and β-endorphin, but not ACTH (10), failed to restore corticosterone production after intercrossing with $Pomc^{-/-}$ mutant mice (Smart, J.L., and M.J. Low, unpublished data).

Activation of the HPA axis

Expression of the *pHalEx2** transgene on the WT genetic background ($Pomc^{+/+}$*Tg*/+) did not result in increased levels of pituitary POMC mRNA and peptides or alterations in either basal or stress-induced plasma ACTH and corticosterone. These findings are consistent with our previous studies of analogous pituitary-specific POMC transgenes (29) and suggest that introduction of the extra *Pomc Tg* allele is balanced by a coordinated regulation of WT and *Tg* allele expression to maintain normal HPA activity with no net gain of function. Similarly, the loss of one functional *Pomc* allele in the heterozygous $Pomc^{+/-}$ mice did not result in a significant decrease in pituitary POMC mRNA levels or decreased basal activity of the HPA axis. Evidently, expression of the remaining normal allele was up-regulated to restore homeostasis at the level of the pituitary. The same was not true for hypothalamic expression of POMC, in which a 50% reduction in α melanocyte-stimulating hormone content was

previously shown (28). Although we observed a statistically nonsignificant trend for reduced levels of POMC mRNA in the ArcN of *Pomc*$^{+/-}$ compared with *Pomc*$^{+/+}$ mice, the quantitation of expression by *in situ* hybridization may have been insufficiently sensitive to detect a biologically meaningful reduction. There were clear phenotypic effects of the heterozygous genotype on both body weight (28) and hypothalamic CRH expression (discussed subsequently) indicating that a reduction in neuronal levels of POMC peptides was disproportionately greater than changes in steady-state levels of POMC mRNA in the *Pomc*$^{+/-}$ mice.

Despite the absence of any detectable gain-of-function phenotype attributable to *pHalEx2** expression in the WT genetic background, transgenic restoration of pituitary *Pomc* expression in *Pomc*$^{-/-}$ mice induced excessive activity of the HPA axis. The phenotype was evident in both sexes but was substantially more severe in males and characterized by increased adrenal cortical mass and elevated basal diurnal levels of corticosterone and plasma ACTH. Other studies have similarly demonstrated the existence of a sexual dimorphism in the HPA activity in rodents (43) dependent on the estrous cycle and likely attributed to the actions of estradiol. Because ovarian steroid effects are partly mediated through the hypothalamus and in particular estrogens modulate ArcN POMC neurons (44), lack of central POMC in *Pomc*$^{-/-}$*Tg*/+ mice can partly explain the differences in the HPA axis responsiveness between males and females.

Based on microscopic observation, hypertrophy of individual zona fasciculata cells appeared to be the primary cause of the increased adrenal mass consistent with previous studies on the effects of chronic ACTH stimulation on rodent adrenals (45–47) and the administration of a potent, long-acting ACTH analog to adult *Pomc*$^{-/-}$ mice (25, 42). Markedly enlarged adrenal glands due to hypertrophy of the adrenal zona fasciculata with concomitant hypercorticosteronemia have also been described secondary to elevated plasma ACTH levels in a rat model overexpressing CRH from transplanted medullary thyroid carcinoma cells (48). Despite blunted ACTH responses after restraint stress, both male and female *Pomc*$^{-/-}$*Tg*/+ mice responded with normal or slightly increased plasma corticosterone levels, suggesting increased adrenal sensitivity to ACTH stimulation. The reasons for this accentuated response in the *Pomc*$^{-/-}$*Tg*/+ mice is unclear but could be mediated through an up-regulation of the adrenal ACTH receptor.

The phenotype of excessive basal HPA activation, together with the observed significant attenuation in stress-induced ACTH secretion in both sexes, also suggested a central nervous system defect in the regulation of the HPA axis in *Pomc*$^{-/-}$*Tg*/+ mice. We therefore examined expression of CRH, the principal secretagogue of ACTH, in the PVH and found that CRH mRNA was inappropriately elevated relative to the hypercorticosteronemia in these mice. Presumably, the increased *Crh* gene expression was accompanied by increased synthesis and secretion of CRH causing the elevated basal ACTH. Previous studies examining the correlation of pituitary POMC mRNA levels and circulating ACTH in *Crh* KO mice have shown discrepancies between the two measures, which suggest independent actions of CRH receptor stimulation and glucocorticoid receptor inhibition on *Pomc* gene expression and ACTH secretion (49, 50). Indeed, a direct inhibitory feedback to *Pomc* transcription in pituitary corticotrophs by the elevated corticosterone in *Pomc*$^{-/-}$*Tg*/+ mice would explain the paradoxically normal levels of basal pituitary POMC mRNA accompanied by increased plasma ACTH. The attenuated increase in ACTH secretion in response to an acute stress is likely due to a combination of receptor desensitization to chronic CRH stimulation, a relative depletion of releasable pools of ACTH secretory granules, and/or feedback inhibition at the pituitary level by the higher than normal circulating corticosterone.

The classical viewpoint of negative feedback regulation of glucocorticoids on PVH CRH expression is sufficient to explain the markedly increased levels of CRH mRNA observed in the hypothalamus of *Pomc*$^{-/-}$ mice by a mechanism of disinhibition (3, 51). However, the fact that we also observed high levels of CRH mRNA in the face of elevated corticosterone suggests that the hormone's ability to inhibit *Crh* expression in the PVH has been disrupted and/or circumvented in *Pomc*$^{-/-}$*Tg*/+ mice. Perhaps equally remarkable, heterozygous *Pomc*$^{+/-}$ mice (with or without *pHalEx2** transgene expression) also had significantly increased CRH mRNA providing additional evidence for an altered homeostatic set point in regulation of the limbic-HPA axis. Recent evidence from the Dallman laboratory and others indicates that corticosterone actually has dual feedback effects in the central nervous system that are dependent on physiological state including energy balance (22, 23). Under normal conditions, corticosterone exhibits an inhibitory action to maintain basal activity of the HPA axis, whereas under chronic stress conditions, corticosterone has a facilitatory effect on HPA activity. Adrenalectomy has been shown to result in a decrease in hypothalamic melanocyte-stimulating hormone content, whereas dexamethasone restores these levels in rats (21), suggesting that hypothalamic POMC peptides may be required for the full suppressive effect of glucocorticoids on PVH CRH expression. In a recent study that used a different strain of global POMC-deficient mice than in our work, chronic administration of corticosterone in the drinking water was apparently sufficient to restore CRH expression to the levels found in wild-type animals (52). However, differences in the plasma corticosterone levels and pattern between the *Pomc*$^{-/-}$*Tg*/+ mice and *Pomc*$^{-/-}$ mice supplemented with glucocorticoids in drinking water, timing for the analysis of CRH expression as well as genetic backgrounds may explain these discrepancies.

Our data demonstrating increased CRH expression in the PVH of mice with a central nervous system specific deficiency of POMC, regardless of whether corticosterone levels were low, normal, or high, implies that POMC neurons are a physiologically important component of the neural circuits regulating the limbic-HPA axis. This role of POMC neurons appears to be specific to control of CRH in the PVH, because CRH expression in the central nucleus of the amygdala was suppressed in *Pomc*$^{-/-}$ mice and up-regulated normally by restoration of circulating corticosterone. There is a direct synaptic projection of POMC neurons from the ArcN to CRH neurons in the PVH, melanocortin-4 receptors have been colocalized to CRH neurons, and melanocortins have functional effects on CRH neurons (14, 15, 17). Our observations

support a net inhibitory effect of POMC on CRH neurons, but we cannot distinguish at this point whether the loss of α melanocyte-stimulating hormone, possibly in combination with the loss of β-endorphin or any of the other POMC-derived peptides is responsible for this. A few studies have described opposite effects of melanocortin agonists on CRH expression or release. In the mouse, central administration of melanocortin agonists activated CRH neurons through melanocortin-4 receptors (14). However, stimulatory effects of AgRP, a melanocortin receptor antagonist, on ACTH and cortisol have been reported and are more consistent with an inhibitory effect of endogenous α melanocyte-stimulating hormone on the HPA axis (53). In *ex vivo* models, α melanocyte-stimulating hormone was shown to either stimulate or suppress CRH release from rat hypothalamic explants (15, 54, 55). Similarly, pharmacological studies of μ receptor agonists administered centrally or applied *in vitro* to hypothalamic preparations have also had mixed effects on CRH secretion depending on dose and other experimental variables (56–58). In addition, the observed lack of effect on CRH mRNA levels in β-endorphin-deficient mice speaks against a critical inhibitory role of the POMC opioid peptides. The reasons for these discrepancies are unclear but may be explained by the differences between acute pharmacological treatments and chronic absence of the endogenous peptides.

It remains possible that there is an indirect or more complex integration of opposing effects from melanocortins and β-endorphin on CRH neurons. One possibility is that the absence or reduction in central POMC might indirectly alter the set point for CRH neuron activity relative to circulating corticosterone levels by a polysynaptic pathway. For example, immunohistochemical analysis has revealed α melanocyte-stimulating hormone and β-endorphin immunoreactivity in the dorsal raphe, a predominantly serotonergic nucleus that is also innervated by CRH projections (8, 59, 60). Furthermore, dorsal raphe serotoninergic neurons reciprocally innervate neuroendocrine control regions in both the PVH (61) and ArcN (62) and activation of 5-HT_{2A} serotonin receptors on CRH neurons stimulates CRH release and increased plasma ACTH levels (63). Inhibition of these serotoninergic neurons by POMC peptides could decrease the serotonergic stimulation of CRH in the PVH, providing an indirect regulation of CRH by POMC neurons via the dorsal raphe nucleus. Additional experiments are needed to further clarify the actual mechanisms responsible for increased CRH tone in the neuronal-specific POMC-deficient mice.

Pituitary tumor induction

Although there was hormonal evidence for abnormal activation of the HPA axis in 3-month-old male *Pomc*$^{-/-}$*Tg*/+ mice based on their elevated corticosterone levels, no gross morphological abnormalities of the pituitary gland were apparent in any genotype until age 10 mo. After that point, we observed an age-dependent, increased incidence of pituitary tumors that appeared to plateau at approximately age 15 months in male and female mice with either a partial (*Pomc*$^{+/-}$) or complete (*Pomc*$^{-/-}$) loss of POMC. Restoration of pituitary POMC expression had no apparent effect on the incidence of tumor development, implicating a loss of neuronal POMC specifically in the pathogenesis of this phenotype. Most of the tumors observed, whether derived from melanotrophs or corticotrophs, were adenomas based on the disruption or loss of the reticulin network and high mitotic index. A common feature of all four mutant genotypes showing adenoma development was their variable increase in hypothalamic CRH expression. Based on our observations and previous data in mice suggesting that hypothalamic factors can profoundly influence pituitary tumor development and progression from other cell types, we hypothesize that chronic exposure of pituitary corticotrophs and melanotrophs to the hypothalamic hormone CRH accounts for the stochastic development of these tumors.

The trophic potential of hypothalamic hormones including GHRH and CRH has been documented previously. Mice that overexpress human GHRH under the control of the metallothionein promoter develop pituitary tumors with hyperplasia (64) and subsequently frank adenomas (65). In humans, GHRH-producing tumors can cause acromegaly associated with somatotroph proliferation and hyperplasia (66, 67). Similarly, patients with long-term exposure to CRH excess from ectopic CRH-producing tumors develop Cushing's syndrome, which has been associated in some cases with corticotroph hyperplasia (45, 68) and adenoma (69).

In the *Pomc* mutant mice, adenomas were associated in most cases with some evidence of hyperplasia that could be a consequence of elevated CRH levels. Indeed, chronic administration of CRH has been associated with an increase in corticotroph and melanotroph cell number (46, 70) due to increased mitogenic activity (71). If chronically increased CRH is sufficient to produce the observed trophic stimulation of the pituitary gland, we would expect that other CRH overexpressing mice would also develop tumors. Two strains of transgenic mice with central overexpression of CRH from different promoters have been independently generated by different laboratories (72, 73). Interestingly, both show an activated HPA axis, but neither strain has been reported to develop pituitary tumors. However, the first strain has a shortened lifespan such that mice rarely reach an age of 10 mo, the minimum age when adenomas were evident in our studies. It is unclear if the second strain has been specifically evaluated for abnormal pituitary histology.

Two distinct types of adenomas were observed depending on genotype of the mice. Both corticotroph and melanotroph tumors were present in *Pomc*$^{-/-}$ mice, whereas only melanotroph tumors developed in the other three mutant genotypes. This difference in tumor cell origin could be secondary to a dose-dependent stimulatory effect of CRH. *Pomc*$^{-/-}$ mice exhibited the highest hypothalamic CRH expression (a 6-fold increase relative to control siblings compared with a more modest 1.5- to 2-fold increase in the other genotypes). A second, but not mutually exclusive, hypothesis relates to the inhibitory feedback actions of glucocorticoids on pituitary corticotrophs but not on melanotrophs because of their cell-specific pattern of glucocorticoid receptor expression (6). Such feedback is completely absent in *Pomc*$^{-/-}$ mice with undetectable plasma corticosterone. Changes in glucocorticoid status have been associated with altered pituitary mitotic and apoptotic activities in animal models. Adrenalectomy in the rat is accompanied by increased mitotic activity

for about 7 d, whereas the frequency of apoptosis is unchanged, resulting in a net increase of trophically sensitive cells. In contrast, glucocorticoid replacement in adrenalectomized animals is associated with a low mitotic index and high occurrence of apoptosis, resulting in a drop in total pituitary cell proliferation (74). Those data suggest that cells undergoing mitosis are sensitive to apoptotic activity in the presence of glucocorticoids, thus protecting against cell proliferation and hyperplasia. In contrast, corticosterone withdrawal induces mitosis without equivalent apoptosis, thus favoring cell division and hyperplasia. As a consequence, normal or elevated levels of corticosterone may prevent tumors of corticotrophs via the established negative effect on corticotroph proliferation (75) in *Pomc*$^{-/-}$*Tg*/+, *Pomc*$^{+/-}$, and *Pomc*$^{+/-}$*Tg*/+ mice. However, melanotrophs that lack functional glucocorticoid receptors could respond continuously to the chronic CRH stimulatory signal.

Lack of production of α melanocyte-stimulating hormone in POMC-deficient mice could also contribute to the development of intermediate lobe tumors. Indeed, a recent set of studies demonstrate that re-expression of the retinoblastoma gene (*Rb*) selectively in melanotroph cells in *Rb*$^{+/-}$ mice protects against melanotroph adenomas but accelerates the progression of noncorticotroph anterior lobe adenomas. Overproduction of α melanocyte-stimulating hormone in the melanotroph cells suggests a paracrine role of melanocortin peptides in regulating cell growth and apoptosis (76) and supports the hypothesis of a protective action of α melanocyte-stimulating hormone against the progression of melanotroph tumors.

Finally, we cannot exclude that other factors such as a potential lack of dopamine tone in POMC-deficient mice induce or modulate the development of pituitary tumors. However, this seems to be an unlikely primary mechanism because dopamine D2 receptor-deficient mice, which have a complete lack of dopamine tone at the intermediate lobe of the pituitary, never developed melanotroph tumors (Low, M.J., unpublished data; and Ref. 77).

In conclusion, our data show that central nervous system and pituitary-derived POMC have unique functions in regulating the HPA axis. Furthermore, pituitary-derived POMC does not compensate for the loss of central nervous system POMC. Although there is evidence for limited transport of POMC peptides across the blood-brain barrier (78), conventional wisdom holds that central nervous system and pituitary-derived POMC constitute separate compartments with distinct biological roles, and the current data further indicate specific roles for POMC peptides in the central nervous system *vs.* pituitary. Additionally, these data show that POMC has dual regulatory roles at multiple levels of the HPA axis, most interestingly regulating CRH expression in the PVH in addition to glucocorticoid secretion from the adrenals.

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