Pituitary hypoplasia and respiratory distress syndrome in Prop1 knockout mice

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Mutations in Prophet of PIT1 (Prop1), one of several homeodomain transcription factors that are required for the development of the anterior pituitary gland, are the predominant cause of MPHD (multiple pituitary hormone deficiency) in humans. We show that deletion of Prop1 in mice causes severe pituitary hypoplasia with failure of the entire Pit1 lineage and delayed gonadotrope development. The pituitary hormone deficiencies cause secondary endocrine problems and a high rate of perinatal mortality due to respiratory distress. Lung atelectasis in mutants correlates with reduced levels of NKX2.1 and surfactant. Lethality of mice homozygous for either the null allele or a spontaneous hypomorphic allele is strongly influenced by genetic background. Prop1-null mice are an excellent model for MPHD and may be useful for testing the efficacy of pharmaceutical intervention for neonatal respiratory distress.

INTRODUCTION

Pituitary-based growth insufficiency is a common birth defect, occurring once in every 4000 births (1). Mutations in the Prophet of Pit1 gene (PROP1) are the most common known cause of multiple pituitary hormone deficiency (MPHD) in humans (reviewed in 2). Despite the frequent deletion of the dinucleotide repeat A301G302, which produces a complete loss of function, the age of onset, effect on pituitary size and number of hormone deficiencies is variable, with evidence for progressive loss. Patients generally lack growth hormone (GH), thyrotropin (TSH), prolactin (PRL) and the gonadotropins (LH and FSH). Acquired ACTH deficiency occurs occasionally. There is no obvious genotype–phenotype correlation, suggesting that other genes influence the severity of the endocrine deficiency.

Many mutant mouse models have led to the identification of the genes that cause human pituitary dysfunction, and generally there has been a satisfying correlation between the mouse and human phenotypes (3). Indeed, the Prop1 gene was discovered by positional cloning of the spontaneous Ames dwarf mouse (df) (4), which quickly led to identification of mutations in humans with MPHD (5). Prop1df contains a Ser83Pro mutation in the paired type homeodomain of PROP1. This mutation produces a phenotype that includes near absence of cells in the Pit1 lineage, resulting in severe GH, TSH and PRL deficiencies, anterior pituitary hypoplasia (6,7) and a 90% reduction in circulating gonadotropins (8). Corticotropes are unaffected (9), although differences in the hypothalamic–pituitary–adrenal (HPA) axis are observed (10).

The Ames dwarf mouse may not be a good model for the most common type of MPHD because two lines of evidence support the idea that the Ser83Pro mutation has residual activity (4,6,7). In order to understand the role of PROP1 in pituitary development and function, we created a null allele by gene targeting. We found that approximately half of the Prop1 knockout mice die of respiratory distress syndrome (RDS) at birth, and additional mutant mice die in the first few weeks of life with failure to thrive. Multiple pituitary hormone deficiencies resulting from lack of Prop1 ultimately lead to lower levels of NKX2.1 (TITF1) and surfactant in the lung, explaining the RDS. Surprisingly, the genetic...
background has a more profound influence on the survival of Prop1 deficient mice than any functional difference between the df and null alleles. These results suggest that mutations in Prop1 may account for some cases of pituitary aplasia, RDS and neonatal death, and support the idea that genetic modifiers influence the clinical features of MPHD patients (11,12).

RESULTS

Generation of Prop1−/− mice

We generated a Prop1 null allele by deleting a portion of the first exon beginning with the initiator ATG codon, the first intron and a portion of exon 2 (Fig. 1). We confirmed the targeting of Prop1 in R1 embryonic stem (ES) cells (13) by PCR and Southern blot analysis. Chimeric mice were generated by injection of a targeted clone into C57BL/6J (B6) blastocysts, and those mice with high ES contribution were bred to B6. Germine transmission produced F1 progeny that were intercrossed to generate F2 Prop1−/− mice of mixed genetic background (129B6) for experiments.

Prop1−/− newborns are cyanotic and have respiratory distress

Heterozygotes were generated with the expected frequency and they appeared no different than wild-type mice. Mice homozygous for the Prop1 null allele were found at approximately half the expected frequency at 2 weeks after birth (14%, P < 0.00001), although they were appropriately represented before birth (22%, P = 0.3; Table 1). Some mutant neonates were cyanotic and died within 30 min to 4 h after birth despite attempts to breathe (Fig. 2). Breathing motions were irregular and associated with gasping, suggesting that the lungs failed to inflate. Swallowed air filled the intestines with bubbles. The penetrance of RDS tended to increase with higher contribution of the 129S1/SvImJ (129S1) background and decrease with higher contribution of B6. After two generations of backcrossing to 129S1, only 6.7% of the progeny surviving to day 2 after birth (14%, P < 0.00001), although they were appropriately represented before birth (22%, P = 0.3; Table 1). Some mutant neonates were cyanotic and died within 30 min to 4 h after birth despite attempts to breathe (Fig. 2). Breathing motions were irregular and associated with gasping, suggesting that the lungs failed to inflate. Swallowed air filled the intestines with bubbles. The penetrance of RDS tended to increase with higher contribution of the 129S1/SvImJ (129S1) background and decrease with higher contribution of B6. After two generations of backcrossing to 129S1, only 6.7% of the progeny surviving to week of age were mutants (P < 0.00001), whereas 14% of the 129B6 F2 mice were mutants. Thus, mutants are 0.43 times as likely to be represented on the 129S1 background than on mixed, P < 0.03. After four generations of backcrossing to B6, there are somewhat fewer Prop1−/− mice at 2 weeks of age than expected (17.5%), but mutants are not significantly underrepresented (P = 0.07). Mutants are 0.33 times as likely to be represented at 2 weeks of age on 129S1 compared with B6, P < 0.007. The expected Mendelian distribution of neonates homozygous for the spontaneous df allele of Prop1 are obtained on the mixed stock on which it is maintained (DF/B), a Mus castaneus backcross, the inbred C3HeB/FeJ (N4) genetic background and B6 (N4) (14). However, a fraction of both Prop1−/− and Prop1dfdf mice exhibited lethargy, wasting and precipitously died between weaning and adulthood (3–7 weeks) on the B6 background (data not shown). The remaining homozygotes lived through adulthood with no evidence of illness through 1 year of age. Thus, the 129S1 background increases the susceptibility to respiratory distress and several mixed backgrounds support viability to adulthood.

Surviving Prop1−/− mice resemble Prop1dfdfmutants

Prop1null homozygotes that survive to adulthood (Fig. 2). The growth rate of Prop1−/− mutants is similar to that of animals homozygous for the hypomorphic Prop1df allele (Prop1dfdf data not shown). Growth retardation is detectable at day 10 after birth, and 2-week-old Prop1−/− mice are smaller than littermates (P < 0.001). Adult Prop1−/− mutants have underdeveloped thyroid glands and gonads that are histologically indistinguishable from those in mice that make no TSH or gonadotropins (15).

In adult Prop1−/− mutants on 129B6 F2 mixed background, the anterior lobe of the pituitary gland consists of only a few cell layers (Fig. 3). The mutant anterior lobes have POMC and LH immunoreactive cells. GH, PRL and TSH cell are readily detectable in wild-type pituitaries, but only an occasional positive TSH or GH cell was detected in Prop1−/− mice. DF/B-Prop1dfdf adult pituitaries have relatively large foci of cells that produce POMC, TSH and PRL, suggesting that there is limited differentiation of the Pit1 lineage (7). GH-positive cells are more abundant than TSH- or PRL-positive cells and can number over 100 per organ. On the B6 background, Prop1df and Prop1−/− adult pituitaries have rare, single, hormone-positive cells of the Pit1 lineage, but no sizeable foci (data not shown). This suggests that the DF/B stock supports the growth of progenitors to form clusters of differentiated cells in the absence of functional Prop1.

Prop1−/− pituitaries develop dorsal overgrowth and ventral hypoplasia

The morphology of the developing pituitary gland in Prop1−/− and Prop1dfdf mice resembles that of normal mice at embryonic day 12.5 (e12.5), but by e14.5–e15 ventral hypoplasia and dorsal dysmorphology are evident in both mutants (Fig. 4). Heterozygotes for either allele exhibit dorsal dysmorphology without ventral hypoplasia at this time, but the dysmorphology resolves by birth (data not shown).

Anterior pituitary hormone deficiencies are evident during gestation in Prop1−/− fetuses. The rostral tip thyrotropes (RTT) arise independent of Pit1 and represent such a small portion of total thyrotropes that they are unlikely to be biologically significant in regulating the pituitary−thyroid axis (9,16). RTT are indistinguishable in Prop1−/− and Prop1++ pituitaries, but there are no TSH- or GH-positive cells in the caudo−median region of the mutant glands at e18.5 (Fig. 4). LH and FSH are detected at e16 and e17, respectively, in wild-type mice, but FSH is reduced (data not shown) and LH is absent at e18.5 in mutant mice. Thus, gonadotrope development is delayed in Prop1 deficient mice on the 129B6 background. Gonadotrope development is indistinguishable in wild-type, Prop1−/−, and Prop1dfdf when compared on the B6 background (data not shown).

We expected that corticotrope function might be reduced in Prop1 null mutants, since some of the human patients develop hypocortisolism (17,18). Immunoreactive POMC is detected in the anterior and intermediate lobes of normal mice at e12.5 and e14.5, respectively (19). The dysmorphology of the mutant gland makes it difficult to rule out subtle developmental delay in melanotrope or corticotrope specification, but
Prop1

\(1^\text{st}/2\) pituitaries have POMC in the prospective anterior and intermediate lobes at e18.5 (Fig. 4). POMC immunoreactivity is also similar in mutant and normal pituitaries examined at earlier developmental stages (data not shown). In addition, at e18 and postnatal day 1 (p1), individually isolated mutant pituitaries have equal ACTH content by western blot analysis compared with wild-type (Fig. 5). Moreover, corticosterone levels are normal in e18 mutants by radioimmunoassay (RIA). After birth, however, mutants exhibit significantly elevated corticosterone. This could be caused by the stress that results from regulating body temperature in the hypothyroid state and/or breathing difficulties (10,20).

**Lung atelectasis and neonatal death in Prop1

\(1^\text{st}/2\) mice**

We observed seven litters of 129B6 F2 mice during birth and for the next 4–6 h and recorded viability. Many Prop1 null newborns gasp for air and are cyanotic, indicating poor blood oxygenation, whereas only one heterozygous animal was cyanotic. The correspondence between the RDS phenotype and the Prop1

\(1^\text{st}/2\) genotype is significant, \(P < 0.01\). Lungs of all cyanotic Prop1

\(1^\text{st}/2\) newborns are ateleatic (no signs of functional inflated alveoli) and contain excess mesenchymal tissue, with cuboidal rather than squamous cells lining the uninflated alveolar surface (Fig. 5). This morphology is similar to that observed in the lungs of human newborn infants with RDS.

To determine the underlying problem with lung function, we confirmed the pituitary specificity of Prop1 expression and analyzed molecular markers of lung maturation and morphogenesis. Prop1 expression has only been reported in the developing pituitary gland (4). To confirm this rigorously, we surveyed Prop1 expression by in situ hybridization assays on wild-type embryos (21). Three sections per slide were mounted and every other slide was examined throughout the entire embryo. Two to three wild-type embryos were examined at e10.5, e12.5 and e14.5, and one embryo at e13.5. We detected Prop1 expression in the developing pituitary gland, weakly at e10.5 and strongly at all other time points. No expression was detected in any other tissue (data not shown). Thus, lung defects must be secondary to the pituitary hormone deficiency caused by lack of Prop1.

By western blot analysis, the level of surfactant B appeared ~2-fold lower in the lungs of Prop1 deficient mice on the
Table 1. Genetic background affects 2 week viability of Prop1 deficient mice

<table>
<thead>
<tr>
<th>Age</th>
<th>++/+</th>
<th>+/-</th>
<th>-/-</th>
<th>Significance</th>
</tr>
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<tbody>
<tr>
<td>A. Mixed genetic background: F2 129B6 Prop1&lt;sup&gt;+&lt;/sup&gt;/&lt;sup&gt;+&lt;/sup&gt; intercross</td>
<td>51</td>
<td>115</td>
<td>47 (22%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P = 0.323</td>
</tr>
<tr>
<td>2 weeks</td>
<td>92</td>
<td>185</td>
<td>45 (14%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P = 0.000049&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. Inbred background: N2 129S1 Prop1&lt;sup&gt;+&lt;/sup&gt;/&lt;sup&gt;+&lt;/sup&gt; intercross</td>
<td>38</td>
<td>74</td>
<td>8 (6.7%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.0000035&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 weeks</td>
<td>49</td>
<td>92</td>
<td>30 (17.5%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P = 0.02</td>
</tr>
<tr>
<td>C. Inbred background: N4 B6 Prop1&lt;sup&gt;+&lt;/sup&gt;/&lt;sup&gt;+&lt;/sup&gt; intercross</td>
<td>14</td>
<td>34</td>
<td>14 (22.6%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P = 0.660</td>
</tr>
<tr>
<td>2 weeks</td>
<td>30</td>
<td>40</td>
<td>17 (19.5%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P = 0.240</td>
</tr>
</tbody>
</table>

Relative survival of mutants by genetic background and allele type at 2 weeks

<table>
<thead>
<tr>
<th>Crosses compared</th>
<th>Mutant representation</th>
<th>Significance&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>B versus A</td>
<td>Null allele on 129 versus 129/B6</td>
<td>0.43 x likely to be represented</td>
</tr>
<tr>
<td>C versus A</td>
<td>Null allele on B6 versus 129/B6</td>
<td>Similar, odds ratio 1.3</td>
</tr>
<tr>
<td>C versus B</td>
<td>Null allele on 129 versus B6</td>
<td>0.33 x likely to be represented</td>
</tr>
<tr>
<td>C versus D</td>
<td>Null allele versus df allele on B6</td>
<td>Similar, odds ratio 1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>P-value from χ<sup>2</sup> test, significant under-representation of mutants relative to normals (**).  
<sup>b</sup>Mutant percentage of the total animals.  
<sup>c</sup>P-value from χ<sup>2</sup> test, significant difference in mutant representation between crosses.

129S1 background (Fig. 5). The transcription factor NKX2.1, also known as TTF1 and TITF1, is an important marker of lung morphogenesis (22). Western blot analysis revealed that NKX2.1 transcription factor levels in Prop1<sup>–/–</sup> are low to almost undetectable in individual lungs at e18 (data not shown) and p1 (Fig. 5), whereas wild-type and heterozygous mice had robust levels of NKX2.1. In addition, there is a correlation between the levels of NKX2.1 in Prop1<sup>–/–</sup> lungs and the ability of mutant newborns to survive RDS at birth.

DISCUSSION

In humans, the major differentiating diagnosis between PROP1 and POU1F1 (PIT1) mutations involves the presence or absence of gonadotropin deficiency, respectively (23). Using Prop1<sup>–/–</sup> mice generated by gene targeting, we show for the first time that Prop1 deficiency can delay gonadotrope differentiation. In contrast, Pit1 deficient mice have enhanced gonadotrope differentiation (24). We propose that promotion of the gonadotrope lineage in humans with POU1F1 mutations and the retardation of the lineage in PROP1 deficient patients are the bases for the diagnostic difference. In mice, at least, Prop1 is not required for gonadotrope development or function because the persistent hypogonadism and infertility of both male and female mutants can be cured by replacement therapy with GH, thyroid hormone and prolactin (8,14).
Mice require thyroid hormone to establish the feedback loops that regulate gonadotrope function (25).

The progression of hormone deficiency in PROPI patients contrasts with the tendency toward congenital hormone deficiency in POU1F1 deficient patients (23). In particular, acquired ACTH deficiency occurs in some PROPI patients. The basis for this distinguishing characteristic is not known. We find no obvious requirement for Prop1 in the initial determination and expansion of corticotrope or melanotrope lineage. Activation of NeuroD1 and Tpit, two transcription factors critical for the production of ACTH, occurs normally (data not shown) (26,27). We discovered, however, that certain genetic backgrounds enhance the limited activation of Pit1 in the absence of Prop1. In addition, there are fewer precursor cells in the anterior lobes of Prop1 deficient fetuses. Taken together these facts support the idea that progressive hormone deficiency in humans with PROPI mutations may result from limited differentiation of precursor cells along the PIT1 lineage in the absence of PROPI, leading to inadequate hormone production initially in babies and young children, and eventual depletion of the progenitor pool resulting in profound hormone deficiency in older children.

The Ser83Pro mutation in Prop1df mice is in position 11 of the first helix of the homeodomain, leaving the recognition helix intact (4). The mutant protein has reduced, but measurable, DNA binding and transactivation properties. Despite this, the phenotypes of Prop1df/+ and Prop1−/− mice are nearly identical when compared on the same genetic background, indicating that the residual activity of the df allele is insignificant in vivo. Genetic background effects on pituitary development have been relatively unexplored, although the severity of the growth hormone deficiency in Pax6 deficient mice is influenced by genetic background (28,29).

Prop1 is exclusively expressed in the developing anterior pituitary (see Results) (4). Thus, the RDS in Prop1 deficient mice is secondary to pituitary hormone deficiencies. Thyroid hormone and corticosterone are both important for lung maturation and development (30). Glucocorticoids stimulate production of surfactants B and C by type II pulmonary cells. These surfactants allow alveoli to expand during inhalation by decreasing surface tension, and their normal level is crucial for preventing lung atelectasis and RDS at birth (31,32). Although surfactant levels were reduced in mutant lungs, we found no evidence for delayed expression or reduced content or secretion of POMC or ACTH in mutant anterior pituitaries. Moreover, circulating glucocorticoids are normal at birth, indicating that circulating ACTH is sufficient to stimulate glucocorticoid production by the zona fasciculata of the adrenal gland. Therefore, decreased corticosterone is not a primary cause of cyanosis and lung atelectasis at birth in Prop1−/− mice.

Thyroid hormone is also important for late stage lung development and surfactant production (12,33). B6 mice normally tend to have large thyroid glands and high thyroid activity, which may contribute to the better survival rate of Prop1−/− newborns on the B6 background relative to 129S1 (34). Thyroid hormone regulates the regional expression of the transcription factor NKX2.1 (also known as TITF1) in the lung, and haploinsufficiency for NKX2.1 causes reduced surfactant production, lung hypoplasia and RDS at birth (33,35–39). We found reduced levels of NKX2.1 in the lungs of late gestation Prop1−/− fetuses and newborns, which explains the excess mesenchymal tissue, lack of mature functional alveoli, reduced surfactant and RDS in mutants. Anecdotal evidence in humans confirms that pituitary hypoplasia patients can present with RDS (11,12,40–42).
Pituitary development is delayed in Prop1 mutants. Mid-sagittal sections of embryos stained with hematoxylin and eosin reveal no obvious dysmorphology in Prop1df/df or Prop1−/− mutants relative to wild-type at e12.5. Similar hypoplasia of the anterior lobe and dorsal dysmorphology is evident in both mutants at e14.5, e18.5 and p1 (data not shown). Cell specification was compared in mutant and wild-type fetuses using immunohistochemical staining for individual pituitary hormones. POMC immunostaining reveals similar development of melanotropes and corticotropes in mutant and wild-type mice at e18.5. TSH immunostaining reveals that rostral tip thyrotropes (RTT) are intact in mutants, but caudo–medial thyrotropes are completely missing at e18.5. The deficiency of somatotropes (GH) and gonadotrope cells (LH) is also obvious in −/− mice at e18.5.
Our research highlights the important medical problem of neonatal hypothyroidism. We show that severe hypothyroidism at birth leads to fatal respiratory distress, a fact that is not emphasized in the medical literature. The mouse model that we have developed should be useful for testing pharmacological intervention strategies that can reduce RDS. Identification of the modifier(s) that enhance lung development in Prop1 deficient mice may be especially relevant for understanding the survival factors important for averting neonatal respiratory distress in the genetically diverse human population.

MATERIALS AND METHODS

Prop1 targeting in ES cells

Mouse genomic DNA for vector construction was isolated from 129P2/OlaHsd P1 genomic library (43,44). We assembled the targeting construct in the pPNT vector, electroporated it into R1 ES cells (passage 16) and used positive–negative selection with G418 and gancyclovir to enrich for homologous recombinants (15). Of 600 clones, four positives were identified by 5′ PCR and confirmed by 3′ PCR, and Southern blot analysis.

Identification of homologous recombinants

5′ PCR: forward primer, outside of the 5′ homology region, 5′-GGCAGACTAGTTG TCTTGACC-3′, ~1.2 kb upstream from the initiator ATG; reverse 2 primer, 5′-CCACTTTGC GTTTCCTCTTG-3′, corresponds to 5′ LacZ sequence. The expected PCR product is 1.2 kb, and is specific only to correctly targeted recombinants. PCR conditions: 95°C 1 min ×1; (94°C 45 s, 60°C 1 min, 68°C 1 min 45 s) × 35, 68°C 10 min ×1, Expand Long kit from Roche. 3′ PCR: forward primer, 5′-CGCTTCTATCGCCTTCTGGAGT TCTT-3′, specific to neo reporter gene (PGK-neo cassette); reverse primer, outside of the 3′ homology region, 5′-CTTACTCC ACCTACTACTCATTCC-3′. The expected PCR product is ~5.6 kb and is specific only to correctly targeted recombinants. PCR conditions: 94°C 2 min ×1, (94°C 45 s, 64°C 1 min, 68°C 8 min 30 s) × 35, 68°C 10 min ×1, Expand Long.

PCR genotyping

PCR primers: forward, 5′-GTAGAGAAAACAG GTATCTA GCT-3′ (specific for both wild-type and targeted alleles); reverse 1, 5′-TTCTGTTGTTCCTCCTGATG-3′ (specific to wild-type allele) and reverse 2 (above, specific for targeted allele only, LacZ-specific) generate 240 bp (targeted) and 270 bp (wild-type) PCR products, respectively. PCR conditions: 93°C 3 min ×1, (94°C 30 s, 55°C 45 s, 72°C 20 s) × 33, 72°C 5 min.

Mice

C57BL/6J mice were mated to chimeras to generate F1 heterozygous animals. The growth curves, pituitary immunohistochemistry and histology and corticosterone measurements were done on F2 null animals and control (Prop1+/− and Prop1+/+) litters. Mice were transferred to C57BL/6J and 129S1/SvImJ (Jackson Laboratory stock 002448) backgrounds, and the survival of Prop1 null animals and p1 lung morphology was studied on each background independently, N4B6 and N2129S1. Our convention for designating mouse ages is such that the morning after conception is embryonic day 0.5 (e0.5) and the day of birth is postnatal day 1 (p1).
Medical Center, Torrance, CA, USA) and used at final dilutions 1:1500–1:2000 with overnight incubation at 4°C. The appropriate VECTASTAIN ABC ECLite kits (Vector Laboratories) were used according to the manufacturer’s protocols.

Western blot analysis

For analysis of ACTH in e18.0 and p1 pituitaries, individual pituitaries were isolated, snap-frozen in separate Eppendorf tubes on dry ice, thawed and minced manually in 20 μl of ice-cold lysis buffer (50 mM HEPES, 250 mM NaCl, 0.5 mM EDTA, 0.5% Igepal, pH 7.6), with 1 tablet of Complete Protease Inhibitor/10 ml buffer (Roche 1836170). Before and 2 h after incubation on ice, pituitaries were vortexed for 1 min to achieve the complete lysis, and the whole lysates were resolved on 16% PAGE with SDS. Anti-hACTH from Pierce (prod. no. 284–290), according to the manufacturer’s instructions. For the analysis of NNX2.1 in lung protein extracts, e18.0 and p1 lungs were isolated and immediately snap-frozen on dry ice. Protein extracts were sonicated in lysis buffer, and 10–15 μg of total protein per sample was loaded on 12% PAGE. Antibodies against NNX2.1 (NeoMarkers, cat. no. MS-699-Po) were used according to manufacturer’s instructions. ACTH and NNX2.1 protein levels were quantitated by phospho-imaging, normalized against β-actin levels and statistically analyzed using the Student’s t-test.

Corticosterone measurement

RIA analysis of corticosterone concentration was done using Double Antibody 125I RIA kit from ICN (cat. no. 07-120102), according to the manufacturer’s instructions. Serum from e18.0 and newborn mice was frozen and thawed only once for analysis.

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