

# Effects on Hippocampus of Lifelong Absence of Glucocorticoids in the Pro-Opiomelanocortin Null Mutant Mouse Reveal Complex Relationship Between Glucocorticoids and Hippocampal Structure and Function

*Dirk Ostwald, Jason Karpac, and Ute Hochgeschwender\**

*Molecular, Cell, and Developmental Biology Program, Oklahoma Medical Research Foundation (OMRF), Oklahoma City, OK 73104*

*Received September 23, 2005; Accepted October 4, 2005*

## Abstract

In humans changes in serum cortisol levels have been observed with aging, stress, and with affective disorders such as major depression and post-traumatic stress disorder. Corticosteroids are known to influence hippocampal structure and function; specifically, plasma corticosteroid levels have been inversely correlated with hippocampal cell proliferation, cell death, and impaired memory function. The relationship between corticosteroids and structure and function of the hippocampus has been studied in experimental systems in adult animals by increasing or decreasing corticosterone levels through pharmacological supplementation and through surgical removal of the adrenal gland. Here, we utilized the genetically engineered pro-opiomelanocortin (POMC) null mutant mouse, which because of the lack of all POMC peptides has no corticosterone from birth throughout life. The effect of this lifelong absence of corticosterone on the dentate gyrus of the hippocampus is a decrease in granule cell density, which correlated with a decrease in cell proliferation but not an increase in cell degeneration. Fine morphology of granule cells was unaltered. Analyses of gene expression revealed no changes in POMC null mutant vs wild-type hippocampus with respect to levels of expression of corticoid receptor genes or genes known to be regulated by corticosterone. Spatial learning as tested by the Morris water maze was not altered in the POMC null mutant mouse. Taken together with findings from other studies of the effects of altered levels of corticosteroids on the hippocampus, our results argue for a complex homeostasis in which disturbances of any one factor can offset the system in varying ways.

DOI 10.1385/JMN/28:03:291

**Index Entries:** Pro-opiomelanocortin; hippocampus; glucocorticoids; dentate gyrus; adrenal; aldosterone; corticoid receptor; Morris water maze; homeostasis.

## Introduction

Pro-opiomelanocortin (POMC) is a polypeptide precursor expressed mainly in the anterior and intermediate lobe of the pituitary and the arcuate nucleus of the hypothalamus. POMC-derived peptides, including adrenocorticotrophic hormone

(ACTH), melanocyte-stimulating hormone, and  $\beta$ -endorphin, have a diverse array of biological activities, as they are involved in the regulation of adrenal gland function, as well as proliferation, pigmentation, energy homeostasis, and pain control (Raffin-Sanson et al., 2003).

\*Author to whom all correspondence and reprint requests should be addressed. E-mail address: [uteh@omrf.ouhsc.edu](mailto:uteh@omrf.ouhsc.edu)

The POMC null mutant mouse lacks all POMC-derived peptides and shows adrenal insufficiency, obesity, and altered pigmentation (Yaswen et al., 1999). The adrenal gland of POMC null mutants regresses with age and, while present, exhibits two defects: It does not release corticosterone at any time, and it releases highly reduced levels of aldosterone (Karpac et al., 2005). Therefore, the POMC null mutant mouse can be used as a model to study the effects of lifelong absence of corticosterone.

Numerous data from clinical studies and from animal studies reveal a relationship between corticosteroids and structure and function of the hippocampus (De Kloet et al., 1998; McEwen, 1999). Increased serum glucocorticoid levels, as seen in aging, stress, or with affective disorders, correlate with slowing of granule cell neurogenesis in the dentate gyrus of the hippocampus (Gould et al., 1991, 1992, 1998; Cameron and Gould, 1994; Ambrogini et al., 2002), dendritic atrophy of CA3 pyramidal cells (Woolley et al., 1990; McEwen et al., 1995; McKittrick et al., 2000), and impaired spatial memory (Montaron et al., 2005). Lack of adrenal steroids after adrenalectomy results in increased proliferation of neural progenitors in the hippocampal granule cell layer (Gould et al., 1991, 1992; Rodriguez et al., 1998; Cameron and McKay, 1999; Nichols et al., 2001) but also in increased cell death of mature granule cells (Sloviter et al., 1989, 1993; Roy et al., 1990; McNeill et al., 1991). Reconstitution with corticosterone or aldosterone can prevent these effects on the hippocampus (Gould et al., 1990; Woolley et al., 1991). Concerning hippocampal function, removal of adrenal steroids has been shown to impair spatial learning (Conrad and Roy, 1993).

Corticosterone promotes its effects on the hippocampus via two intracellular receptors, the high-affinity mineralocorticoid receptor (MR) and the low-affinity glucocorticoid receptor (GR) (Sousa and Almeida, 2002). It has been shown that disruption of the *MR* gene, but not the *GR* gene, results in dentate granule cell degeneration in mice (Gass et al., 2000).

Glucocorticoids influence expression of a variety of genes in the hippocampus, including genes involved in cellular metabolism, signal transduction, and neuronal plasticity (Vreugdenhil et al., 2001). Transcription of two growth factors, brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF), have been reported to be modulated by corticosterone depletion (Hansson et al., 2000).

The POMC null mutant mouse provides a new model to study the effects of lifelong complete absence of corticosterone. In this report we characterize the phenotype of the POMC null mutant mouse dentate gyrus regarding its morphology, assess the expression of corticosteroid receptors and corticosteroid-responsive genes in the hippocampus, and analyze the impact of altered corticosteroid levels on hippocampus-mediated spatial learning of the POMC null mutant mouse.

## Materials and Methods

### Animals

POMC wild-type and mutant mice in the 129SvEv background (Yaswen et al., 1999) were housed under a 12-h light/12-h dark cycle, with standard laboratory diet (PMI5053, Purina Mills, Richmond, IN), provided *ad libitum* unless otherwise stated. Mice were genotyped by PCR analysis of tail DNA (Yaswen et al., 1999). All procedures described below follow NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

### Corticosteroid Radioimmunoassays

Blood was collected from the mouse tail vein using a heparinized capillary tube, for 2–3 min. Blood was allowed to clot at room temperature for 15 min and was centrifuged afterward for 15 min at 4°C, 3500 rpm. Serum was transferred to new microcentrifuge tubes, flash frozen, and stored at –80°C until further processing. Levels of corticosterone and aldosterone were determined in serum samples using radioimmunoassay (RIA) kits according to the manufacturer's specifications (Corticosterone RIA, ICN Biomedicals, Irvine, CA; Coat-A-Count Aldosterone RIA, Diagnostic Products Corporation, Los Angeles, CA). The limits of detection for these RIAs are 25 ng/mL for corticosterone and 25 pg/mL for aldosterone, respectively.

### Histology and Immunocytochemistry

Animals were killed under deep anesthesia by transcardiac perfusion with 4% (w/v) paraformaldehyde, and the brain was dissected carefully and post-fixed in 10% formalin for 2–12 h. After embedding in paraffin, 4- to 6- $\mu$ m sections were cut, mounted on slides, and stored at room temperature until further processing. Cell counts were performed blindly to the genotype.

For Nissl analysis, brain sections were cut at 6  $\mu$ m. Nissl staining was performed using cresyl

violet (0.5% [w/v]) for up to 10 s. Sections at the level of the dorsal hippocampus (bregma, 2.06 mm, as matched with the Franklin/Paxino mouse brain atlas [Franklin, 1998]) were selected for analysis. Five appropriate hippocampal sections were selected for each brain from three animals, resulting in analysis of  $n = 15$  sections per genotype and age group. Sections were pictured using the Zeiss Axiocam at  $\times 20$  magnification, under a light microscope. On these pictures the middle upper blade of the dentate gyrus was marked at a length of 500  $\mu\text{m}$ . Pictures were printed, and all cells counted encompassing the 500- $\mu\text{m}$  mark.

A proliferating cell nuclear antigen (PCNA) staining kit (Zymed Laboratories, South San Francisco, CA) was used according to the provider's instructions to stain for proliferating cells. Hippocampal sections at the level of the dorsal hippocampus were selected, and dark brown-stained nuclei were counted.

The NeuroTACS II kit (Trevigen, Gaithersburg, MD) was used according to the provider's instructions to stain for apoptotic cells (TUNEL staining). A positive control was generated using TACS nuclease to artificially fragment DNA.

For immunohistochemical staining with antibodies against p-ERK and calretinin, sections were cut at 6  $\mu\text{m}$ . Antibodies were used in the following dilutions: rabbit IgG against p-ERK (Tyr-204), 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-calretinin polyclonal antibody, 1:500 (Chemicon International, Temecula, CA). Immunoreactivity was visualized using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and DAB as the color reagent.

### ***Golgi Impregnation and Analysis***

For Golgi staining the FD Rapid Golgi stain kit (FD Neuro Technologies, Baltimore, MD) was used according to the provider's instructions, except for decreased staining times. Tissue was flash frozen in precooled methyl butane on dry ice and cut at 100  $\mu\text{m}$  using a cryomicrotome. For each brain five suprapyramidal granule neurons with a single primary dendrite were selected to match the following criteria: (1) location of the cell body in the middle third of the section; (2) relative isolation from neighboring impregnated cells; and (3) dark and consistent impregnation throughout the neuron. Dendrite lengths were measured, and dendritic branching points counted blindly to the genotype on live pictures at  $\times 40$  magnification, using the Zeiss Axioplan 2i light microscope, the Zeiss Axiocam MRc, and Axiovision software (Zeiss).

### ***In situ Hybridization***

For cRNA probes of the corticosteroid receptors a 500-bp fragment for each receptor was selected (*MR*, accession no. XM\_356093/Nr3c2, position 2701–3207; *GR*, accession no. NM\_008173/Nr3c1, position 1551–2062), and PCR primers flanking this fragment designed. Using mouse brain cDNA, fragments were amplified by PCR and cloned into the pDrive vector (Qiagen PCR cloning kit, Qiagen, Valencia, CA). Sequence-confirmed plasmids were linearized. Sense and antisense digoxigenin (DIG)-labeled riboprobes were synthesized using the DIG RNA labeling kit (SP6/T7; Roche Diagnostics, Indianapolis, IN). Labeled RNA was quantified with quantification strips (Roche), and probe sizes confirmed by RNA gel electrophoresis.

Mice were perfused transcardially with 4% paraformaldehyde, and the brains dissected immediately and postfixed in 4% paraformaldehyde at 4°C overnight. Brains were incubated in 30% sucrose at 4°C for 12 h before being frozen in TBS medium in precooled methyl butane on dry ice. Blocks were stored at  $-20^\circ\text{C}$  overnight. Sections (8  $\mu\text{m}$ ) were cut on a cryostat at  $-22^\circ\text{C}$  and dried overnight at room temperature. Prior to hybridization, sections were treated with 1%  $\text{H}_2\text{O}_2$  in methanol, 0.3% Triton X-100, and DEPC-PBS in a 65°C shaking water bath. Tissue was permeabilized with 1.5  $\mu\text{g}/\text{mL}$  RNase-free proteinase K at 37°C in buffer for 30 min and postfixed for 5 min in DEPC-PBS with 4% paraformaldehyde at 4°C. Sections were acetylated for 10 min in 0.1 M TEA buffer with 0.25% acetic anhydride and incubated in prehybridization buffer (4 $\times$  SSC, 50% [v/v] deionized formamide). Sections were hybridized with both sense and antisense probe. For overnight hybridization at 55°C, 180 ng DIG RNA probe/100  $\mu\text{L}$  hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1 $\times$  Denhardt's solution, 4 $\times$  SSC, 10 mM DTT, 100  $\mu\text{g}/\text{mL}$  yeast tRNA, 1 mg/mL denatured and sheared salmon sperm DNA) were used. The following day, sections were washed in decreasing concentrations of SSC and treated with 20  $\mu\text{g}/\text{mL}$  RNase A. Anti-DIG AP Fab fragments (Roche), diluted 1:400, were applied to the hybridized sections, and NBT/BCIP (Roche) was used to visualize the signal.

### ***RNA Extraction***

Total RNA was isolated from hippocampal tissue using Trizol (Invitrogen, Gaithersburg, MD) and the RNeasy kit (Qiagen), following the manufacturers' instructions.

### Northern Blot

RNA was quantified by spectrophotometry. Denaturing electrophoresis was carried out on a 1.0% agarose gel made with denaturing gel buffer (NorthernMax; Ambion, Austin, TX) and using 1× MOPS as running buffer. Total RNA (15 µg) was dissolved in H<sub>2</sub>O and loading buffer (NorthernMax) and heated to 65°C for 15 min prior to gel loading. Transfer to a BrightStar-Plus positively charged nylon membrane (Ambion) was carried out by capillary transfer using 20× SSC as transfer buffer. After transfer, RNA was cross-linked by baking the membrane at 80°C in vacuum for 30 min. Hybridization was carried out in Ultrahyb solution (Ambion). A prehybridization of 2 h was followed by an overnight hybridization at 42°C with <sup>32</sup>P-end-labeled oligonucleotide probes. After hybridization, membrane was washed twice in an aqueous solution of 2× SSC, 2× SSPE, and 10% SDS for 10 min at room temperature and once with the same solution at 42°C for 2 min. Membrane was exposed to film (Biomax MS, Kodak) at -80°C for 48–72 h. Before the next hybridization with another probe, membrane was stripped with boiling water, three times for 5 min.

Oligonucleotide antisense-probes (bFGF, 5'-GAA GCC GCC GTT CTT GCA GTA GAG-3'; BDNF, 5'-GCT CAA AAG TGT CAG CCA GTG ATG-3'; glucose-6-phosphate dehydrogenase ([G6PDH], 5'-CAT CAG CTC ATC TGC CTC TG-3') were end-labeled with <sup>32</sup>P using polynucleotide kinase (Roche). After incubation for 1 h, unincorporated label was removed using a MicroSpin column (Amersham Biosciences, Piscataway, NJ).

### Behavioral Testing: Morris Water Maze

The water maze consisted of a round pool, 150 cm in diameter, which was divided into four quadrants (A, B, C, and D). A translucent platform (20 cm high, 12 cm in diam.) was placed in the middle of quadrant A. The platform remained in the same quadrant throughout the training period. The pool was filled 0.5 cm above the platform with water at room temperature, and powdered milk was added to the pool to make the platform's location undetectable. Four extrinsic black-and-white cues (an ellipse, a star, a triangle, and bars) were affixed on the inside of the pool and not moved during the experiment. The investigator was visible to the mice but sat in the same location throughout testing. The maze training began 2–3 h into the light cycle. Mice were given a pretesting trial 1 d before training. During the pretrial, mice were placed on the platform and allowed

to swim in the pool for 15 s. The mice were given five training trials a day for five consecutive days (25 trials total). Mice were started from four starting points at the wall in the middle of each quadrant in a pseudorandom manner, different each day. By the end of each day, the mice had started from different positions the same number of times but never from the same starting point twice in a row. The latency from release facing the wall of the pool to the time the mouse touched the platform was recorded. If the mouse did not reach the platform within 60 s, it was guided to the platform. All mice were allowed to sit for 10 s on the platform and were then transferred to the next starting point. On the sixth day, mice were tested in a probe trial. After one more training trial with the platform present, the platform was removed from the pool. All mice started from the same starting point and were allowed to look for the platform for 60 s. The time the mice spent in quadrant A, which formerly contained the platform, and in all other quadrants was measured.

## Results

### *Lifelong Lack of Corticosterone and Reduction of Aldosterone Serum Levels in POMC Null Mutant Mice*

Four to 12 mice per age group were used. The data displayed here confirm and extend findings published in previous analyses of the POMC null mutant mouse (Yaswen et al., 1999; Coll et al., 2004; Karpac et al., 2005). Corticosterone serum levels were below detectable levels (25 ng/mL in the test used) in all POMC null mutant mice tested, regardless of the age of the mouse (Fig. 1A). Aldosterone levels in POMC null mutant mice were consistently reduced to ~25% of those of wild-type littermates, as long as adrenal structures were present (Fig. 1B). We did not find adrenal structures or measurable levels of aldosterone past 8 mo of age.

### *Decrease of Granule Cell Density in the Dentate Gyrus With Decrease in Cell Proliferation but No Increase in Cell Degeneration*

A total of 12 wild-type and 12 mutant mice were used, each grouped into four different age groups. POMC null mutant mice display a decrease in dentate gyrus cell density (Fig. 2A–C), most prominent in older animals. In addition to the cell loss, some POMC null mutant mice show an increase in granule cell size (Fig. 2B). No changes could be observed in cornu ammonis subfields in any age group (data not shown).

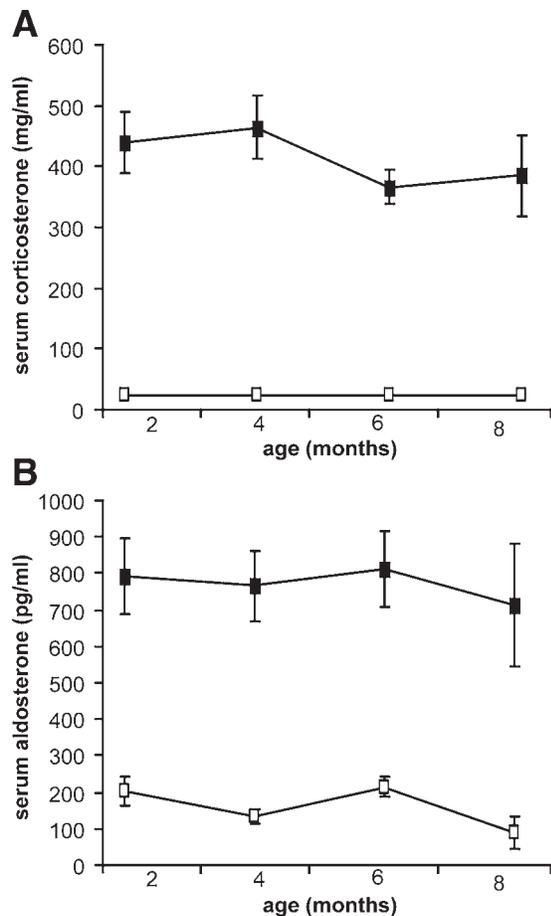


Fig. 1. Corticosterone (A) and aldosterone (B) serum levels in POMC null mutant mice and wild-type controls over 8 mo. Serum corticosterone was below detectable levels (<25 ng/mL) in all POMC null mutant mice tested. ■, Wild type; □, mutant.

Three mice of each genotype at the age of 3.5 mo were used for PCNA staining. Immunocytochemical staining for PCNA was carried out as a marker for neurogenesis. PCNA staining revealed less proliferating cells in the POMC null mutant dentate gyrus (Fig. 3).

Three mice of each genotype, 3.5 mo of age, were used for TUNEL staining, and two mice of each genotype, aged 3 and 6.5 mo, were used for immunohistochemical staining with antibodies against p-ERK and calretinin. Direct evidence for cell degeneration could not be obtained, as neither TUNEL staining for apoptotic cells nor immunocytochemical staining for p-ERK, a marker of activated astrocytes, revealed positive cells in either wild-type or mutant mice (data not shown). An increase in the number of hilus neurons as reported in the MR<sup>-/-</sup> mouse (Gass et al., 2000) could not be observed. Immunocytochemical staining for

calretinin, a marker for a subset of GABAergic hilus neurons, showed equal numbers of positive cells in wild-type and mutant dentate gyri (data not shown).

### ***Fine Morphology of Granule Cells is Not Affected***

Adrenalectomy has been shown to affect the morphology of dentate gyrus granule cells (Hashimoto et al., 1989; Gould et al., 1990). Brains of four 4.5-month-old mice of each genotype were processed for Golgi staining. Quantitative analysis of Golgi-impregnated granule cells of the POMC null mutant mouse revealed no differences in either total dendrite length or dendrite branching (Table 1).

### ***Expression of MR and GR Genes in the Dentate Gyrus is Not Changed***

MRs and GRs promote the effects of corticosterone on granule cells. *In situ* hybridization of sections from 4-month-old mice with DIG-labeled RNA antisense probes showed expression of both receptors in the POMC null mutant dentate gyrus. No signal was detected using a sense probe (Fig. 4). Qualitative analysis revealed no striking differences in expression levels among wild-type and mutant mice.

### ***Growth Factor Expression in the Hippocampus is Not Altered***

Northern blot analysis of total hippocampal RNA collected from four mice of each genotype, 5 mo old, was carried out to assess expression levels of bFGF and BDNF. Qualitative analysis revealed no differences in the levels of expression among wild-type and mutant mice. G6PDH was used as loading control (Fig. 5).

### ***Spatial Learning is Not Impaired***

A total of 10 wild-type and nine POMC null mutant male mice were tested in the Morris water maze when they were 3.5–5.5 mo old. POMC-null mutants display no major impairment of performance in the Morris water task. Mean escape latencies for wild-type and mutant mice during the 5-d training are shown in Fig. 6. Both mutant and wild-type mice were able to decrease their escape latencies from day 1 to 3 but showed no further improvement of performance at days 4 and 5. In the probe trial, both groups spent more time in the quadrant that formerly contained the platform, but the difference was statistically significant only for the wild type.

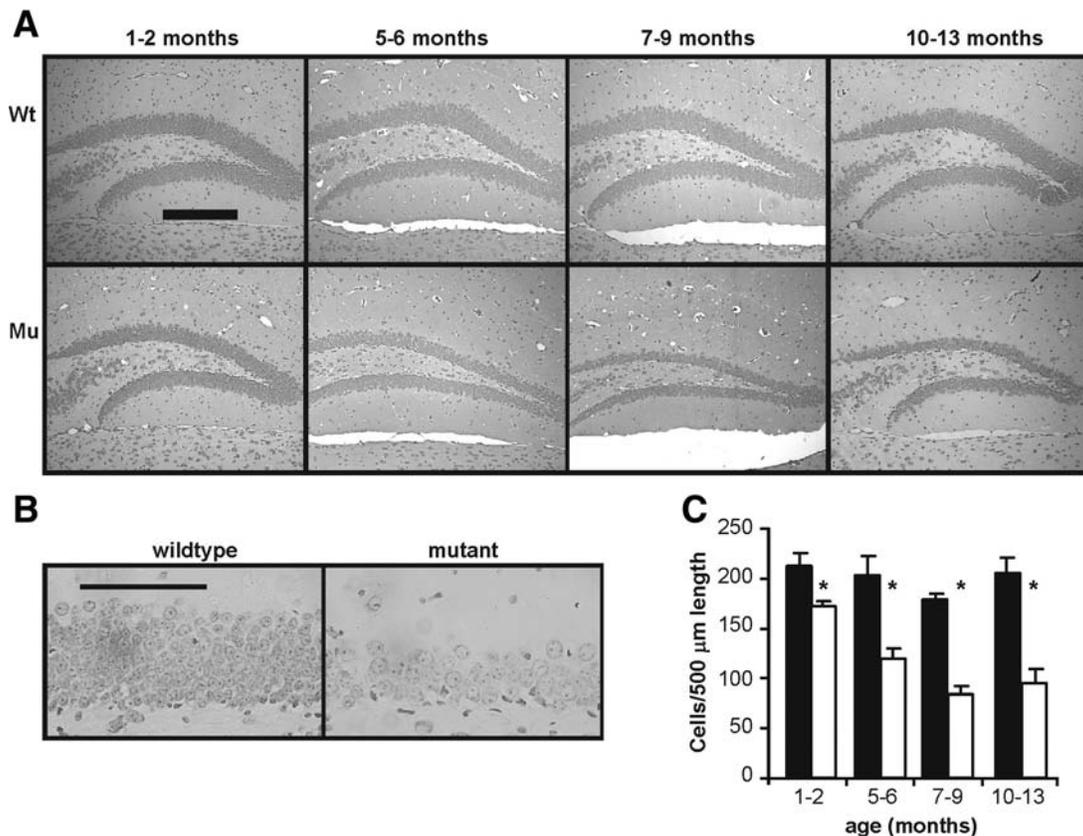


Fig. 2. Nissl analysis of the dentate gyrus in POMC null mutant mice and wild-type controls. (A) Nissl-stained sections of dentate gyri from POMC wild-type (Wt) and null mutant (Mu) mice at indicated ages. The 500- $\mu\text{m}$  scale bar in the first picture applies to all images. (B) Cell loss and different cell sizes in the POMC null mutant dentate gyrus. Shown are images of the dentate gyrus' upper blades in the 10- to 13-mo age group in A at higher magnification. The 100- $\mu\text{m}$  scale bar in the first picture applies to both. (C) Mean granule cell numbers in the upper blade of the dentate gyrus counted per 500- $\mu\text{m}$  length in sections from ages indicated. Solid bar, wild type; open bar, mutant. (\*)  $p < 0.05$  for all mean differences (ANOVA single-factor analysis).

## Discussion

The POMC null mutant mouse has no corticosterone throughout life. The effect of this absence of corticosterone on the dentate gyrus of the hippocampus is a decrease in granule cell density, which is most prominent in older mutant animals. The number of proliferating cells is decreased, whereas neurodegeneration could not be demonstrated, most likely implying that the decrease in neurogenesis causes the overall reduction in granule cells of the dentate gyrus. Fine morphology of granule cells remains unaltered. Expression levels of the MR, GR, bFGF, and BDNF are not changed. Spatial learning of the POMC null mutant mouse is not impaired in the Morris water maze.

In the following text we will put these results in the context of findings from other studies of the

effects of increased or decreased levels of corticosteroids on the hippocampus. These studies investigated the acute or chronic effects of increased corticosterone by administering corticosterone to rats, and those of decreased corticosterone by adrenalectomy, or clamping, in rats and by genetic deletion of corticosteroid-binding receptors in mice.

The effect of increased corticosterone levels on granule cell neurogenesis in the dentate gyrus of the hippocampus is a slowing down of cell birth (Gould et al., 1991, 1992, 1998; Cameron and Gould, 1994; Ambrogini et al., 2002), whereas adrenalectomy, i.e., absence of corticosterone, increases the proliferation of progenitor cells (Gould et al., 1991, 1992; Cameron and Gould, 1994; Rodriguez et al., 1998; Cameron and McKay, 1999; Nichols et al., 2001). In general, the rate of granule cell proliferation is inversely related to basal glucocorticoid levels. However,

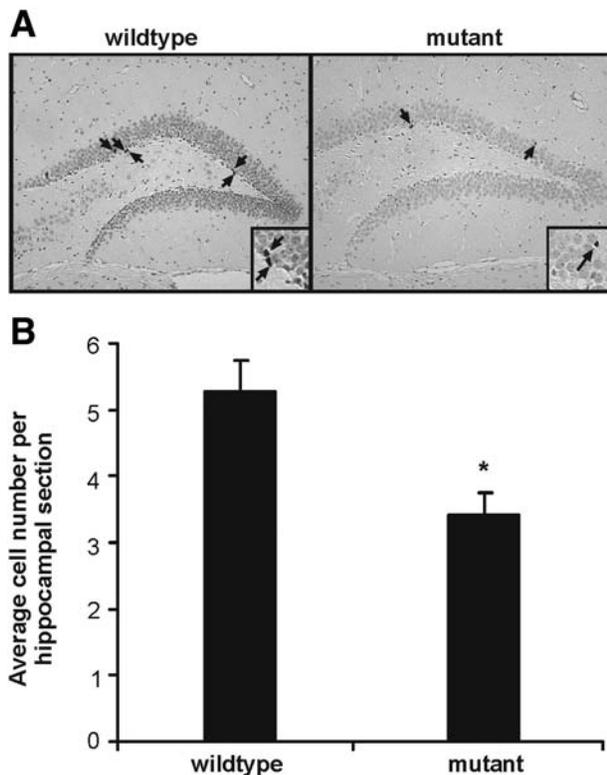


Fig. 3. Neurogenesis in the dentate gyrus of POMC null mutant mice and wild-type controls. (A) PCNA-positive cells (arrows) in the dentate gyrus. These sections are examples from the quantitative analysis reported in B. The inserts show positive cells at higher magnification. (B) Average number of PCNA-positive nuclei per hippocampal section. A total of  $n = 31$  sections of three wild-type animals and  $n = 48$  sections of three mutant animals were analyzed. (\*)  $p < 0.05$  for the mean difference (ANOVA single-factor analysis).

adrenalectomy in early postnatal rats, followed by supplementation with chronically low corticosterone throughout life (clamped rats), had no effect on granule cell proliferation (Brunson et al., 2005). Complete lifelong absence of corticosterone in POMC null mutants resulted in a decrease of cell proliferation (see above, Results). If glucocorticoid levels determine the rate of neurogenesis in the adult hippocampus, then granule cell production should be significantly increased in chronically clamped rats and in POMC null mutant mice compared with age-matched controls. That this is not the case necessitates modification of the model of inverse correlation of glucocorticoids and hippocampal neurogenesis.

Although corticosterone affects proliferation of granule cell precursors, it affects death of mature granule cells. There is an increase in cell death and a severe loss of granule cells with lack of cortico-

Table 1  
Analysis of Granule Cell Morphology  
in the Dentate Gyrus of POMC Null Mutant  
Mice and Wild-Type Controls

Morphological variable	Wild type	Mutant
Total dendrite length ( $\mu\text{m}$ )	$659.9 \pm 33.1$	$652.7 \pm 29.0$
No. of dendritic branch points	$6.1 \pm 0.3$	$6.1 \pm 0.3$

Quantitative Golgi analysis was performed of 20 individual granule cell neurons with a single primary dendrite within a 100- $\mu\text{m}$ -thick section for each genotype. Values represent mean  $\pm$  S.E.

sterone, which can be prevented by both aldosterone and corticosterone. In the first report on granule cell degeneration following adrenalectomy, Sloviter et al. (1989) demonstrate nearly complete cell loss in the dentate gyrus 3–4 mo after adrenalectomy. In subsequent studies granule cell loss was consistently observed after adrenalectomy, although at varying degrees (Sapolsky et al., 1991; Sloviter et al., 1993). In agreement with earlier reports that even low doses of corticosterone prevent granule cell loss after adrenalectomy (Sloviter et al., 1989), no change in cell death was observed over time in the clamped rat model (low corticosterone [Brunson et al., 2005]). In the POMC null mutant mouse, cell loss over lifetime was not as dramatic as in some adrenalectomized animals after shorter periods but occurred reliably and was most pronounced in older mutant animals. However, we did not see increased cell death of hippocampal granule cells. Thus, the most likely explanation for the observed overall cell loss in the POMC null mutant dentate gyrus is the decrease in granule cell neurogenesis. Supplementing adrenalectomized rats with either corticosterone or aldosterone prevents granule cell death (Sloviter et al., 1989; Gould et al., 1990). In one study aldosterone completely protected granule cells against adrenalectomized cell death, whereas a glucocorticoid agonist protected only partially (Woolley et al., 1991). Thus, the low levels of aldosterone present in POMC null mutant mice might be sufficient to protect from cell death. Interestingly, both the clamped rat and the POMC null mutant mouse do not show increased cell death, with the former having low corticosterone and no aldosterone, and the latter no corticosterone and low aldosterone.

Lack of corticosterone also resulted in a decrease in the number of dendritic branch points of granule cells, as reported for 7 d (Gould et al., 1990) and for 3 wk (Hashimoto et al., 1989) after adrenalectomy.

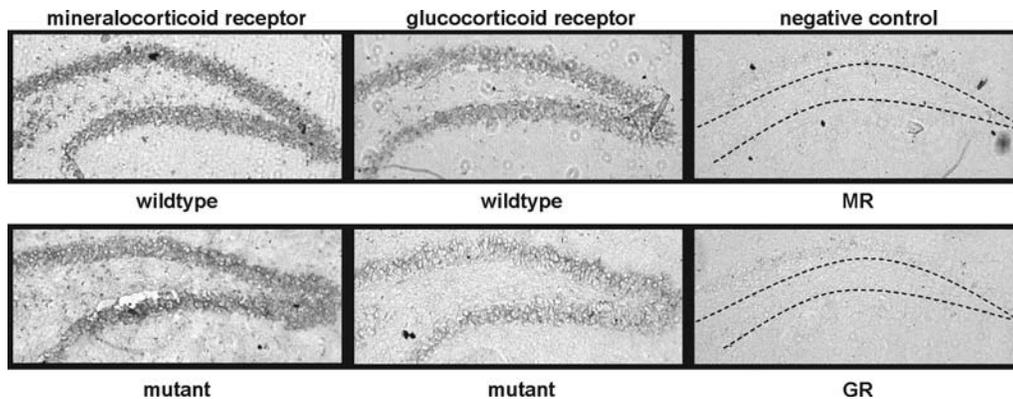


Fig. 4. *MR* and *GR* expression in the dentate gyrus of POMC null mutant mice and wild-type controls. *In situ* hybridization of hippocampal sections of 4-mo-old mice using DIG-labeled antisense (*MR*, *GR*) and sense (control) riboprobes.

We did not observe this in 4.5-mo-old POMC null mutant mice. This could mean that either the fine structural changes in our model are only transient or they do not occur at all. Total dendrite lengths in both cases, after adrenalectomy and in the POMC null mutant mouse, stayed the same.

Corticosterone and aldosterone exert their actions through the high-affinity MR and the low-affinity GR. The expression levels of these receptors are inversely correlated with corticosterone levels. High levels of corticosterone result in down-regulation of MR and GR in the hippocampus, whereas adrenalectomy has been reported to up-regulate the expression level of both the MR and the GR in the hippocampus and the dentate gyrus specifically, on both mRNA and protein levels (Patchev et al., 1994; Chao et al., 1998; Herman and Spencer, 1998; Kalman and Spencer, 2002). However, mRNA expression for *GR* in granule cells of clamped, low-corticosterone rats was not changed and was only transiently increased during postnatal development for *MR* (Brunson et al., 2005). In the POMC null mutant mouse, the mRNA for neither receptor is increased, as demonstrated by our *in situ* hybridizations. The effects seen after adrenalectomy are changes in gene expression resulting from the acute loss of adrenal steroids. In the POMC null mutant mouse, however, the absence of corticosterone is perpetual, and constant up-regulation of receptor expression might not be economic. Supporting this, Reul et al. (1989) demonstrated that receptor mRNA levels in the dentate gyrus/hippocampus increase on the first day after adrenalectomy but return to intact levels over the course of 16 d.

Interestingly, complete lack of GR in brain-specific GR null mutant mice has no effect on neuro-

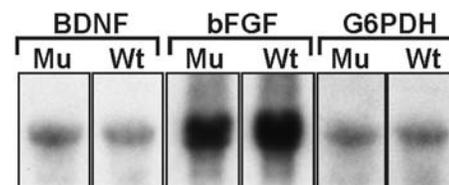


Fig. 5. Expression of corticoid-regulated genes in POMC null mutant mice and wild-type controls. Northern blot analysis of total hippocampal RNA from wild-type (Wt) and mutant (Mu) mice using oligonucleotide probes for BDNF and bFGF. G6PDH was used as loading control.

genesis or cell death in the dentate gyrus, whereas MR null mutant mice show cell loss more prominent and developing earlier than in our POMC null mutant mouse (Gass et al., 2000). Gass et al. (2000) also reported a reduction in neurogenesis in the adult *MR<sup>-/-</sup>* mouse dentate gyrus, which is comparable to our findings in the POMC null mutant mouse. However, these investigators suggested the increased corticosterone levels in MR null mutant mice as the cause for the reduced neurogenesis.

BDNF and bFGF are two growth factors involved in neuronal differentiation and plasticity (Sousa and Almeida, 2002). Adrenalectomy has been reported to modulate BDNF and bFGF mRNA levels in the hippocampus (Barbany and Persson, 1992; Chao and McEwen, 1994; Hansson et al., 2000). In the POMC null mutant mouse no changes in expression of these neurotrophic factors could be detected by Northern blot analysis of total hippocampal RNA. Here again, the difference might be attributable to the time points of analysis. Although the studies cited focus on short-term changes in expression, analysis in our model assessed the effects of long-term corticosterone depletion.

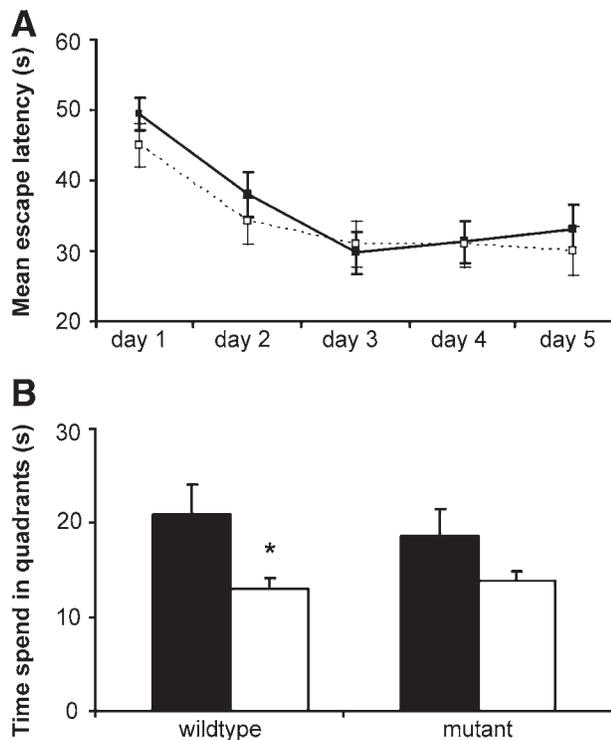


Fig. 6. Spatial learning in POMC null mutant mice and wild-type controls. **(A)** Escape latencies in the Morris water task (in seconds). ■, Wild type; □, mutant. Mean escape latencies represent 5 trials of 10 (wild-type) or 9 (mutant) animals per training day. **(B)** Performance in the probe trials. Solid bar, time in seconds spent in quadrant A, which during training contained the platform; open bar, mean time, in seconds, spent in all other quadrants. The increase in time spent in quadrant A is statistically significant (\*)  $p < 0.05$  for the wild-type but not for the mutant group ( $p = 0.15$ ).

The correlation of plasma glucocorticoids and granule cell neurogenesis has led to the hypothesis that age-related elevated plasma glucocorticoid levels are responsible for decreased neurogenesis in the aging hippocampus and further that the decreased neurogenesis might contribute to age-related memory deficits (Gould et al., 1992; Cameron and McKay, 1999; Drapeau et al., 2003; Montaron et al., 2005). On the other hand, human patients with severe or long-standing adrenal insufficiency also show impairment of memory in 5%–20% of cases (Leigh and Kramer, 1984). In rodents the Morris water task has been used to study the impact of corticosterone depletion on hippocampus-mediated memory function. Adrenalectomized rats learn the location of the platform slower than control groups do (Conrad and Roy, 1993) and, at least in one report, perform worse in a probe trial (Islam et al., 1995).

However, in a different study, no impairment in learning and probe trial was reported 3 mo after surgery in middle-aged rats (Conrad and Roy, 1995). A distinctive cell loss appears to be necessary to impair performance in the water maze (Armstrong et al., 1993). Lifelong low levels of corticosterone (clamped rats) did not result in any impairment in memory function (Brunson et al., 2001). Similarly, the POMC null mutant mouse shows no impairment in learning and probe trial compared with wild-type controls. A reason might be that at the age we tested the mice (3.5–5.5 mo), the damage to the dentate gyrus was too small to result in functional consequences. However, our results show that the lifelong absence of corticosterone *per se* does not impair spatial learning.

Table 2 summarizes the most pertinent findings in the POMC null mutant mouse model in comparison with other models with respect to corticosterone levels and effects on the dentate gyrus of the hippocampus. It is obvious that there is no simple correlation between corticosterone levels and structure and function of the hippocampus. How can we explain these results? The first aspect to consider is methodology: Different experimental approaches were used to detect cell proliferation or cell death, to measure cell numbers, or to test learning in the Morris water task. Although such differences might account for some of the results, it is unlikely that all findings can be explained by differences in methods. Another factor is the use of different species: Some observations and correlations were made in humans, most experimental results are from rats, and some are from mice. Although all are vertebrates, there are differences even between the two rodent species. A more significant factor to consider is the time course of the different studies: Effects can be expected to be different between short- and long-term modifications, such as the continued absence of corticosterone starting at birth versus the acute effects of adrenalectomy in the adult animal. Adult adrenalectomized animals spend their entire lifetime, including their early development, with normal corticosteroid serum levels. From the time point of adrenalectomy on, they are challenged with the acute absence of corticosterone and most likely urged to compensate for the loss, if compensation is possible (e.g., up-regulation of corticosteroid receptor expression). The clamped rat, however, has low levels of corticosterone from early postnatal development onward (Brunson et al., 2005), and the POMC null mutant mouse is without corticosterone from the day of birth.

Table 2  
Summary of Findings in POMC Null Mutant Mouse Model vs Other Models: Corticosterone Levels, Homeostasis, and Effects on Hippocampus

	Corticosterone levels		None		
	Model	High Extra corticosterone	Low Clamping	POMC <sup>-/-</sup>	Adrenalectomy
Homeostatic context	Corticosterone	↑	↓	⊙	⊙
	Aldosterone	~	⊙	↓	⊙
	CRH	↓	↑	↑	↑
	ACTH	↓	↑	⊙	↑
Hippocampal phenotype	Neurogenesis	↓	~	↓	↑
	Cell death	↓	~	~	↑
	Size of GC layer	~	~	↓	↓
	Memory function	↓	~	~	↓ or ~

↑, increased; ↓, decreased; ⊙, not present at all; ~, unchanged.

Brunson et al. (2005) suggested that early-life onset reduction in plasma corticosterone results in adaptive mechanisms that overcome the relationship of corticosterone and neurogenesis seen in experiments with adult-onset reduction. Probably the most significant aspect to consider is the homeostatic context in the different studies. For example, aldosterone is absent in adrenalectomized animals, low in POMC null mutants, and normal in corticosterone-supplemented animals with high corticosterone levels. Aldosterone has been shown to completely prevent cell death caused by absence of corticosterone. Low levels of aldosterone might explain the lack of cell death seen in the POMC null mutant mouse. Other factors contributing to the homeostatic context are ACTH and corticotropin-releasing hormone (CRH), which are part of the hypothalamic-pituitary-adrenal (HPA) axis counter-regulatory response to changing corticosterone levels. Absence of corticosterone in POMC null mutants results in increased levels of CRH, but because of the deletion of the POMC gene ACTH serum levels are zero (Yaswen et al., 1999). In adrenalectomized animals, however, the HPA axis reacts to the loss of corticosterone with compensatory upregulation of both CRH and ACTH (Eberwine and Roberts, 1984). Thus, different ACTH levels might also account for some of the differences between adrenalectomized animals and the POMC null mutant mouse.

A way to reconcile the observed effects of varying corticosterone levels on hippocampal structure and function is to assume a scenario in which the effects

of high levels of corticosterone are not the opposite of those of low levels, but rather a reflection of the disturbance of the overall homeostatic equilibrium of the system. As the experimental models become more refined and sophisticated, they reveal the biological phenomenon under study as increasingly complex. It is likely that this moves us rather closer to the truth, which might well be a delicately balanced homeostatic equilibrium in which disturbances of any one factor can offset the system in varying ways.

## Acknowledgments

We thank Stephanie Bui, Peggy Hunnewell, and OMRF's Imaging Facility for their technical assistance, Mary Flynn for artwork, and the German National Academic Foundation for supporting D. O.

## References

- Ambrogini P., Orsini L., Mancini C., Ferri P., Barbanti I., and Cuppini R. (2002) Persistently high corticosterone levels but not normal circadian fluctuations of the hormone affect cell proliferation in the adult rat dentate gyrus. *Neuroendocrinology* **76**, 366–372.
- Armstrong J. N., McIntyre D. C., Neubort S., and Sloviter R. S. (1993) Learning and memory after adrenalectomy-induced hippocampal dentate granule cell degeneration in the rat. *Hippocampus* **3**, 359–371.
- Barbany G. and Persson H. (1992) Regulation of neurotrophin mRNA expression in the rat brain by glucocorticoids. *Eur. J. Neurosci.* **4**, 396–403.
- Brunson K. L., Baram T. Z., and Bender R. A. (2005) Hippocampal neurogenesis is not enhanced by lifelong reduction of glucocorticoid levels. *Hippocampus* **15**, 491–501.

- Brunson K. L., Eghbal-Ahmadi M., Bender R., Chen Y., and Baram T. Z. (2001) Long-term, progressive hippocampal cell loss and dysfunction induced by early-life administration of corticotropin-releasing hormone reproduce the effects of early-life stress. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8856–8861.
- Cameron H. A. and Gould E. (1994) Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience* **61**, 203–209.
- Cameron H. A. and McKay R. D. (1999) Restoring production of hippocampal neurons in old age. *Nat. Neurosci.* **2**, 894–897.
- Chao H. M. and McEwen B. S. (1994) Glucocorticoids and the expression of mRNAs for neurotrophins, their receptors and GAP-43 in the rat hippocampus. *Brain Res. Mol. Brain Res.* **26**, 271–276.
- Chao H. M., Ma L. Y., McEwen B. S., and Sakai R. R. (1998) Regulation of glucocorticoid receptor and mineralocorticoid receptor messenger ribonucleic acids by selective agonists in the rat hippocampus. *Endocrinology* **139**, 1810–1814.
- Coll A. P., Challis B. G., Yeo G. S., Snell K., Piper S. J., Halsall D., et al. (2004) The effects of proopiomelanocortin deficiency on murine adrenal development and responsiveness to adrenocorticotropin. *Endocrinology* **145**, 4721–4727.
- Conrad C. D. and Roy E. J. (1995) Dentate gyrus destruction and spatial learning impairment after corticosteroid removal in young and middle-aged rats. *Hippocampus* **5**, 1–15.
- Conrad C. D. and Roy E. J. (1993) Selective loss of hippocampal granule cells following adrenalectomy: implications for spatial memory. *J. Neurosci.* **13**, 2582–2590.
- De Kloet E. R., Vreugdenhil E., Oitzl M. S., and Joels M. (1998) Brain corticosteroid receptor balance in health and disease. *Endocr. Rev.* **19**, 269–301.
- Drapeau E., Mayo W., Aurousseau C., Le Moal M., Piazza P. V., and Abrous D. N. (2003) Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 14385–14390.
- Eberwine J. H. and Roberts J. L. (1984) Glucocorticoid regulation of pro-opiomelanocortin gene transcription in the rat pituitary. *J. Biol. Chem.* **259**, 2166–2170.
- Franklin K. P. G. (1998). *The Mouse Brain in Stereotaxic Coordinates*, Academic Press, San Diego, CA.
- Gass P., Kretz O., Wolfer D. P., Berger S., Tronche F., Reichardt H. M., et al. (2000) Genetic disruption of mineralocorticoid receptor leads to impaired neurogenesis and granule cell degeneration in the hippocampus of adult mice. *EMBO Rep.* **1**, 447–451.
- Gould E., Cameron H. A., Daniels D. C., Woolley C. S., and McEwen B. S. (1992) Adrenal hormones suppress cell division in the adult rat dentate gyrus. *J. Neurosci.* **12**, 3642–3650.
- Gould E., Tanapat P., McEwen B. S., Flugge G., and Fuchs E. (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3168–3171.
- Gould E., Woolley C. S., and McEwen B. S. (1990) Short-term glucocorticoid manipulations affect neuronal morphology and survival in the adult dentate gyrus. *Neuroscience* **37**, 367–375.
- Gould E., Woolley C. S., Cameron H. A., Daniels D. C., and McEwen B. S. (1991) Adrenal steroids regulate postnatal development of the rat dentate gyrus. II. Effects of glucocorticoids and mineralocorticoids on cell birth. *J. Comp. Neurol.* **313**, 486–493.
- Hansson A. C., Cintra A., Belluardo N., Sommer W., Bhatnagar M., Bader M., et al. (2000) Gluco- and mineralocorticoid receptor-mediated regulation of neurotrophic factor gene expression in the dorsal hippocampus and the neocortex of the rat. *Eur. J. Neurosci.* **12**, 2918–2934.
- Hashimoto H., Marystone J. F., Greenough W. T., and Bohn M. C. (1989) Neonatal adrenalectomy alters dendritic branching of hippocampal granule cells. *Exp. Neurol.* **104**, 62–67.
- Herman J. P. and Spencer R. (1998) Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo. *J. Neurosci.* **18**, 7462–7473.
- Islam A., Henriksson B., Mohammed A., Winblad B., and Adem A. (1995) Behavioural deficits in adult rats following long-term adrenalectomy. *Neurosci. Lett.* **194**, 49–52.
- Kalman B. A. and Spencer R. L. (2002) Rapid corticosteroid-dependent regulation of mineralocorticoid receptor protein expression in rat brain. *Endocrinology* **143**, 4184–4195.
- Karpac J., Ostwald D., Bui S., Hunnewell P., Shankar M., and Hochgeschwender U. (2005) Development, maintenance, and function of the adrenal gland in early postnatal proopiomelanocortin-null mutant mice. *Endocrinology* **146**, 2555–2562.
- Leigh H. and Kramer S. I. (1984) The psychiatric manifestations of endocrine disease. *Adv. Intern. Med.* **29**, 413–445.
- McEwen B. S. (1999) Stress and hippocampal plasticity. *Annu. Rev. Neurosci.* **22**, 105–122.
- McEwen B. S., Albeck D., Cameron H., Chao H. M., Gould E., Hastings N., et al. (1995) Stress and the brain: a paradoxical role for adrenal steroids. *Vitam. Horm.* **51**, 371–402.
- McKittrick C. R., Magarinos A. M., Blanchard D. C., Blanchard R. J., McEwen B. S., and Sakai R. R. (2000) Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites. *Synapse* **36**, 85–94.
- McNeill T. H., Masters J. N., and Finch C. E. (1991) Effect of chronic adrenalectomy on neuron loss and distribution of sulfated glycoprotein-2 in the dentate gyrus of prepubertal rats. *Exp. Neurol.* **111**, 140–144.
- Montaron M. F., Drapeau E., Dupret D., Kitchener P., Aurousseau C., Le Moal M., et al. (2005) Lifelong corticosterone level determines age-related decline in neurogenesis and memory. *Neurobiol. Aging* (Epub ahead of print 13 June).
- Nichols N. R., Zieba M., and Bye N. (2001) Do glucocorticoids contribute to brain aging? *Brain Res. Brain Res. Rev.* **37**, 273–286.

- Patchev V. K., Brady L. S., Karl M., and Chrousos G. P. (1994) Regulation of HSP90 and corticosteroid receptor mRNA by corticosterone levels in vivo. *Mol. Cell. Endocrinol.* **103**, 57–64.
- Raffin-Sanson M. L., de Keyser Y., and Bertagna X. (2003) Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. *Eur. J. Endocrinol.* **149**, 79–90.
- Reul J. M., Pearce P. T., Funder J. W., and Krozowski Z. S. (1989) Type I and type II corticosteroid receptor gene expression in the rat: effect of adrenalectomy and dexamethasone administration. *Mol. Endocrinol.* **3**, 1674–1680.
- Rodriguez J. J., Montaron M. F., Petry K. G., Aourousseau C., Marinelli M., Premier S., et al. (1998) Complex regulation of the expression of the polysialylated form of the neuronal cell adhesion molecule by glucocorticoids in the rat hippocampus. *Eur. J. Neurosci.* **10**, 2994–3006.
- Roy E. J., Lynn D. M., and Bemm C. W. (1990) Individual variations in hippocampal dentate degeneration following adrenalectomy. *Behav. Neural Biol.* **54**, 330–336.
- Sapolsky R. M., Stein-Behrens B. A., and Armanini M. P. (1991) Long-term adrenalectomy causes loss of dentate gyrus and pyramidal neurons in the adult hippocampus. *Exp. Neurol.* **114**, 246–249.
- Sloviter R. S., Sollas A. L., Dean E., and Neubort S. (1993) Adrenalectomy-induced granule cell degeneration in the rat hippocampal dentate gyrus: characterization of an in vivo model of controlled neuronal death. *J. Comp. Neurol.* **330**, 324–336.
- Sloviter R. S., Valiquette G., Abrams G. M., Ronk E. C., Sollas A. L., Paul L. A., and Neubort S. (1989) Selective loss of hippocampal granule cells in the mature rat brain after adrenalectomy. *Science* **243**, 535–538.
- Sousa N. and Almeida O. F. (2002) Corticosteroids: sculptors of the hippocampal formation. *Rev. Neurosci.* **13**, 59–84.
- Vreugdenhil E., de Kloet E. R., Schaaf M., and Datson N. A. (2001) Genetic dissection of corticosterone receptor function in the rat hippocampus. *Eur. Neuropsychopharmacol.* **11**, 423–430.
- Woolley C. S., Gould E., and McEwen B. S. (1990) Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. *Brain Res.* **531**, 225–231.
- Woolley C. S., Gould E., Sakai R. R., Spencer R. L., and McEwen B. S. (1991) Effects of aldosterone or RU28362 treatment on adrenalectomy-induced cell death in the dentate gyrus of the adult rat. *Brain Res.* **554**, 312–315.
- Yaswen L., Diehl N., Brennan M. B., and Hochgeschwender U. (1999) Obesity in the mouse model of proopiomelanocortin deficiency responds to peripheral melanocortin. *Nat. Med.* **5**, 1066–1070.