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ABCB1 (MDR1)-Type P-Glycoproteins at the Blood–Brain Barrier Modulate the Activity of the Hypothalamic–Pituitary–Adrenocortical System: Implications for Affective Disorder

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Multidrug-resistance gene 1-type P-glycoproteins (ABCB1-type P-gps) protect the brain against the accumulation of many toxic xenobiotics and drugs. We recently could show that the access of the endogenous glucocorticoids corticosterone and cortisol to the brain are regulated by ABCB1-type P-gps *in vivo*. ABCB1-type P-gp function, therefore, is likely to exert a profound influence on the regulation of the hypothalamic–pituitary–adrenocortical (HPA) system. Hyperactivity of the HPA system is frequently observed in human affective disorder, and a considerable amount of evidence has been accumulated suggesting that normalization of the HPA system might be the final step necessary for stable remission of the disease. To examine whether blood–brain barrier (BBB) function influences neuroendocrine regulation, we investigated HPA system activity in *abcb1ab* (−/−) mice under basal conditions and following stress. *Abcb1ab* (−/−) mice showed consistently lower plasma ACTH levels and lower evening plasma corticosterone levels. CRH mRNA expression in the hypothalamic paraventricular nucleus was decreased and pituitary POMC mRNA expressing cells were significantly reduced in number in *abcb1ab* (−/−) mutants; however, they showed a normal activation of the HPA system following CRH stimulation. Lower doses of dexamethasone were required to suppress plasma corticosterone levels in mutants. Our data thus provide evidence for a sustained suppression of the HPA system at the hypothalamic level in *abcb1ab* (−/−) mice, suggesting that BBB function significantly regulates HPA system activity. Whether naturally occurring polymorphisms in the human ABCB1 gene might result in persistent changes in the responsiveness and regulation of the HPA system will be the subject of future investigations, correlating both genetic information with individual characteristics of the neuroendocrine phenotype.
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INTRODUCTION

Glucocorticoid hormones, secreted by the adrenal cortex, are potent modulators of neuronal activity and function: they control the excitability of neuronal networks that underlie emotions and cognitive processes, such as learning and memory (Belanoff *et al*, 2001). In addition, corticosteroids play extremely important roles in modulating fear and anxiety-related behavior (Korte, 2001). In concert with other components of the stress hormone system, glucocorticoids maintain basal activity of the hypothalamic–pituitary–adrenocortical (HPA) system and control the sensitivity or threshold of the HPA system's response to stress (for a review, see de Kloet *et al*, 1998). Glucocorticoids help to terminate stress-induced HPA system activation via negative feedback inhibition at the level of the hypothalamic paraventricular nucleus and the hippocampus (Erkut *et al*, 1998). Within the HPA system, corticotropin-releasing hormone (CRH) is the primary hypothalamic hypophysiotropic factor regulating basal and stress-induced release of pituitary corticotropin (ACTH; for a review, see Owens and Nemeroff, 1991; Aguilera, 1998). CRH triggers the immediate release of corticotropin (ACTH) from the anterior pituitary, subsequently leading to release of glucocorticoid hormones (GC, cortisol in humans and corticosterone in rodents) from the adrenal cortex.

Hyperactivity of the HPA system is observed in a substantial percentage of depressed patients, and a considerable amount of evidence has been accumulated suggesting that normalization of the HPA system might be the final step necessary for stable remission of the disease (for a review, see Holsboer, 1999, 2000). Additional evidence

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for this HPA system hyperactivity stems from human *post-mortem* studies, showing that depressed patients display an increased expression of hypothalamic CRH (Raadsheer *et al*, 1994, 1995). Antidepressant drugs, in turn, have been shown to attenuate and normalize those HPA system abnormalities (for a review, see Holsboer and Barden, 1996). Physiological control of the access of endogenous glucocorticoids into the brain, therefore, is an important issue. The question of whether increased levels of circulating glucocorticoids might exert detrimental effects on the central nervous system has been discussed controversially (for a review, see Joels, 2001): recent investigations, which examined the human hippocampus in both the clinical condition of major depression and following treatment with glucocorticoids for possible structural alterations, revealed no evidence of any major hippocampal cell death or damage due to elevated plasma glucocorticoid levels (Lucassen *et al*, 2001; Müller *et al*, 2001a), as described following prolonged and fatal stress in nonhuman primates (Sapolsky *et al*, 1985; Uno *et al*, 1989).

Recently, the ABCB1-type P-glycoprotein (ABCB1-type P-gp), a 170 kDa glycoprotein that is the gene product of the multidrug-resistance gene (also called MDR1-type P-gps) and that belongs to a phylogenetically highly conserved superfamily of ATP-binding cassette (ABC) transport proteins (for a review, see Croop *et al*, 1989), has been found to be responsible for differences in brain penetration of antidepressant drugs (Uhr *et al*, 2000, 2003). ABCB1-type P-gp actively transports substrates against a concentration gradient. Several investigations have confirmed an important role for ABCB1-type P-gps in the blood–brain barrier (BBB), protecting the central nervous system against the accumulation of a wide range of toxic xenobiotics, and also various drugs (for review: Schinkel, 1998). Both murine *abcb1a* and *abcb1b*, which together embody all functions displayed by one single human ABCB1 (Devault and Gros, 1990), are expressed on the luminal surface of cerebral endothelial cells or the brain parenchyma (Regina *et al*, 1998; Kwan *et al*, 2002). Although there is a considerable overlap between the expression of *abcb1a* and *abcb1b* (Croop *et al*, 1989), the overall distribution of these two genes coincides with that of the single ABCB1 gene in humans, suggesting that both *abcb1a* and *abcb1b* together function in the same manner as human ABCB1 (van de Vrie *et al*, 1998).

Using mice deficient for both murine *abcb1a* and *abcb1b* P-gps [*abcb1ab* (−/−)], we most recently provided first evidence that the access of the endogenous steroid hormones corticosterone and hydrocortisone (cortisol) is regulated by ABCB1-type P-gps *in vivo* (Uhr *et al*, 2002). Control of the access of endogenous corticosteroids to the brain by ABCB1-type P-gps is likely to exert a profound influence on the activity and regulation of the HPA system both under basal conditions and during stress, where peripheral glucocorticoid levels rapidly increase. Additional evidence for a physiological role of ABCB1-type P-gps in modulating HPA system function arises from the finding that murine *abcb1b* P-gp contains a glucocorticoid-responsive element in its promoter region (Cohen *et al*, 1991; Altuvia *et al*, 1993). We therefore hypothesized that ABCB1-type P-gps at the BBB might exert an important influence on HPA system activity, and that genetic or acquired variability or alterations of ABCB1-type P-gp function, in turn, could lead to either stable or transient individual differences in neuroendocrine regulation.

We therefore investigated the HPA system regulation in mice deficient for both murine *abcb1a* and *abcb1b* P-gps under basal conditions and following stress. To elucidate which level of HPA system regulation (ie hypothalamic, pituitary, or adrenal level) is affected by ABCB1-type P-gp deficiency, we performed detailed neuroendocrine analyses, including dexamethasone suppression and CRH stimulation tests. By means of *in situ* hybridization, we investigated hypothalamic and pituitary expression levels of HPA system-related peptides.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany, and according to the NIH guidelines.

Male *abcb1ab* (−/−) and FVB/N wild-type mice (age: 2–4 months) were housed 4 to 6 per cage in the breeding unit of the Max Planck Institute of Psychiatry under standard conditions with a 12 h light : 12 h dark cycle (lights on from 0600 to 1800; $22 \pm 1^{\circ}$C, 40–60% humidity) and received standard pelleted food and water *ad libitum*. *Abcb1ab* double knockout mice, originally created by Schinkel *et al* (1997) by sequential gene targeting in 129/Ola E14 embryonic stem cells and backcrossed seven times (N7) to FVB/N from the C57BL/6 × 129 chimera, and FVB/N wild-type mice were obtained from Taconic (Germantown, USA; FVB/Tac-[KO]Pgy2 N7).

Blood Collection and Stress Experiments

At 2 weeks before the experiments, animals were separated and housed singly to avoid uncontrolled stress reactions.

Basal Hormone Levels

To determine the basal morning plasma levels of ACTH and corticosterone, mice ($n = 10$ per genotype) were left undisturbed throughout the night before the experiment. Blood sampling was performed in the early morning (0700–0800) by rapid retro-orbital bleeding (Müller *et al*, 2000a). Blood was sampled in prechilled EDTA tubes. To examine the circadian rhythm of stress hormone secretion, we further took blood samples for measurement of plasma corticosterone in the evening (1730) shortly before the onset of the dark phase, that is, the maximum rise in plasma corticosterone concentrations.

Poststress Levels of Hormones

Forced swim test. The same animals were subjected to the forced swim stress as a predominantly physical stressor: on the day of testing, between 0800 and 1200, each mouse ($n = 10$ per genotype) was placed for 5 min in a glass beaker filled with tap water (21°C; diameter 12.5 cm, height 14 cm). Subsequently, the animals were returned to their home-cages, and blood collection was performed by retro-orbital bleeding 5 min after the end of stress exposure.

Dexamethasone-suppression test. Different dosages of dexamethasone (0, 10, 25, or 50 ng/g body weight, $n=7$ per genotpye) were injected subcutaneously into *abcb1ab* (−/−) mice and wild-type littermates (injection time: 0800). At 1400, mice were killed by decapitation and trunk blood was collected in prechilled EDTA tubes. Plasma corticosterone and ACTH concentrations were measured by radioimmunoassay.

CRH challenge test. After 1 week of daily handling, male *abcb1ab* (−/−) mutants and wild-type littermates ($n=8$ per genotype) were subcutaneously injected with either vehicle (0.9% saline) or 1 μg of CRH (Ferring, Pharmaceuticals Ltd, Malmö, Sweden), injection time between 1345 and 1415. Blood samples were collected by rapid retro-orbital bleeding as described above, 30 min after the injection for measurement of plasma ACTH and corticosterone.

Treatment of Blood Samples and Hormone Analysis

Blood samples were collected in prechilled tubes containing EDTA and a protease inhibitor (10 μl aprotinin, Trasylol™, Bayer, Germany) and centrifuged (10 min, 3500 rpm, 4°C). Plasma samples were stored at −80 and −20°C until assay. Plasma ACTH (50 μl) and corticosterone (10 μl) levels were measured using commercially available kits (DRG, Marburg, Germany) according to the respective protocols.

In Situ Hybrization

Animals ($n=5$ per genotype) were killed with an overdose of halothane. All animals for *in situ* hybridization experiments were killed in the morning between 0800 and 1000. Thereafter, brains and pituitary glands were quickly removed. Tissue was frozen on dry ice and stored at −80°C. For subsequent *in situ* hybridization experiments, brains and pituitary glands ($n=6$ per genotype) were sectioned in a cryostat (18 μm). All brains were cut in five parallel series (coronal sections, spanning the region of the PVN from bregma −0.70 mm to bregma −1.22 mm), with every fifth section being thaw-mounted on the same glass slide. Sections were stored at −20°C until use.

POMC in situ *hybridization histochemistry.* The following oligonucleotide DNA probe was used for *in situ* hybridization: *POMC* (48-mer): 5′-CTC GGA GAA GGC AGA CTC AGG GTC GCA GGC GGG GTC GGT GCG GCA GCC (directed against nucleotides 890–937 of the rat POMC sequence).

All *in situ* hybridization experiments were carried out as previously described in detail (Müller *et al*, 2000a, b), and all sections were run in the same experiment under identical conditions. Briefly, the synthesized oligonucleotide was labeled at the 3′ end with α-[^{35}S]dATP (NEN DuPont) using terminal dexoynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany). Radiolabeled probe (10^6 cpm/200 μl/slide) was diluted into hybridization buffer consisting of 1 × Denhardt's solution, 0.25 mg/ml yeast tRNA (Sigma, Deisenhofen, Germany), 0.5 mg/ml salmon sperm DNA (Sigma, Deisenhofen, Germany), 10% dextran sulfate, 10 mM dithiothreitol, and 50% formamide, applied to the slides and incubated for 20 h at 42°C. Following hybridization, the slides were washed in 1 × SSC (55°C), four times for 15 min each, dehydrated in ethanol and air-dried. Finally, the slides were dipped in Kodak NTB2 emulsion diluted 1 : 1 in distilled water, exposed for 3–5 days (POMC) and for 4 weeks in the case of CRH and finally developed in Kodak D19 solution. The developed slides were lightly counterstained with cresyl violet and examined using a Leica microscope with both bright- and dark-field condensors.

CRH in situ *hybridization histochemistry.* The CRH cRNA riboprobe was produced from a 356 bp 5′ fragment (nucleotides 1308–1664) of mouse CRH cDNA (Seasholtz *et al*, 1991) in pCR II TOPO vector (Invitrogen, Karsruhe, Germany) using either SP6 (antisense probe) or T7 (sense probe) transcription systems in a standard labeling reaction mixture consisting of: 1.5 μg of linearized plasmid, 1 × transcription buffer, 0.12 mCi of ^{35}S-UTP, 1 mM NTPs, 16.7 mM dithiothreitol, 40 U of RNase inhibitor, and 20 U of the appropriate polymerase. The reaction was incubated at 37°C for 3 h, the labeled probe being separated from free nucleotides over Qiagen spin columns. The specificity of the probe was confirmed by the absence of a signal in sections labeled with sense probe and sections pretreated with Rnase prior to hybridization with antisense (cRNA) probe.

Optical Densitometry

Representative areas of the hypothalamic PVN (in the case of CRH mRNA) or the pituitary gland (POMC) were scanned by a digital camera under dark–light conditions. Care was taken to scan all images under identical light conditions. Quantitative analysis of mRNA expression was performed blind to the animals' genotype as previously described (Müller *et al*, 2000b), using the Macintosh-based public domain image analysis program NIH image, version 1.6.1. (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov./nih-image). At least three parallel tissue sections per animal and region were analyzed, and the mean values for each animal and region were calculated.

CRH mRNA. Levels of mRNA expression (optical density) were determined by measuring the mean gray value on inverted (*in situ* hybridization signal: black) images in the PVN. The region of interest was outlined according to adjacent sections that had been stained with cresyl violet to determine orientation and the precise anatomical localization and borders of the nuclei. As the three-dimensional structure of the hypothalamic paraventricular nucleus varies from rostral to caudal, at least three parallel sections per animal and region were analyzed, spanning the section containing the highest signal intensity.

POMC mRNA. As corticotrope cells expressing POMC mRNA are scattered throughout the anterior lobe of the pituitary gland, semiquantitative analysis of POMC mRNA expression was performed as an automatic count of labeled objects (= cells)/area on thresholded and binary images. The means for the number of labeled cells/mm^2 were calculated for each animal, and statistical differences between the two groups (*abcb1ab* (−/−) mutants and wild-type littermates) were determined.

Statistical Analysis

Results are presented as means + SEM. Statistical analysis was performed with a software package (GB Stat™, version 6.5 PPC, Dynamic Microsystems Inc., Silver Spring, MD, USA). Statistical significance of differences between groups was determined by ANOVA (one-way ANOVA, factor: *genotype* or two-way ANOVA, factors *genotype* and *treatment*, where appropriate), followed by *post hoc* Newman–Keuls test, $p<0.05$ was considered statistically significant.

RESULTS

Plasma ACTH Levels are Consistently Lower in *abcb1ab* (−/−) Knockout Mice

Two-way ANOVA with repeated measures revealed a significant effect of both genotype ($F_{1,18}=31.9$; $p<0.0001$) and treatment condition ($F_{1,18}=257.2$; $p<0.0001$) with a significant interaction of both factors ($F_{1,18}=7.8$; $p=0.012$). *Post hoc* analysis (Newman–Keuls test) revealed significantly lower plasma ACTH levels in *abcb1ab* (−/−) mutants compared to wild-type mice both under basal conditions (0800; wild-type mice: 274.1 ± 122.8 pg/ml; *abcb1ab* (−/−): 129.6 ± 30.1 pg/ml; $p<0.05$) and following forced swim stress (wild-type mice: 1090.2 ± 141.57 pg/ml; *abcb1ab* (−/−): 684.38 ± 143.69 pg/ml; $p<0.01$; Figure 1a). In both wild-type and *abcb1ab* (−/−) animals, a significant increase in plasma ACTH *vs* basal levels was observed following 5 min of forced swim stress ($p<0.01$; Figure 1a).

Plasma Corticosterone Levels at the Circadian Peak and Following Stress are Reduced in *abcb1ab* (−/−) Mutants

By means of two-way ANOVA with repeated measures, a significant effect of both genotype ($F_{1,36}=10.9$; $p<0.0039$) and treatment condition ($F_{1,36}=168.2$; $p<0.0001$) with a significant interaction of both factors ($F_{1,36}=7.4$; $p=0.02$) was detected. *Post hoc* analysis (Newman–Keuls test) revealed no difference in basal morning plasma corticosterone levels (wild-type mice: 6.2 ± 3.8 ng/ml; *abcb1ab* (−/−): 9.4 ± 8.6 ng/ml; Figure 1b); however, basal evening levels were significantly lower in *abcb1ab* (−/−) mutants compared to wild-type mice (wild-type mice: 86.3 ± 20.5 ng/ml; *abcb1ab* (−/−): 51.7 ± 9.8 ng/ml; p<0.01; Figure 1d)

Similarly, stress-induced plasma corticosterone levels were reduced in *abcb1ab* (−/−) mutants in comparison to those of wild-type mice (wild-type mice: 103.8 ± 16 ng/ml; *abcb1ab* (−/−): 88.2 ± 15.3 ng/ml; $p<0.05$; Figure 1b). In both wild-type and *abcb1ab* (−/−) animals, a significant increase in poststress plasma corticosterone *vs* basal levels was observed ($p<0.01$; Figure 1b).

Dexamethasone-Suppression Test

Plasma ACTH concentrations are consistently lower in abcb1ab *(−/−) mutants.* Two-way ANOVA revealed a significant effect of both genotype ($F_{1,48}=38.1$; $p<0.0001$) and treatment condition ($F_{1,48}=4.1$; $p<0.01$). *Post hoc* Newman–Keuls test revealed significantly lower plasma ACTH concentrations in *abcb1ab* (−/−) mice when compared to wild-type littermates following administration of vehicle (wild-type mice: 338.5 ± 103.7 pg/ml; *abcb1ab* (−/−): 166.9 ± 46.9 pg/ml; $p<0.01$), 10 ng dexamethasone/g body weight (wild-type mice: 311.1 ± 112.6 pg/ml; *abcb1ab* (−/−): 135.8 ± 84.8 pg/ml; $p<0.05$), or 50 ng dexamethasone/g body weight (wild-type mice: 217.5 ± 86.7 pg/ml; *abcb1ab* (−/−): 46.55 ± 10.7 pg/ml; $p<0.05$; Figure 2a).

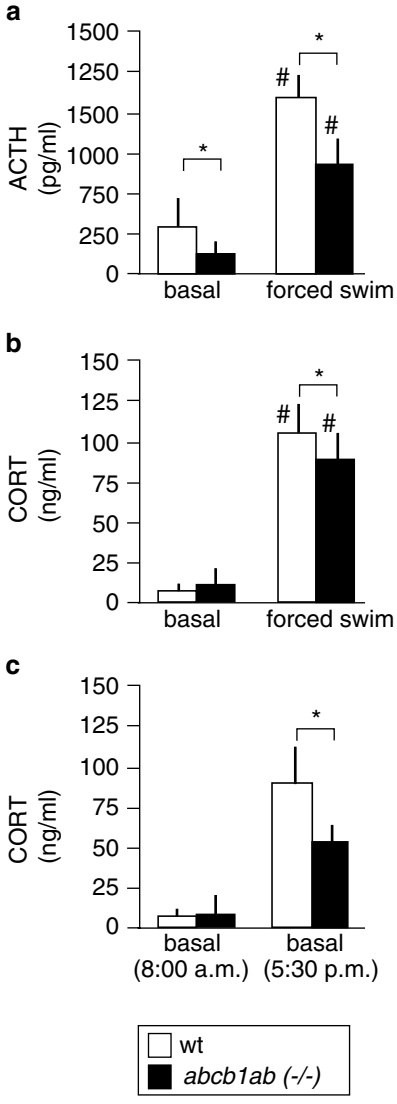

Figure 1 Plasma ACTH (a) and corticosterone (b, c) concentrations in wild-type mice (wt) and *abcb1ab* (−/−) mutants under basal conditions (basal morning levels: a, b and basal evening levels: c) and following forced swim stress. Data are expressed as means + SEM ($n=10$). *Denotes statistically significant differences between wild-type and mutant mice. #Indicates a significant effect of the experimental condition *vs* basal levels within the same group, $p<0.05$.

Endogenous corticosterone release is suppressed at lower dexamethasone concentrations in abcb1ab *(−/−) mutants.* Two-way ANOVA with repeated measures revealed a significant effect for both genotype ($F_{1,48}=13.4$; $p<0.0006$) and treatment condition ($F_{1,48}=8.8$; $p<0.0001$). Plasma corticosterone levels were indistinguishable between wild-type mice and *abcb1ab* (−/−) mutants following administration of either 0 ng dexamethasone/g body weight (vehicle condition; wild-type mice: 84.8 ± 34.2 ng/ml; *abcb1ab* (−/−): 56.5 ± 14.5 ng/ml; $p>0.05$), 10 ng dexamethasone/g

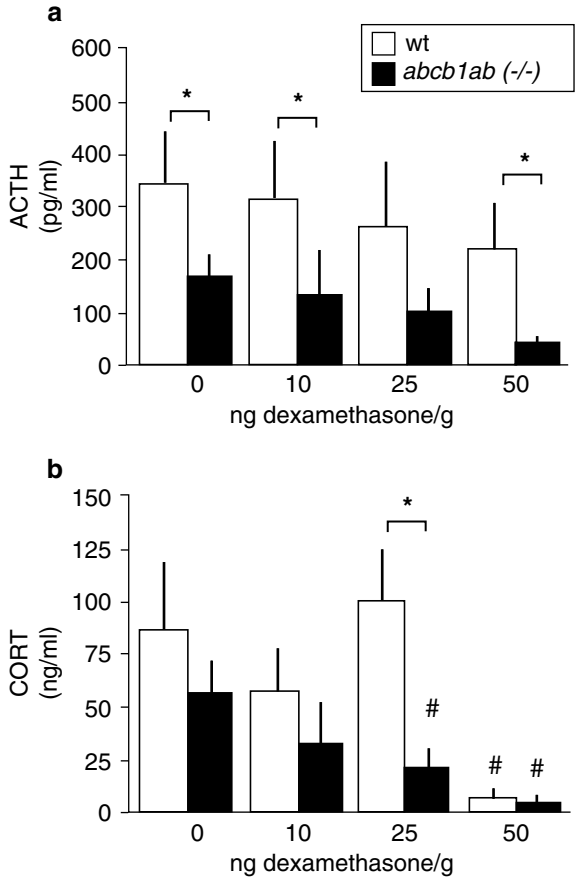

Figure 2 Results of the dexamethasone suppression test: 0, 10, 25, or 50 ng/g body weight dexamethasone were injected subcutaneously into wild-type mice and *abcb1ab* (−/−) mutants at 0800 ($n=7$). At 1400, mice were killed and trunk blood was collected. Plasma corticosterone and ACTH concentrations were measured by radioimmunoassay. Data are expressed as means + SEM ($n=8$–10). Plasma ACTH concentrations are consistently lower in *abcb1ab* (−/−) mutants. Moreover, endogenous corticosterone release is suppressed at lower dexamethasone concentrations in *abcb1ab* (−/−) mutants. *Indicates a significant difference between mutant and control mice, $p<0.05$.

body weight (wild-type mice: 55.3 ± 20.5 ng/ml; *abcb1ab* (−/−): 33.1 ± 20.2 ng/ml; $p>0.05$), or 50 ng dexamethasone/g body weight (wild-type mice: 4.8 ± 4.6 ng/ml; *abcb1ab* (−/−): 5.1 ± 3.2 ng/ml; $p>0.05$). Following administration of 25 ng dexamethasone/g body weight, however, a significant difference in plasma corticosterone levels could be detected between *abcb1ab* (−/−) mutants and wild-type littermates (wild-type mice: 98.6 ± 24.1 ng/ml; *abcb1ab* (−/−): 21.4 ± 10.2 ng/ml; $p<0.01$; Figure 2b).

CRH Stimulation Induces Normal Activation of the HPA System in *abcb1ab* (−/−) Mice

Plasma ACTH. By means of two-way ANOVA with repeated measures, a significant effect of both genotype ($F_{1,22}=109.6$; $p<0.0001$) and treatment condition ($F_{1,22}=6.7$; $p=0.01$) was detected (Figure 3). For plasma corticosterone, two-way ANOVA with repeated measures revealed a significant effect of both genotype ($F_{1,18}=384.1$; $p<0.0001$) and treatment condition ($F_{1,18}=4.9$; $p=0.03$; Figure 3). *Post hoc* analyses detected a significant difference in both plasma ACTH and corticosterone levels between wild-type mice and *abcb1ab* (−/−) mutants following vehicle stimulation, with mice deficient for both *abcb1a* and *abcb1b* P-gps consistently displaying lower plasma hormone levels (*ACTH*: wild-type mice: 367 ± 74.21 pg/ml; *abcb1ab* (−/−): 130.4 ± 10 pg/ml; $p<0.01$; *corticosterone*: 78.3 ± 18.9 ng/ml; *abcb1ab* (−/−): 32.1 ± 6 ng/ml; $p<0.05$; Figure 3). Both wild-type animals and *abcb1ab* (−/−) *mutants* showed a significant increase in plasma ACTH and corticosterone *vs* basal hormone levels following CRH stimulation (*ACTH*: wild-type mice: 1058.5 ± 271.5 pg/ml; *abcb1ab* (−/−): 1019.2 ± 244.5 pg/ml; $p<0.01$ *vs* the respective basal levels; *corticosterone*: 276.1 ± 18.3 ng/ml; *abcb1ab* (−/−): 261.1 ± 62.1 ng/ml; $p<0.01$ *vs* the respective basal level). There was no statistically significant difference in CRH-induced plasma ACTH or corticosterone levels between wild-type animals and mutants.

The Number of POMC mRNA Expressing Cells in the Anterior Pituitary of *abcb1ab* (−/−) Knockout Mice is Significantly Reduced

One-way ANOVA with *post hoc* Newman–Keuls tests revealed a significantly reduced number of POMC mRNA expressing corticotrophic cells/mm^2 in the anterior pituitary (wild-type mice: 96.7 ± 9.9 cells/mm^2; *abcb1ab* (−/−): 54 ± 9.3 cells/mm^2; $p<0.01$; Figures 4 and 5).

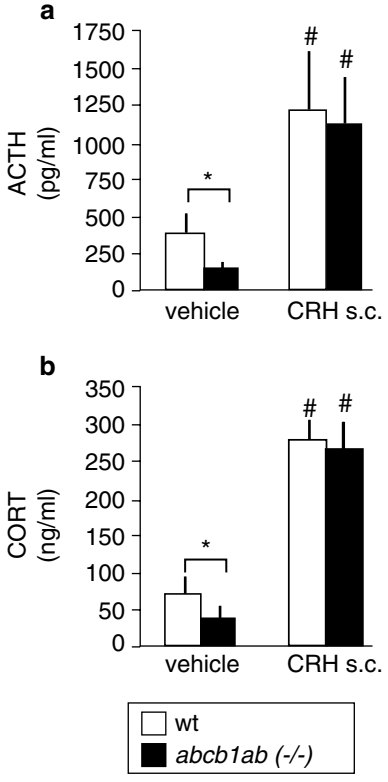

Figure 3 CRH challenge test reveals normal activation of the HPA system in *abcb1ab* (−/−) mice following CRH stimulation: plasma ACTH (a) and corticosterone (b) response to s.c. administration of either 1 μg CRH or vehicle. Data are expressed as means + SEM ($n=8$). *Indicates a significant difference between mutant and control mice. # Indicates a significant effect of CRH administration *vs* vehicle condition within the same group, $p<0.05$.

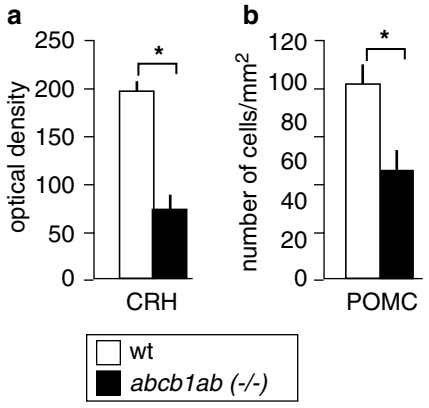

Figure 4 Results of semiquantiative measurements of CRH (a) mRNA expression in the PVN (a) as well as POMC mRNA levels in the anterior pituitary (b) of wild-type mice and *abcb1ab* (−/−) knockout mice. Data are expressed as mean optical density (mean gray levels) + SEM ($n = 5$ per group). *Indicates a significant difference between mutant and control mice, $p < 0.05$.

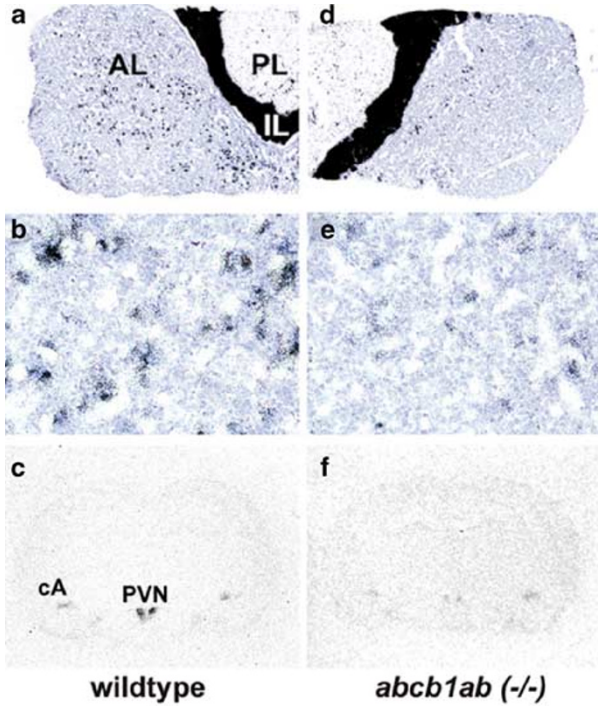

Figure 5 Brightfield photomicrographs showing a significantly reduced expression of POMC mRNA in the anterior lobe of the pituitary gland of *abcb1ab* (−/−) mutants (d) compared to wild-type mice (a). (b, e) Shows the difference in POMC expression in pituitary corticotropes of wild-type (b) and mutant mice (e) in more detail at higher magnification. Note the marked reduction of pituitary POMC mRNA in *abcb1ab* (−/−) mutants. *In situ* hybridization with a riboprobe against CRH reveals a significant suppression of hypothalamic CRH mRNA in *abcb1ab* (−/−) mutants (f) when compared to wild-type CRH expression (c). AL = anterior lobe, IL = intermediate lobe, PL = posterior lobe, cA = central amygdaloid nucleus, PVN = paraventricular nucleus.

CRH mRNA Expression in the PVN of *abcb1ab* Knockout Mice is Significantly Reduced

One-way ANOVA with *post hoc* Newman–Keuls tests revealed that CRH mRNA expression in the hypothalamic paraventricular nucleus was significantly reduced in *abcb1ab* (−/−) knockout mice compared to wild-type controls (optical density (mean gray level): wild-type mice: 195.9 ± 10.9; *abcb1ab* (−/−): 75.2 ± 13.8; $p < 0.01$; Figures 4 and 5).

DISCUSSION

The present data indicate that the absence of ABCB1-type P-gp function at the BBB as examined by use of a knockout mouse model leads to profound changes in the activity and regulation of the HPA system under both basal and stress conditions.

Naturally occurring, functionally relevant polymorphisms of the human MDR gene that significantly influence both the expression levels and the functional properties of ABCB1-type P-gps have been described recently (Hoffmeyer *et al*, 2000). Extrapolating our data to the human situation, we hypothesize that both genetically determined and acquired alterations in ABCB1-type P-gp function could lead to stable or transient individual differences in HPA system regulation. Such variability could be either drug-induced (Schinkel *et al*, 1996; Doze *et al*, 2000; Pariante *et al*, 2003) or ABCB1-type P-gp function could be altered under certain pathophysiological conditions.

Absence of ABCB1-type P-gps at the BBB Profoundly Alters HPA System Activity

Based on our previous finding that the endogenous glucocorticoid hormone corticosterone is a substrate of ABCB1-type P-gps *in vivo* (Uhr *et al*, 2002), our data provide the first *in vivo* evidence that modulation of BBB function by the presence or absence of major transport proteins profoundly influences neuroendocrine activity and regulation. Mice deficient for both *abcb1b* and *abcb1b* P-gp display characteristics of chronically increased negative feedback at the hypothalamic level due to an enhanced penetration of corticosterone into the central nervous system. In the present series of experiments, plasma ACTH levels were significantly lower in *abcb1ab* (−/−) mutants both under basal conditions and following forced swim stress. Basal plasma corticosterone levels were indistinguishable from wild-type levels during the circadian trough. However, *abcb1ab* (−/−) mutants displayed significantly reduced plasma corticosterone concentrations both during the circadian peak and following forced swim stress. Suppression of peripheral stress hormones is likely to reflect the consequences of increased glucocorticoid feedback on hypothalamic paraventricular neurons in *abcb1ab* (−/−) mutants. Consistent with this hypothesis, we found CRH mRNA expression in the hypothalamic paraventricular nucleus to be significantly downregulated in these animals. Glucocorticoid-induced feedback on paraventricular CRH neurons is mediated by both direct actions on hypothalamic neurosecretory neurons and by glucocorticoid-dependent regulation of afferent input to the dorsal part of the medial parvocellular part of the PVN (Erkut *et al*, 1998; for a review, see Kovács, 1998; Tanimura and Watts, 2001). This negative feedback is a fundamental way in which the HPA

system is restrained during stress and activity (for a review, see de Kloet *et al*, 1998).

We further investigated whether chronically reduced stimulation of anterior pituitary corticotropes due to suppressed hypothalamic CRH expression in *abcb1ab* (−/−) mutants affects anterior pituitary pro-opiomelanocortin (POMC) gene expression. Consistent with the suppression of plasma ACTH levels, we found anterior pituitary POMC mRNA expression to be significantly reduced in *abcb1ab* (−/−) mutants. The release of pituitary hormones derived from the POMC gene is under multihormonal and tissue-specific control in the anterior and intermediate lobes of the pituitary. CRH stimulates the immediate release of POMC-derived peptides in the pituitary gland (Vale *et al*, 1981), and is the main positive physiological regulator of POMC gene transcription in anterior pituitary corticotropes both *in vitro* and *in vivo* (Gagner and Drouin, 1987; Jin *et al*, 1994). Exogenous stimulation with CRH revealed similar increases in plasma ACTH and corticosterone in both *abcb1ab* (−/−) mutants and wild-type controls. We can, therefore, rule out the possibility that the differences in neuroendocrine regulation observed in *abcb1ab* (−/−) mutants are due to an altered responsiveness of pituitary corticotropes. The CRH dosage applied has been previously shown to stimulate the HPA system within a physiological range, eliciting a stress hormone release that is comparable to the effects of a severe stressor (Barden *et al*, 1997; Müller *et al*, 2001b). We cannot exclude the possibility that following application of a supramaximal dose of CRH, a difference in pituitary ACTH release might be observed between *abcb1ab* (−/−) mutants and wild-type littermates; however, we doubt whether this might be of any functional relevance for the *in vivo* situation because it does not mirror physiological conditions. The results of our CRH challenge test are in support of our hypothesis that the sustained suppression of the HPA system in *abcb1ab* (−/−) mutants is mainly an effect of increased negative feedback via corticosterone at the level of hypothalamic neurosecretory neurons.

Using mice deficient for murine *abcb1a* (single knockout), previous investigations found the synthetic glucocorticoid dexamethasone to be a substrate of ABCB1-type P-gps (Schinkel *et al*, 1995; Karssen *et al*, 2001). However, Karssen *et al* (2001) most recently failed to identify the endogeneous rodent glucocorticoid hormone corticosterone as being a substrate of ABCB1-type P-gps in *abcb1a*-knockout mice. At first glance, this finding seems to contradict our previous results showing that not only dexamethasone, but also corticosterone significantly accumulates in the brain of *mrd1a/1b* (−/−) mice, with [^{3}H]corticosterone levels in the brain of mutant mice being twice as high as the respective levels in wild-type littermates (Uhr *et al*, 2002). However, both murine *abcb1a* and *abcb1b* P-gps are expressed at the BBB in mice (Croop *et al*, 1989), and murine *abcb1b P-gp*, in particular, has been shown to have the capacity to transport corticosterone (Wolf and Horwitz, 1992). In all major tissues, at least some RNA of both *abcb1a* and *abcb1b* is detectable by RNAse protection (Schinkel *et al*, 1997). Heterozygous animals (*abcb1a* +/−) do not display any overt phenotype; *abcb1b*-type P-gp, however, is markedly upregulated in both heterozygous and homozygous *abcb1a* knockout mice. This points towards a compensatory upregulation of *abcb1b* that takes over the function of *abcb1a*. It is, therefore, most likely that the presence of *abcb1b* in *abcb1a* (−/−) mutants confounded the findings of Karssen *et al* (2001). By the use of *abcb1ab* (−/−) double knockout mice, we can exclude that similar compensatory mechanisms might have influenced our data. In addition, the present data provide functional evidence for a sustained suppression of the HPA system in *abcb1ab* (−/−) mice, most probably mediated by increased intracerebral concentrations of corticosterone (Uhr *et al*, 2002).

Previous investigations have already provided some, albeit indirect, evidence that BBB function and/or ABCB1-type P-gps might play a crucial role in controlling the access of endogenous steroid hormones to the central nervous system. Endogenous steroid hormones, in turn, have been shown to regulate ABCB1-type P-gp function: the expression of murine *abcb1b*-Pgp has been described to be enhanced by steroid hormones in a feedback regulatory mechanism *in vitro*, an effect which could be blocked by administration of 2-aminoglutethimide, an inhibitor of steroid synthesis (Altuvia *et al*, 1993).

Whether in the presence of ABCB1-type P-gp the synthetic glucocorticoid dexamethasone penetrates the BBB at biologically significant concentrations has been the subject of controversy (Fink *et al*, 1988; Meijer *et al*, 1998; Pariante *et al*, 2002). The results from our dexamethasone suppression test provide additional *in vivo* evidence that the penetration of this synthetic glucocorticoid into the central nervous system is regulated by ABCB1-type P-gps: lower doses of dexamethasone are required to completely suppress endogenous plasma corticosterone levels in *abcb1ab* (−/−) mice.

Modulation of HPA System Feedback Mechanisms ABCB1-Type P-gps: Implications for Affective Disorder

The cumulative evidence derived from both basic and clinical studies makes a strong case implicating HPA system dysregulation in the pathogenesis of affective disorders (for a review, see Keck and Holsboer, 2001). Impairment of corticosteroid receptor signaling and feedback inhibition, in particular, has been suggested to play a key role in the development of neuroendocrine changes associated with human affective disorders (Holsboer, 2000; Pariante and Miller, 2001; Müller *et al*, 2002). Data supporting the notion that glucocorticoid-mediated feedback inhibition is impaired in major depression come from studies revealing nonsuppression of cortisol secretion following administration of dexamethasone (Modell *et al*, 1997) and investigations showing an exaggerated ACTH response to CRH following dexamethasone pretreatment in the combined dexamethasone-CRH-challenge test (eg Heuser *et al*, 1994). Antidepressant-induced upregulation of corticosteroid-receptors in the rat brain, in turn, has been shown to occur after chronic antidepressant treatment and has been suggested to precede normalization of HPA system dysregulation (Holsboer, 2000; Pariante and Miller, 2001). Most recently, Pariante *et al* (2003) hypothesized that antidepressants, by inhibiting membrane steroid transport, could directly increase the access of endogenous glucocorticoids into the brain and, consequently, induce corticosteroid receptor activation and enhance negative feedback on

the HPA system. The present data are in support of this notion, demonstrating that the absence of ABCB1-type P-gps leads to an increased penetration of endogenous corticosteroid hormones into the central nervous system, which, in turn, enhances central negative feedback inhibition of stress hormone secretion.

Taken together, our data add to the increasing evidence that the ABCB1-type P-gp transport system may provide a general mechanism for communication and cross-talk between the central nervous system and the periphery by controlling the bi-directional transport of both centrally and peripherally acting peptides and hormones at the BBB. In addition, ABCB1-type P-gps are likely to play an important role in modulating HPA system function by controlling the access of endogenous glucocorticoid hormones to the central nervous system. In this respect, we hypothesize that ABCB1-type P-gps play a crucial role in 'restraining' the HPA system following stressful stimuli and physiological activation, but are not mandatory for basal maintenance of the stress hormone system, as basal corticosterone levels are indistinguishable between wild-type and knockout animals.

A recent investigation provided evidence that polymorphisms in the human ABCB1-gene have a major impact on the intestinal absorption of drugs, thus significantly influencing their tissue concentrations and therapeutic efficacy (Hoffmeyer *et al*, 2000). With respect to the present data, those naturally occurring and functionally relevant polymorphisms of ABCB1 are also likely to affect and modulate the penetration of endogenous glucocorticoid hormones into the central nervous system. Genetic variability of the ABCB1 gene, therefore, may alter an innate setpoint for susceptibility to stress-associated psychiatric disorders (Meaney *et al*, 1996; Brunson *et al*, 2001). Whether this might result in persistent changes in the responsiveness and regulation of the HPA system will be the subject of future investigations, correlating both genetic information with individual characteristics of the neuroendocrine phenotype.

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