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Penetration of Dexamethasone into Brain Glucocorticoid Targets Is Enhanced in mdr1A P-Glycoprotein Knockout Mice*

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ABSTRACT

Mice with a genetic disruption of the multiple drug resistance (mdr1a) gene were used to examine the effect of the absence of its drug-transporting P-glycoprotein product from the blood-brain barrier on the distribution and cell nuclear uptake of [^{3}H]-dexamethasone in the brain. [^{3}H]-dexamethasone (4 μg/kg mouse) was administered sc to adrenalectomized mdr1a (−/−) and mdr1a (+/+) mice. One hour later, the mice were decapitated, and the radioactivity was measured in homogenates of cerebellum, blood, and liver following extraction of the radioactive steroid. The frontal brain was cut in sections for autoradiography.

In the cerebellum of the mdr1a mutants, the amount of [^{3}H]-dexamethasone relative to blood was about 5-fold higher than observed in the controls, whereas the ratio in blood *vs.* liver was not different. Using autoradiography, it was found that brain areas expressing the glucocorticoid receptor (GR) in high abundance, such as the hippocampal cell fields and the paraventricular nucleus (PVN), showed a 10-fold increase in cell nuclear uptake of radiolabeled steroid. The amount of retained steroid increased toward levels observed in the pituitary, which contains a similar density of GRs. The [^{3}H]-dexamethasone concentration in pituitary was not affected by mdr1a gene disruption. The GR messenger RNA expression pattern in hippocampus was not different between the wild types and mdr1a mutants, which rules out altered receptor expression as a cause of the enhanced dexamethasone uptake.

In conclusion, the present study demonstrates that the brain is resistant to penetration by dexamethasone because of mdr1a activity at the level of the blood-brain barrier. The data support the concept of a pituitary site of action of dexamethasone in blockade of stress-induced ACTH release. Dexamethasone poorly substitutes for depletion of the endogenous glucocorticoid from the brain and therefore, in this tissue, may cause a condition resembling that of adrenalectomy. (*Endocrinology* **139:** 1789–1793, 1998)

THE HIPPOCAMPUS is one of the most sensitive targets for the action of circulating naturally occurring glucocorticoids such as corticosterone (1–4). If tracer amounts of [^{3}H]-corticosterone are injected into adrenalectomized (ADX) rats, the pyramidal and granular neurons in the hippocampus accumulate and retain the radiolabeled steroid (5–7). This marked localization in hippocampus reflects the cell nuclear retention of [^{3}H]-corticosterone after binding to mineralocorticoid receptors (MR), which display a very high affinity for this steroid (8) similar to that of aldosterone (9, 10). Glucocorticoid receptors (GR), which are also present in these neurons (11), have a 10-fold lower affinity for corticosterone but display high affinity for the synthetic glucocorticoid dexamethasone (9). In spite of this high affinity for GR, [^{3}H]-dexamethasone administered to ADX rats does not lead to cell nuclear retention of the tracer in the hippocampal neurons, whereas the uptake and retention of dexamethasone is very high in pituitary corticotrophs (12–15). The question why dexamethasone poorly penetrates into the brain, whereas it readily enters other targets containing similar amounts of GR, remained unresolved during the past two decades.

Recently, a mouse line was generated with a genetic disruption of the multiple drug resistance (mdr1a) gene (mdra −/−), which encodes a drug-transporting P-glycoprotein (16, 17). The mdr1a gene product is expressed in the apical membranes of the endothelial cells of the blood-brain barrier and functions as an extrusion pump that limits access of its substrate molecules to the brain. Mdr1a encoded P-glycoprotein therefore protects the brain against accumulation of specific xenobiotic agents (18). It was shown that mdr1a (−/−) knockout mice exhibit increased accumulation of [^{3}H]-dexamethasone in brain as compared with wild-type control (mrd1a +/+) mice (17). These measurements were made in a brain homogenate of adrenally intact animals and provided the first evidence that mdr1a-endoced P-glycoprotein limits access of dexamethasone to the brain.

In the present study, we have tested the hypothesis that mrd1a P-glycoprotein is responsible for the poor cell nuclear retention of dexamethasone in rat hippocampus and other glucocorticoid targets in brain. We have tested this hypothesis by *in vivo* autoradiography after injection of tracer amounts of [^{3}H]-dexamethasone into ADX mdr1a (−/−) mice and mdr1a (+/+) controls. We find that access of dexamethasone to central GR-containing glucocorticoid tar-

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gets (*e.g.* hippocampus PVN is substantially enhanced in mdr1a (−/−) mutants.

Materials and Methods

Animals and surgery

The generation of the mdr1a (−/−) mutants has been described previously. The disruption of the mdr1a gene did not produce overt changes in phenotype (16). Individually housed wild-type mdr1a (+/+) (n = 9) and mdr1a (−/−) mice (n = 8), body weight about 40 g, were anaesthetized by ip injection with a mixture of Hypnorm (JanssenPharmaceutica, Beerse, Belgium): Dormicum (Hoffman-La Roche, Mÿchrecht, The Netherlands): H_2O (1:1:2; 0.07 ml/10 g body weight) and bilaterally adrenalectomized by dorsal approach. After adrenalectomy (ADX) the animals had access to 0.9% saline. These ADX mice were used for the [^{3}H]-dexamethasone uptake experiments. Adrenally intact mice (n = 4) were used for the *in situ* hybridization of GR messenger RNA (mRNA). All experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC; all efforts were made to minimize animal suffering during the experiments. The protocols were approved by the animal care committee of the Faculty of Medicine, University of Leiden (Leiden, The Netherlands).

In vivo [^{3}H]-dexamethasone distribution study and autoradiography

Two days after ADX, the animals were injected sc with tritiated steroids for *in vivo* autoradiography. All steroids were dissolved in 2% ethanol/0.9% saline. Mdr1a (+/+) (n = 6) and (−/−) mice (n = 6) were injected with 10 μCi/10 g (1, 2, 4, 6, 7) [^{3}H]-dexamethasone (Amersham, UK, specific activity 100 Ci/mmol). As a positive control, wild-type and null mice were treated with 5 μCi/10 g (1, 2, 6, 7) [^{3}H]-corticosterone (Amersham, Buckinghamshire, UK; specific activity 70 Ci/mmol). As a control for nonspecific uptake, one mouse of the four groups was pretreated with either 100-fold excess unlabeled dexamethasone or 100-fold excess corticosterone 30 min before injection of the [^{3}H]-dexamethasone or [^{3}H]-corticosterone tracer, respectively. One hour after injection of the tracer, the mice were decapitated. Trunk blood was collected in EDTA-coated tubes for determination of tracer concentration in the plasma. Liver, cerebellum, forebrain, and pituitary were dissected, frozen on dry ice, and stored at −40 C until further use. Liver and cerebellum were homogenized using Soluene-350 (Packard), diluted in Hionic-Fluor (Packard) and tissue radioactivity was determined in a Tricarb β-counter(Packard). Ten-micrometer sections of brain and pituitary were cut on a cryostat and thaw-mounted on Polysine microscopic slides (Menzel-Gläser, Braunschweig Germany). The slides were put in an x-ray exposure holder (Kodak) under Ultrofilm (Leica, Heerbrugg, Switzerland) and exposed for 15 weeks.

In situ hybrdization of GR mRNA

In situ hybridization was performed using ^{35}S-labeled riboprobes. For GR, a 0.52-kb *Sal*I-*Hin*dIII fragment of exon 2 of the mouse GR gene in pBluescript was used (generously provided by Prof. Dr. G. Schütz, German Cancer Research Institute, Heidelberg, Germany). Antisense and sense probes were generated from linearized plasmids using with T3 and T7 polymerases using a standard protocol. Twenty-micrometer sections containing dorsal hippocampus were cut on a cryostat, thaw-mounted on poly-L-lysine coated microscope slides, and kept at −80 C until further use. The *in situ* hybridization procedure was started by postfixation of the sections in 4% paraformaldehyde in PBS (pH 7.2) for 1 h (room temperature). Sections were rinsed twice for 5 min in PBS, permeabilized by incubation with proteinase K (1 μg/ml in 0.1 M Tris, pH 8.0; 10 min, 37 C), rinsed briefly in DEPC-treated water, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0, 10 min, room temperature), rinsed for 10 min in 2 × SSC, pH 7.0 (SSC = 0.15 M NaCl and 0.015 M sodium citrate) and dehydrated in a graded ethanol series. A hybridization mix containing 70% formamide, 10% dextran sulfate, 3 × SSC, 0.06 M sodium phosphate buffer (pH 7.4), 1 × Denhardt's solution, 10 mM DTT, 0.1 mg/ml yeast transfer RNA, and 0.1 mg/ml salmon sperm DNA was prepared. Riboprobes were added to this mix to a concentration of 40×10^6 dpm/ml. One hundred microliters of this mix was applied to each slide, which was then covered with a standard microscopic coverslip and put in a moist chamber for overnight hybridization at 53 C. The next day, the coverslips were removed and the slides were washed in 2 × SSC (10 min), treated with RNAse A (2 mg/100 ml in 0.5 M NaCl, 0.01 M Tris; 15 min at 37 C), and washed at 65 C in 2 × SSC (10 min), 2 × SSC/50% formamide (15 min at 65 C), 2 × SSC/50% formamide (5 min at room temperature), 1 × SSC (10 min at 65 C) and 0.1 × SSC (10 min at 65 C). The slides were dehydrated in a graded ethanol series, put in a cassette and a X-OMAT AR film (Kodak, Rochester, NY) was exposed to the sections for 14 days.

Image analysis

The optical density of radiolabeled steroid and of GR mRNA in different brain areas was quantified using an Olympus image analysis system (Paes B.V, Zoeterwoude, The Netherlands) equipped with a Cue CCD camera. Film background was subtracted after shading correction. The optical densities were quantified on the basis of a standard curve calculated with the use of [^{3}H]- and [^{14}C] microscales (Amersham, UK). From each brain, six sections were measured by outlining the different brain regions. Data were evaluated by Student's *t* test, and significance was taken at $P < 0.05$. Data are presented as the mean ± SEM

Results

Table 1 depicts the absolute amounts of radioactivity measured in the plasma, liver, and cerebellum in mutants and wild types. At 60 min following [^{3}H]-dexamethasone administration to the ADX mice, the plasma radioactivity concentrations of the mdr1a (−/−) and (+/+) mice were comparable, and the concentrations in the liver homogenates were not significantly different. However, in the cerebellum, the amount of [^{3}H]-dexamethasone was about 5-fold higher in mdr1a (−/−) mice than in controls. These data demonstrate that the uptake in brain tissue, but not in liver, is enhanced after ablation of the mdr1a gene.

The autoradiograms (Fig. 1) show the effect of mdr1a gene disruption on the regional distribution of [^{3}H]-dexamethasone in the brain. In neural tissue of mdr (+/+) animals, [^{3}H]-dexamethasone labeling is negligible (Fig. 1a), whereas the label is visible in pituitary, cerebral ventricles and to a lesser extent in the arcuate nucleus. In contrast, in mdr1a (−/−) mutants, [^{3}H]-dexamethasone labeling is clearly visible (Fig. 1, b and c). In particular, brain areas that express GR in high abundance, such as hippocampal cell fields and PVN, show a substantial increase in labeling. Mdr1a (−/−) mice that were pretreated with unlabeled dexamethasone show no labeling of neural structures (data not shown). Accordingly, mdr1a-Pgp apparently hampers access of [^{3}H]-dexamethasone to the GRs in brain.

[^{3}H]-Corticosterone injection into ADX mice leads to strong labeling of hippocampal pyramidal and granular neurons both in mdr1a (+/+) wild types and in mdr1a (−/−) mutants (Fig. 1d). Our previous studies have shown that this

TABLE 1. Radioactivity 1 h after administration of [^{3}H]-dexamethasone (n = 5) in tissue homogenates and blood

	DEX (+/+)	DEX (−/−)
Blood (dpm/μl)	220 ± 30	254 ± 30
Liver (dpm/mg × 10^{-3})	23.3 ± 1.6	25.5 ± 2.5
Cerebellum (dpm/mg)	65 ± 5	322 ± 20[a]
Brain/blood	31 ± 3	130 ± 10[a]
Liver/blood (×10^{-3})	11 ± 2	10 ± 1
Brain/liver (×10^3)	2.8 ± 0.2	13.4 ± 1.7[a]

[a] $P < 0.05$.

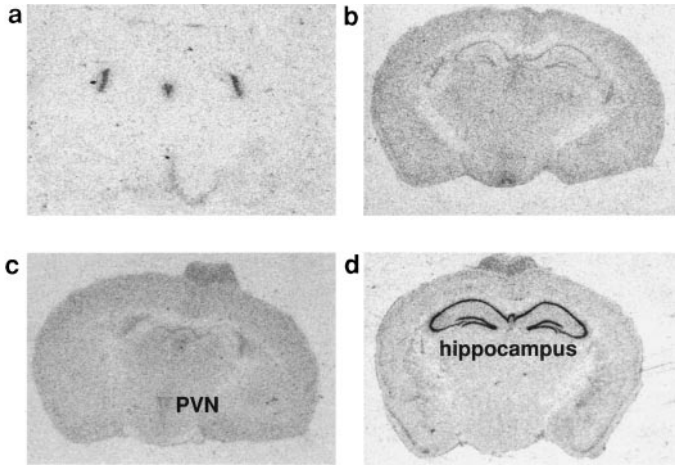

FIG. 1. Representative autoradiograms of 10-μm coronal sections of the brain of wild type and mdr1a (−/−) mice. Autoradiograms show labeling with [^{3}H]-dexamethasone of the following groups. a, Wild type treated with [^{3}H]-dexamethasone, hippocampus level. The dark spots represent transversal section of the cerebroventricular space and adjacent ventricular walls. b, Mutant treated with [^{3}H]-dexamethasone, hippocampus level. c, Mutant treated with [^{3}H]-dexamethasone, PVN level, note the pituitary mounted on top of the brain. d, Mutant treated with [^{3}H]-corticosterone, hippocampus level. Note the pituitary mounted on top of the brain.

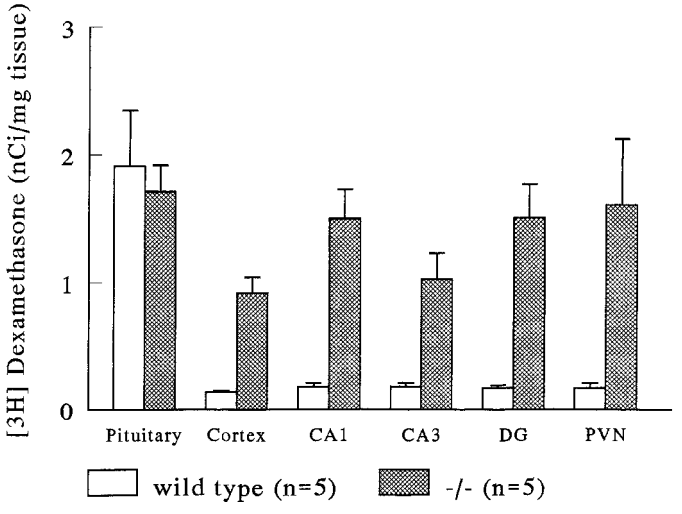

FIG. 2. Quantification of the autoradiograms of [^{3}H]-dexamethasone in wild type and mdr1a (−/−) mice. There are no differences between the wild types and mutants for pituitary. Brain nuclei that contain GR show an increased cell nuclear retention of [^{3}H]-dexamethasone. Data are of n = 5 animals ± SEM. Four brain sections per animal were measured.

labeling represents binding of [^{3}H]-corticosterone to MR (8). Pretreatment with excess cold corticosterone leads to disappearance of the hippocampal signal in wild types and mutants, underscoring that the signal represents receptor bound steroid (data not shown).

Quantification of the autoradiograms is presented in Fig. 2. The data demonstrate the large difference in cell nuclear concentration of [^{3}H]-dexamethasone in the pituitary *vs.* the brain of wild-type ADX animals. In mdr1a mutants, retention is enhanced about 10-fold in PVN, dentate gyrus, and CA1 cell fields, and about 8-fold in cortex and CA3 cell field, reaching values observed in pituitary. Dexamethasone uptake in pituitary is not affected by disruption of the mdr1a gene. Regarding [^{3}H]-corticosterone, the retention of this naturally occurring steroid does not seem to be different between wild types and mutants in hippocampus (data not shown).

In situ hybridization showed that mdr1a gene disruption did not affect the distribution and level of GR mRNA in dentate gyrus, CA1, and CA3 cell fields of the mouse hippocampus (Fig. 3). In arbitrary OD units, the average values (n = 4) were in dentate gyrus, 1.06 *vs.* 1.18; CA1, 1.41 *vs.* 1.46; CA3, 0.62 *vs.* 0.52 for wild types and mutants, respectively. Sense controls in the range of 0.14 to 0.32 arbitrary OD units.

Discussion

Using the mdr1a (−/−) mice, we have demonstrated in the present study that disruption of mdr1a gene enhances penetration of dexamethasone in the brain of ADX mice. This finding resolves an enigma ever since it was observed that the *in vivo* cell nuclear uptake of dexamethasone in hippocampus was 10-fold lower than in the anterior pituitary, whereas both tissues contained similar amounts of GR. *In vitro* cell nuclear uptake in tissue slices (12), and autoradiography of *in vitro* labeled of brain sections showed the expected efficient labeling of brain GR sites (19). Therefore, it was reasoned that some factor in the blood-brain barrier blocked access of synthetic glucocorticoids to the brain (12, 20). This factor most likely is the P-glycoprotein extrusion pump located in the apical membrane of the endothelial cells (18) because ablation of the encoding mdr1a gene in the

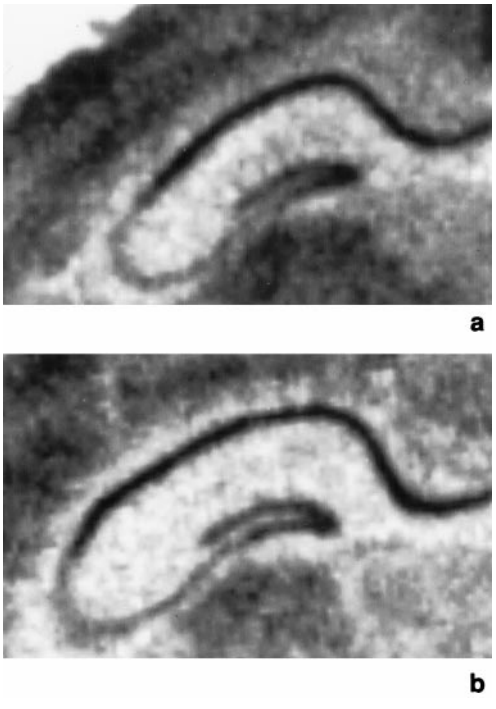

FIG. 3. *In situ* hybridization of the GR mRNA in hippocampus of wild-type and mdr1a mutants. There is no difference in GR mRNA expression in the hippocampus or other brain regions between the genotypes. a, Hippocampus of wild-type mouse. b, Hippocampus of mutant mouse.

mouse enhanced the uptake of dexamethasone in glucocorticoid target regions of the brain about 10-fold. The retention of dexamethasone in these central targets (PVN and hippocampus) rises toward the level observed in the pituitary, where dexamethasone uptake was not different between mutants and wild types. The level and pattern of hippocampal GRmRNA was not affected by mdr1a gene disruption, which minimizes the possibility that the enhanced dexamethasone uptake is due to overexpression of GRs.

Previously, Schinkel *et al.* (17) reported a 3-fold higher concentration of radioactivity in brain homogenate of adrenally intact mdr1a mutants at 30 and 60 min after administration of labeled dexamethasone (0.2 mg/kg mouse) as compared with wild types. The enhanced uptake in the mdr1a (−/−) brain disappeared in Schinkel's study when the dose of dexamethasone was raised to 1 mg/kg mouse, indicating a limited capacity of the P-glycoprotein transport system. Furthermore, it was verified that the P-glycoprotein actively transports the dexamethasone substrate across a polarized kidney epithelial cell layer *in vitro* (17).

We have extended these original observations in a number of ways. First, the present study was performed after ADX, which depletes the corticosteroid receptors of endogenous ligand and permits to quantitate receptor-mediated retention of the labeled steroid. The use of tracer amounts (4 μg dexamethasone/kg mouse) administered to ADX animals revealed a more enhanced retention of dexamethasone then previously observed in the adrenally intact animals (17) with a 50-fold higher dose of dexamethasone. Second, autoradiography allows precise anatomical localization of the radiolabeled dexamethasone and revealed enhanced retention in GR abundant glucocorticoid targets. Third, the mdr1a gene disruption selectively enhanced brain uptake, without altering blood, liver, cerebroventricular, and pituitary concentration of the synthetic steroid. Because the concentration of labeled dexamethasone did not seem to be different in the cerebroventricular area, it is unlikely that the clearance of dexamethasone from CSF to blood was affected by mdr1a gene disruption.

In previous studies using high resolution autoradiography, it was proposed that [^{3}H]-dexamethasone gained access to the brain through the cerebroventricular system (7, 13, 14). Indeed, some brain regions partly outside the blood-brain barrier (*e.g.* arcuate nucleus) retained rather high amounts of dexamethasone both in mutants and in controls. The access to these neurons in the medial-basal hypothalamus could have been through the pericapillary space in the median eminence in which compounds can freely exchange between liquor, blood, and nerve tissue (21). It might well be that also entrance from the cerebroventricular system into the brain is hampered by P-glycoprotein present in epithelial cells of the ventricular ependyma and in glial cells. In support of this, it was previously found that icv and systemic administration had a similar dose-response curve in the suppression of stress-induced pituitary ACTH release (22). These data suggest that the mdr1a-P-glycoprotein gene product is a potential target to achieve enhanced delivery of synthetic glucocorticoids to the central nervous system.

The uptake of [^{3}H]-dexamethasone observed in the hippocampus pyramidal cell fields and dentate gyrus was about 10-fold lower than observed after injection of [^{3}H]-corticosterone tracer in the ADX mice, as was previously found in rats (12). Our preliminary observations suggest that the retention of the naturally occurring glucocorticoid is hardly affected by the P-glycoprotein. Yet, as we demonstrated previously, retention of low amounts of corticosterone occurs via the high affinity MRs (8), to which dexamethasone displays *in vitro* a 10-fold lower affinity (9, 10). Moreover, in a 100-fold excess, it does not compete *in vivo* for retention of corticosterone in hippocampus (23). There are two reasons why dexamethasone even in excess does not compete. First, in rats and mice dexamethasone is retained by GRs rather than MRs. Second, as shown here dexamethasone poorly penetrates the blood-brain barrier. Thus, although in mdr1a (−/−) dexamethasone uptake is enhanced in hippocampus toward levels observed for corticosterone, this is for the synthetic steroid rather due to binding to GR than to MR. For the localization, this does not make a difference because MRs and GRs were found to be colocalized in clusters within hippocampal neuronal nuclei using dual labeling immunocytochemistry and confocal microscopy (11).

Dexamethasone's poor penetration in brain supports the concept of a pituitary rather than a central site of action in its suppression of stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis (21). After dexamethasone treatment, a condition of "chemical adrenalectomy" is created and the brain therefore becomes depleted of endogenous corticosterone, which is poorly substituted. The corticosteroid depletion of the brain may explain a number of features revealed by dexamethasone administration. For instance, the depletion of corticosterone from MRs may explain why chronic dexamethasone treatment produces apoptotic cells in the dentate gyrus of the hippocampus (24, 25), a phenomenon that is well documented after ADX (26, 27). It also provides a rationale for the use of the combined CRH-dexamethasone suppression test, which is presently the most precise laboratory test assisting diagnosis of depression (28). Through depletion of the brain corticosteroids, dexamethasone is thought to amplify the CRH hyperdrive characteristic for these patients (29). This CRH hyperdrive is also evoked by chronic blockade of brain (hypothalamic) GRs with glucocorticoid antagonists (30, 31).

Mice express two genes encoding drug-transporting P-glycoproteins, mrd1a and mrd1b (32), which both embody all functions displayed by one single human MDR1 (18). The mrd1a is predominantly expressed in intestines, liver, testis, and brain capillary endothelial cells, whereas mrd1b occurs in adrenal, ovaries, placenta, and uterus (33). The latter gene, if overexpressed in murine thymoma cell line confers resistance to dexamethasone, which can be prevented by treatment with P-glycoprotein blockers such as verapamil (34). Because the synthetic glucocorticoids are also substrates for the P-glycoprotein cellular extrusion pump, they potentially could induce their own resistance. In yeast the ATP binding cassette transport protein LEM1 has strong homology with the P-glycoprotein detoxification proteins and also actively exports dexamethasone. Resistance to xenobiotic substances seems therefore an evolutionary conserved principle (35).

In conclusion, the brain appears protected against moderate amounts of synthetic glucocorticoids such as dexa-

methasone by a drug-exporting mdr1a encoding P-glycoprotein in the blood-brain barrier. This phenomenon provides an additional level of glucocorticoid resistance to brain target cells and supports the concept of a pituitary site of action of dexamethasone on stress-induced HPA activation. Moderate amounts of dexamethasone, therefore, may create a condition of chemical adrenalectomy in the brain, which is expected to destabilize neuronal homeostasis and lead to dysfunction of discreetly localized nerve cells in hippocampus through underexposure to the vitally important corticosteroids.

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