

The role of the efflux transporter P-glycoprotein in brain penetration of prednisolone

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Abstract

In the present study, we have investigated the role of the multidrug resistance (mdr) P-glycoprotein (Pgp) at the blood-brain barrier in hampering the access of the synthetic glucocorticoid, prednisolone.

In vivo, a tracer dose of [³H]prednisolone poorly penetrated the brain of adrenalectomised wild-type mice, but the uptake was more than threefold enhanced in the absence of Pgp expression in *mdr1a* (–/–) mice. *In vitro*, in stably transfected LLC-PK1 monolayers the human MDR1 P-glycoprotein was able to transport prednisolone present at a micromolar concentration. A specific Pgp blocker, LY 335979, could block this polar transport of

[³H]prednisolone. Human Pgp does not transport all steroids, as cortexolone was not transported at all and aldosterone was only weakly transported.

The ability of Pgp to export the synthetic glucocorticoid, prednisolone, suggests that uptake of prednisolone in the human brain is impaired, leading to a discrepancy between central and peripheral actions. Furthermore, the ensuing imbalance in activation of the two types of brain corticosteroid receptors may have consequences for cognitive performance and mood.

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Introduction

The synthetic glucocorticoids prednisolone and dexamethasone are widely used as anti-inflammatory and immunosuppressive drugs, because of their potent glucocorticoid actions in combination with their low mineralocorticoid (salt-retaining) actions. These actions are mediated by glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) respectively (De Kloet *et al.* 1998). Both types of corticosteroid receptors are present in the brain. Glucocorticoid treatment, therefore, has major effects on cognitive function (Belanoff *et al.* 2001*b*), whereas blockade of GR action seems to be a promising anti-depression strategy (Belanoff *et al.* 2001*a*). Glucocorticoids are commonly believed to cross endothelial barriers with relative ease because of their highly lipophilic nature and their small size. However, we recently demonstrated that the penetration of dexamethasone into mouse brain is hampered because the multidrug resistance 1a (*mdr1a*) P-glycoprotein (Pgp) excludes this high affinity GR ligand from the brain (Schinkel *et al.* 1995, Meijer *et al.* 1998). The drug-transporting Pgp is expressed at the luminal membranes of endothelial cells of the blood-brain barrier (BBB) (Cordon-Cardo *et al.* 1989, Thiebaut *et al.* 1989). This transmembrane protein is

encoded by the *mdr1a* gene in rodents and by the highly homologous MDR1 gene in humans (Jette *et al.* 1995, Van de Vrie *et al.* 1998). We hypothesised that the synthetic GR ligand, prednisolone, is also a substrate of this efflux transporter and is thus hampered in its ability to enter the glucocorticoid target areas in the brain. A poor penetration of the BBB by this synthetic glucocorticoid may have important implications for its actions on brain function.

Pgp-mediated transport is not a common feature of steroids. We have examined Pgp-mediated transport of several naturally occurring corticosteroids. Recently, we have shown that there is a large difference in transport between the mixed MR/GR agonists cortisol and corticosterone (Karssen *et al.* 2001). Cortisol is transported by human MDR1 Pgp and, in line with the presence of MDR1 Pgp at the BBB, the levels of cortisol in human brain are decreased towards those of corticosterone. In contrast, the latter compound freely crosses the BBB in rodents as well as in man (Karssen *et al.* 2001). We now demonstrate the lack of robust Pgp-mediated transport for two other corticosteroids which are circulating in human plasma, i.e. the high affinity MR ligand, aldosterone and the precursor of cortisol, 11-deoxycortisol (cortexolone).

We have tested our main hypothesis about the role of Pgp at the BBB in reducing the penetration of

prednisolone into the brain in two ways. First, we tested whether *mdr1a* Pgp at the mouse BBB limits *in vivo* brain penetration of prednisolone. For this purpose we have injected adrenalectomised *mdr1a* ($-/-$) and wild-type mice with [^3H]prednisolone with or without pretreatment with excess unlabelled prednisolone. Secondly, we have investigated *in vitro* whether the human homologue, MDR1 Pgp, is also able to transport prednisolone. Therefore, we measured polar transport in monolayers of pig kidney epithelial cells stably transfected with human cDNA of MDR1 Pgp compared with nontransfected host cells.

Materials and Methods

In vivo distribution and autoradiography

The *in vivo* distribution experiments were carried out as described previously (Meijer *et al.* 1998, Karssen *et al.* 2001) with some modifications. Male *mdr1a* ($-/-$) and wild-type Friends virus B (FVB) mice were bred under specific pathogen free conditions at TNO (Leiden, The Netherlands). Male mice at the age of 18–23 weeks were used for this study. All experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC and with approval from the animal care committee of the Faculty of Medicine, Leiden University (Leiden, The Netherlands).

After transport, the mice were housed individually at our laboratory, at ambient temperature and under a 12 h/12 h lighting schedule (lights on at 0700 h, lights off at 1900 h) with free access to food and water.

To remove the source of endogenous corticosterone, mice were bilaterally adrenalectomised under gas anaesthesia (isoflurane) by a dorsal approach. After adrenalectomy (ADX), the animals had free access to 0.9% saline. At the time of the experiment the animals weighed 28 ± 2.0 g (mean \pm S.D.). Two days after ADX, the animals were subcutaneously injected with tritiated prednisolone (dissolved in 2% ethanol/0.9% saline) for *in vivo* distribution. Wild-type ($n=9$) and mutant mice ($n=6$) were injected with $3.5 \mu\text{Ci}/10$ g [^3H]prednisolone (Amersham Pharmacia Biotech, UK, specific activity (SA) 48 Ci/mmol). For *in vivo* autoradiography, mice ($n=2$) were treated with $13.5 \mu\text{Ci}/10$ g [^3H]prednisolone in a separate but similar experiment. As a control for non-specific retention, one mouse of each genotype was pretreated with a 100-fold excess of unlabelled prednisolone (Sigma-Aldrich, Germany). One hour after injection the animals were decapitated. Trunk blood was collected in EDTA-coated tubes and centrifuged for determination of radioactivity and of remaining corticosterone in the plasma using a ^{125}I -corticosterone radioimmunoassay kit (ICN Biomedicals, Inc., Costa Mesa, CA, USA). The brain was dissected and quickly frozen in isopentane precooled on dry ice/ethanol. Liver, testis,

intestine and cerebellum were dissected and frozen on dry ice. All tissues were stored at -80°C until further use.

All organ tissues studied, except for the brain, were homogenised using Soluene-350 (Packard Bioscience, Groningen, The Netherlands). Hionic-Fluor (Packard Bioscience) was added to tissue homogenates, and plasma and radioactivity was determined in a Tricarb β -counter (Packard Instruments, Meriden, USA). Twelve-micrometre coronal sections of brain were cut on a cryostat and thaw-mounted on poly-L-lysine (Sigma Chemical Co., St Louis, MO, USA)-coated microscopic slides. The slides were put in an X-ray exposure holder (Amersham Pharmacia Biotech) and apposed to Ultrafilm (Leica Corp., Heerbrugg, Switzerland) for 18 months.

Transepithelial transport and inhibition studies

In order to examine the interactions of the glucocorticoid prednisolone, the mineralocorticoid aldosterone and the antiglucocorticoid cortexolone with the human P-glycoprotein, we used monolayers of the porcine kidney epithelial cell-line LLC-PK1 and LLC-PK1 cells stably transfected with cDNA of the human MDR1 gene (LLC-PK1:MDR1). Cells obtained from the American Type Culture Collection (Manassas, VA, USA) were kindly provided by the Dutch Cancer Institute (Amsterdam, The Netherlands) (Schinkel *et al.* 1995). Human P-glycoprotein has been shown before to be specifically expressed on the apical surface of LLC:PK1:MDR1 cells in these monolayers (Ueda *et al.* 1992). Therefore, Pgp substrates entering these cells from the basal side will be translocated to the apical compartment, while those entering the apical membrane will be pumped back into the medium, thus resulting in polarised transport of substrates. This system models the way Pgp is likely to function in the BBB in excluding drugs from the brain (Yamazaki *et al.* 2001).

Cells were cultured at 37°C in the presence of 5% CO_2 in complete medium, which consisted of DMEM (Biowhittaker Europe, Verviers, Belgium) supplied with 25 mM HEPES and 4.5 g/l glucose and supplemented with 100 000 U/l penicillin, 100 mg/l streptomycin, 2 mM L-glutamine and 10% (vol/vol) foetal calf serum. The LLC-PK1 and LLC-PK1:MDR1 cell lines were subcultured by trypsinization every 3 to 4 days and medium was replaced twice a week.

During the experiments complete medium was used. The LLC-PK1 and LLC-PK1:MDR1 cells were seeded on microporous polycarbonate membrane filters ($0.4 \mu\text{m}$ pore size, 12 mm diameter, Transwell, Costar, Corning BV, Schiphol-Rijk, The Netherlands) at a density of 120×10^3 cells/cm 2 . The cells were grown for 5–6 days in complete medium with a medium replacement at day 3. Two hours before the start of the experiment, the medium was replaced with 800 μl fresh medium at both the apical and basal side of the monolayer. In the inhibition

experiments, one hour later the potent and selective P-glycoprotein blocker LY 335979 (1 μ M in water; kindly provided by Eli Lilly & Co (USA)) or water was added at the basal side. To measure the transepithelial transport from the apical to the basal side or from the basal to the apical side 8 μ l of a 100 times stock of tritiated steroid in ethanol were added in triplicate at the apical or basal side respectively, at the start of the experiment ($t=0$). We have tested [3 H]prednisolone, and [1,2,6,7- 3 H]aldosterone (Amersham Pharmacia Biotech, UK; SA 64 Ci/mmol), (1,2(n)-[3 H]deoxycortisol (=cortisolone; NEN Life Science Products, Boston, USA; SA 57 Ci/mmol) and, as a positive control, [1,2,4,6,7- 3 H]dexamethasone (Amersham Pharmacia Biotech, UK; SA 91 Ci/mmol). As we were interested whether MDR1 Pgp was able to transport prednisolone even at high concentrations we supplemented [3 H]prednisolone with 1 μ M unlabelled prednisolone. The starting concentrations for each experiment are mentioned in the legends of the corresponding Figures. Over the four hours of study, 75 μ l aliquots were taken once every hour from both compartments. Eight microlitre samples of the 100 times stock, and samples from the compartment opposite the one to which activity was added, were counted in a Tricarb β -counter after adding 3 ml Emulsifier Safe (Packard). Basal-to-apical and apical-to-basal transport is presented as a percentage of total radioactivity added at the beginning of the experiment. Transepithelial electrical resistance was measured before and after the experiments to check the integrity of the monolayers (Gaillard & De Boer 2000).

Statistical analysis

Mouse data were evaluated by Student's *t*-test. The results of the monolayer experiments were analysed by repeated measures ANOVA. Significance was taken at $P<0.05$.

Results

Differences in brain uptake of [3 H]prednisolone in *mdr1a* (-/-) and wild-type mice

One hour after administration of [3 H]prednisolone to ADX mice, the uptake of radioactivity in brain showed a clear difference between *mdr1a* (-/-) mice and wild-type mice. After injection with 2.5 μ g/kg radiolabelled prednisolone the amount of radioactivity in cerebellum homogenates was 3.2-fold higher in mutants than in *mdr1a* (+/+) mice (Table 1). The plasma levels of radioactivity were similar and the concentrations in the liver or any other peripheral tissue examined were not significantly different between the genotypes (Table 1). Comparable results were obtained after administration of 10 μ g/kg [3 H]prednisolone (Table 1). Although absolute levels in all tissues and blood were obviously higher after

Table 1 Uptake of radioactivity in tissue homogenates and blood 1 h after administration of [3 H]prednisolone at two dose levels. Results are means \pm S.E.M.

	Wild-type	<i>mdr1a</i> (-/-)
[3H]Prednisolone (2.5 μg/kg)		
<i>n</i>	9	6
Cerebellum (nCi/mg)	0.009 \pm 0.001	0.030 \pm 0.001*
Plasma (nCi/ μ l)	0.044 \pm 0.003	0.043 \pm 0.001
Liver (nCi/mg)	1.443 \pm 0.076	1.565 \pm 0.147
Testis (nCi/mg)	0.030 \pm 0.001	0.033 \pm 0.003
Intestine (nCi/mg)	1.040 \pm 0.246	1.146 \pm 0.325
Brain/blood ratio	0.225 \pm 0.022	0.725 \pm 0.026*
[3H]Prednisolone (10 μg/kg)		
<i>n</i>	2	2
Cerebellum (nCi/mg)	0.050 \pm 0.010	0.225 \pm 0.021*
Plasma (nCi/ μ l)	0.247 \pm 0.035	0.337 \pm 0.034
Brain/blood ratio	0.202 \pm 0.014	0.668 \pm 0.004*

* $P<0.05$, Student's *t*-test, pretreated animals are not excluded from the analysis.

Table 2 Uptake of radioactivity in tissue homogenates and blood after pretreatment with unlabelled prednisolone

	Wild-type	<i>mdr1a</i> (-/-)
Pretreated with 0.25 mg/kg prednisolone followed by 2.5 μg/kg [3H]prednisolone		
<i>n</i>	2	1
Cerebellum (nCi/mg)	0.009 \pm 0.001	0.031
Plasma (nCi/ μ l)	0.040 \pm 0.003	0.038
Liver (nCi/mg)	1.244 \pm 0.018	1.234
Testis (nCi/mg)	0.027 \pm 0.002	0.026
Intestine (nCi/mg)	1.951 \pm 0.533	2.191
Brain/blood ratio	0.222 \pm 0.002	0.645
Pretreated with 1 mg/kg prednisolone followed by 10 μg/kg [3H]prednisolone		
<i>n</i>	1	1
Cerebellum (nCi/mg)	0.061	0.204
Plasma (nCi/ μ l)	0.282	0.303
Brain/blood ratio	0.216	0.673

this higher dose, the brain-to-blood ratios were not significantly different from the ratios obtained after administration of the lower dose (Table 1). Remarkably, pretreatment with unlabelled prednisolone does not lead to any change in uptake of radioactivity in the cerebellum (Table 2). Thus, disruption of the *mdr1a* gene leads to enhanced uptake of prednisolone into the brain.

The autoradiograms also clearly demonstrate the difference between knockouts and controls. The *mdr1a* (+/+) animals showed negligible labelling of brain tissue after administration of [3 H]prednisolone (Fig. 1A). Labelling in brain sections was restricted to the cerebral ventricles. Some radioactive label seemed to have penetrated into the brain tissue around the ventricles, but the amount was considerably lower compared with the amount taken up

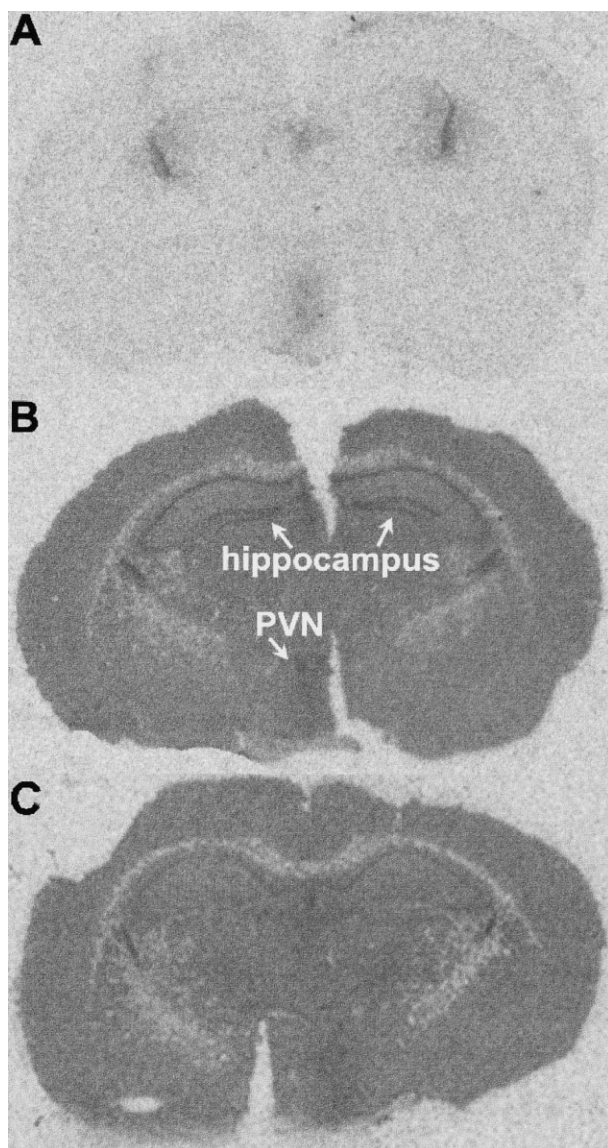


Figure 1 Representative autoradiograms of 12- μ m coronal sections of the brain demonstrate the large difference in uptake of [3 H]prednisolone in wild-type (A) and *mdr1a* (-/-) mouse (B) brains one hour after administration of 13.5 μ Ci/10 g body weight. The dark spots in (A) represent transverse sectioning of the cerebroventricular space and adjacent ventricular walls. Pretreatment with unlabelled prednisolone abolishes hippocampal labelling in *mdr1a* (-/-) mouse brain but does not affect the labelling of the rest of the brain (C).

by the brain of *mdr1a* (-/-) mice. These mutant mice showed increased labelling of whole brain (Fig. 1B). In particular, radioactivity was retained in the paraventricular nucleus (PVN) and hippocampal cell fields. These brain areas abundantly express GRs. Pretreatment with a 100-fold excess of unlabelled prednisolone prevents this specific labelling (Fig. 1C). Remarkably, this pretreatment does

not affect the labelling of the rest of the brain as was also shown in the cerebellum homogenates. Accordingly, these data clearly demonstrate that the presence of *mdr1a* Pgp in the BBB hampers the access of prednisolone to the mouse brain, particularly to the target areas within the brain that abundantly express GR.

Trans epithelial transport of prednisolone in monolayers of LLC-PK1 and LLC-PK1:MDR1 cells

To test whether the human homologue of the *mdr1a* Pgp, MDR1 Pgp, is also able to transport prednisolone, we studied the transport capabilities of monolayers of pig kidney cells stably transfected with the human MDR1 cDNA compared with untransfected cells. Unlabelled prednisolone (1 μ M) supplemented with a tracer dose of [3 H]prednisolone was added to the basal or apical compartment. Prednisolone was transported in a clear polarised fashion in the MDR1-transfected monolayers, but not in monolayers of the parental cells (Fig. 2A), indicating that the human Pgp is able to transport prednisolone, even at the high concentration tested. Polarised transport in MDR1 monolayers of prednisolone was abolished in the presence of LY 335979, a potent and selective Pgp blocker (Starling *et al.* 1997, Dantzig *et al.* 1999), resulting in similar fractions transported as in untransfected cells (Fig. 2B). This confirms that prednisolone transport is largely mediated by human P-glycoprotein.

As a positive control, we also assessed transepithelial transport of dexamethasone in our monolayers and demonstrated that MDR1 Pgp also efficiently transports this synthetic glucocorticoid (data not shown), as has been shown previously (Ueda *et al.* 1992, Schinkel *et al.* 1995).

Trans epithelial transport of naturally occurring steroids in monolayers of LLC-PK1 and LLC-PK1:MDR1 cells

Furthermore, we tested corticosteroids that are naturally occurring in humans. Previously, we published the marked difference between transport of cortisol and corticosterone (Karssen *et al.* 2001). Now we focused on two additional corticosteroids, cortexolone and aldosterone. Cortexolone transport in the MDR1-transfected monolayers was not different from transport in monolayers of control cells (Fig. 3A), although polarised transport was observed in both cell lines which is probably caused by other renal transporters. This indicates the absence of human MDR1 Pgp-mediated transport of cortexolone. Administration of LY 335979 did not change the fraction of cortexolone translocated through the membrane (Fig. 3B), confirming the lack of Pgp contribution to the transport of cortexolone.

Examination of the transport of aldosterone shows that, although aldosterone displayed polarised transport in both transfected and parental monolayers, there was a small but

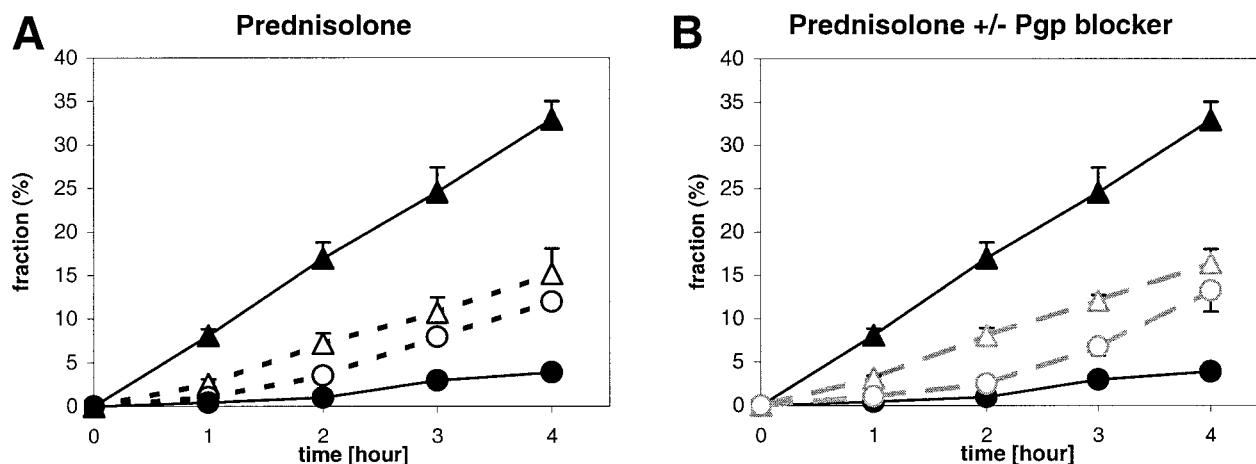


Figure 2 (A) Activity of [3 H]prednisolone present in medium at different time points after adding 1 μ M prednisolone supplemented with [3 H]prednisolone to the opposite compartment at $t=0$. Transepithelial transport from the basal to apical (Δ , \blacktriangle) and from the apical to basal (\circ , \bullet) compartment was measured in wild-type LLC-PK1 (broken line) or MDR1-transfected LLC-PK1 (solid line) monolayers. (B) Transepithelial transport of prednisolone was measured in MDR1-transfected LLC-PK1 monolayers after adding 1 μ M LY 335979 (broken line) or vehicle (water, solid line) 1 h before the start of the experiment. Results are presented as the fraction of the dose of radioactivity, which is 7 nM, added to the respective compartment. Each point represents the mean of three monolayers \pm S.E.M. Repeated measures ANOVA showed a significant interaction of time*cell type*transport for prednisolone in both (A) and (B) ($P<0.0001$).

significant MDR1 Pgp contribution (Fig. 3C). This Pgp-mediated transport could be partly blocked by LY 335979 (Fig. 3D). These results demonstrate that aldosterone is only weakly transported by MDR1 Pgp.

Discussion

The present study indicates that the efflux transporter Pgp at the level of the BBB decreases the degree of brain exposure to the synthetic glucocorticoid prednisolone. Our *in vivo* autoradiography data show that the *mdr1a* Pgp present in BBB hampers the penetration of [3 H]prednisolone into the mouse brain, whereas our results with monolayers of human MDR1 cDNA-transfected LLC-PK1 cells suggest that Pgp exports prednisolone from the human brain as well. We further demonstrated that aldosterone is transported by Pgp to a much smaller extent, while corticosterone is not transported at all.

Cells expressing P-glycoproteins are able to exclude a wide variety of structurally and functionally unrelated drugs, a phenomenon called multidrug resistance (Van de Vrie *et al.* 1998). It is now well established that Pgp is expressed in many normal tissues including the intestinal epithelium, the adrenals and brain capillary endothelial cells (Schinkel 1999). Several steroids such as dexamethasone and cortisol but not corticosterone are among its substrates (Ueda *et al.* 1992, Bourgeois *et al.* 1993, Ueda *et al.* 1996, Karssen *et al.* 2001). In the last decade, several studies have established the Pgp-mediated transport of the widely used synthetic glucocorticoid, dexamethasone. Pgp overexpressing cells accumulate reduced amounts of

dexamethasone (Barnes *et al.* 1996), while the steroid is transported in a polarised fashion in monolayers of LLC-PK1 cells transfected with MDR1 (Ueda *et al.* 1992, Schinkel *et al.* 1995, this study). Furthermore, dexamethasone penetration into *mdr1a* ($-/-$) mouse brain is enhanced compared with wild-type brain (Schinkel *et al.* 1995), increasing the access to the glucocorticoid receptor (Meijer *et al.* 1998). While dexamethasone transport by Pgp has thus been convincingly demonstrated, information about Pgp-mediated transport of prednisolone is sparse. Bourgeois *et al.* (1993) have shown that murine thymoma cells expressing *mdr1b* Pgp are resistant to prednisolone (and dexamethasone)-induced apoptosis, but this second murine multidrug resistance Pgp is not expressed at the BBB. Our study clearly shows that both mouse *mdr1a* and human MDR1 Pgp transport prednisolone, as has been shown previously for dexamethasone and cortisol. This implies that any GR expressing cell type which also expresses the efflux transporter Pgp is resistant to these glucocorticoids, which is in line with the recent results of Pariente *et al.* (2001).

In contrast to these glucocorticoids, Pgp does not transport corticosterone (Karssen *et al.* 2001), a glucocorticoid that also circulates in human plasma but at 10-fold lower levels than cortisol and some other naturally occurring steroids. In line with the presence of MDR1 Pgp at the BBB, the levels of cortisol in human brain are decreased towards the brain levels of corticosterone as measured in post mortem specimens with liquid chromatography-mass spectrometry (Karssen *et al.* 2001). We now demonstrate that Pgp is also unable to transport corticosterone, which is a weak partial agonist/antagonist at

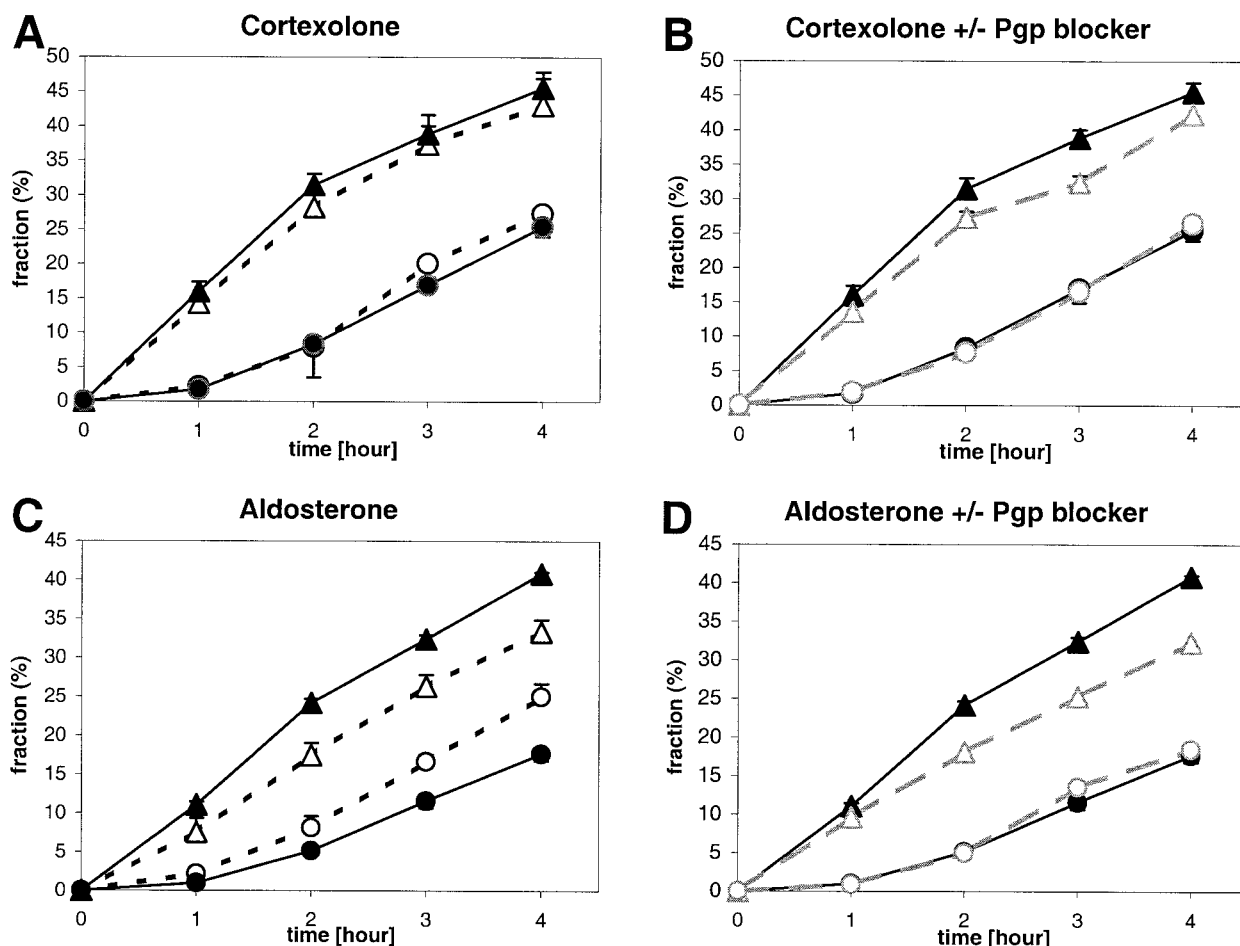


Figure 3 Activity of [^3H]cortisolone (A, B) and [^3H]aldosterone (C, D) present in medium at different time points after adding [^3H] steroid to the opposite compartment at $t=0$. (A, C) Transepithelial transport from the basal to apical (Δ , \blacktriangle) and from the apical to basal (\circ , \bullet) compartment was measured in wild-type LLC-PK1 (dotted line) or MDR1-transfected LLC-PK1 (solid line) monolayers. (B, D) Transepithelial transport from the basal to apical (Δ , \blacktriangle) and from the apical to basal (\circ , \bullet) compartment was measured in MDR1-transfected LLC-PK1 monolayers after adding 1 μM LY 335979 (broken line) or water (solid line) one hour before the start of the experiment. Results are presented as the fraction of the dose of radioactivity, which is 4 to 5 nM for both steroids, added to the respective compartment. Each point represents the mean of three monolayers \pm s.e.m. In the case of aldosterone the experiment was repeated three times with similar results. Repeated measures ANOVA did not show a significant interaction of time*cell type*transport for either corticosteroid.

the GR *in vitro* (Kaiser & Mayer 1980, Schmidt & Davidson 1987) and *in vivo* (Acs & Stark 1975, Duncan & Duncan 1979, Kaiser & Mayer 1980). In addition, the high affinity MR ligand aldosterone is only weakly transported by Pgp as demonstrated by our data. These results agree with several *in vitro* studies on Pgp-mediated transport of aldosterone. Using comparable monolayers to those in this study, Ueda *et al.* (1992) have demonstrated that aldosterone is moderately transported by the human MDR1 Pgp, while Bourgeois *et al.* (1993) showed that cortisolone was not and aldosterone was only weakly transported by *mdr1b* Pgp. The weak transport of aldosterone by Pgp cannot explain why this mineralocorticoid

seems to play a limited role in limbic functioning relative to corticosterone, while both steroids bind with similar affinity to MR *in vitro* (Veldhuis *et al.* 1982, De Kloet 1991). Moreover, upon administration of tracer amounts of [^3H]cortisolone and [^3H]aldosterone to adrenalectomised rodents both steroids are retained very well in limbic brain structures that abundantly express MR (Birmingham *et al.* 1984). However, in adrenal-intact animals only a small amount of aldosterone is extracted from hippocampal cell nuclei relative to corticosterone, probably because the latter steroid circulates in one hundred to one thousand times higher concentrations in the blood (Yongue & Roy 1987). Cells conferring aldosterone

selectivity are present in the periventricular brain areas involved in salt appetite, regulation of the electrolyte balance and autonomic outflow (Seckl 1997, Van Acker *et al.* 2002). This aldosterone selectivity is due to an 11β -steroid dehydrogenase that breaks down corticosterone allowing aldosterone access to MR (Seckl 1997). In the hippocampus this reductase activity is absent (Robson *et al.* 1998). Further studies with *mdr1a* ($-/-$) mice are necessary to directly examine the involvement of Pgp in aldosterone uptake in brain.

Although prednisolone is one of the most common clinically used glucocorticoids, this study is the first that shows the fate of prednisolone in brain, whereas other frequently occurring glucocorticoids have been extensively studied before (for review see McEwen *et al.* 1986). In contrast to uptake into the brain of both corticosterone and aldosterone (McEwen *et al.* 1968, 1976, De Kloet *et al.* 1975, De Nicola *et al.* 1981, Coutard *et al.* 1987), access of dexamethasone and cortisol to brain is impaired (De Kloet *et al.* 1974, 1975, Rees *et al.* 1975, McEwen *et al.* 1976, Karssen *et al.* 2001), which has been shown to be caused by the presence of Pgp at the BBB (Meijer *et al.* 1998, Karssen *et al.* 2001). We now demonstrate that prednisolone is also hampered in its ability to reach the brain due to the presence of Pgp at this barrier. The active exclusion from the brain provides an explanation for the long-established puzzling phenomenon that GR in the brain is not labelled after *in vivo* administration of tracer doses of synthetic GR ligands.

In *mdr1a* null mice the high affinity GR ligand prednisolone is retained by hippocampal and paraventricular neurons as expected based on localisation of GR expressing cells (Van Eekelen *et al.* 1987). Among the hippocampal subfields, the CA3 pyramidal layer retained the lowest amount of label, in accordance with the neuro-anatomical distribution of hippocampal GR as measured with immunocytochemistry and *in situ* hybridisation (Van Eekelen *et al.* 1987, 1988). Pretreatment of mice with 100-fold excess of unlabelled prednisolone abolishes this selective retention in hippocampal cells and the PVN, but does not affect the overall uptake elsewhere in the brain. This indicates that the uniform labelling of the rest of the brain may be due mainly to non-receptor bound, freely moving prednisolone masking specific nuclear retention of low abundantly expressed GR in these areas.

Prednisolone, like dexamethasone, can bind to MR *in vitro*, but the affinity to this receptor is much lower than to GR to which these steroids bind with very high affinity (<1 nM) (Lan *et al.* 1981, 1982, De Kloet *et al.* 1984). In contrast, corticosterone and cortisol bind with high affinity to MR (<1 nM) and with tenfold lower affinity to GR. In line with this low affinity to the MR, prednisolone has only minor effects on salt retention in the kidney (Karssen & De Kloet 2000). Using *in vivo* autoradiography, only very high affinity receptors can be visualised due to the

low doses used (De Kloet 1991); e.g. in *mdr1a* ($-/-$) mice only MR can be made visible with [3 H]cortisol autoradiography as is the case with [3 H]corticosterone in both wild-type and mutant mice (Karssen *et al.* 2001). In this study, prednisolone is only able to visualise GR, while the MR has too low an affinity for this steroid for detection.

The lack of effect of pretreatment of unlabelled prednisolone on uptake of [3 H]prednisolone in wild-type brain indicates that the capacity of Pgp to expel prednisolone from brain is rather high. Furthermore, the monolayer results using $1\ \mu\text{M}$ prednisolone also suggest that even at high concentrations Pgp is able to reduce prednisolone concentrations in the brain. Therapeutically, prednisolone is used in high doses in the treatment of diverse medical conditions, including pulmonary, rheumatological, neurological and autoimmune diseases and immune suppression following organ transplantation. Our results suggest that in humans treated with prednisolone, the resultant glucocorticoid levels in brain would be considerably lower than plasma levels. As most peripheral tissues are not protected by a Pgp expressing barrier, peripheral effects would therefore be relatively more potent than central effects, although this does not preclude central effects of prednisolone.

Hippocampal-dependent memory impairment after long-term high dose treatment with prednisone (which is quickly converted to prednisolone *in vivo*) has been described (Keenan *et al.* 1996). As hippocampal GR is involved in memory performance (Oitzl & De Kloet 1992), these effects could be attributed to activation of this receptor. On the other hand, an imbalance in central MR and GR activation as a result of prednisolone treatment could provide an alternative explanation. Differential effects mediated by MR and GR activation on cognitive function have been proposed (De Kloet *et al.* 1999, Lupien & Lepage 2001). Although mainly based on animal studies, De Kloet and colleagues (De Kloet 1991, De Kloet *et al.* 1999) have postulated that a balance between MR- and GR-mediated effects critically determines human cognitive functioning. Whether prednisolone reaches the brain or not, due to the prednisolone-induced suppression of pituitary-adrenal activity and thus of adrenocortical secretion, the brain becomes deprived of the endogenous glucocorticoids, corticosterone and cortisol. As a consequence, the ratio of MR/GR occupation will shift towards GR occupation with consequences for cognitive performance, mood and regulation of the behavioural stress response.

As many different drugs used in the clinic are Pgp substrates as well, one has to be aware of undesired side effects when prednisolone (or dexamethasone) is used in conjunction with these drugs. Cotreatment may be able to enhance the brain uptake of the synthetic glucocorticoid. The potent immunosuppressants FK506 and cyclosporine A have been shown to potentiate dexamethasone-

but not corticosterone-mediated transcriptional activity, apparently due to inhibition of a MDR pump similar to Pgp (Medh *et al.* 1998). The facilitation of prednisolone's poor penetration into the brain by anti-cancer drugs may also give a rationale to the reported success of combination therapy in treatment of different types of brain tumours (Shibamoto *et al.* 1999, Wu *et al.* 1999, Maipang & Janjindamai 2000). Many anti-cancer drugs (e.g. Vinca alkaloids, anthracyclines, and taxanes) are known to be Pgp substrates. In fact, the first reports of Pgp expression dealt with tumour cells developing multidrug resistance after treatment with a single cytotoxic drug. Further treatment of these tumours is difficult. Provided that the tumour does not disrupt the BBB, brain tumours are even intrinsically resistant to these drugs, as they are behind the BBB (Regina *et al.* 2001). A combination of drugs including prednisolone, which are all Pgp substrates, may mutually increase their active brain levels by saturating Pgp.

It is remarkable that, in spite of its broad spectrum of substrates (Schinkel *et al.* 1994), Pgp distinguishes subtle differences in steroid structure. The differences shown in this study reflect the importance of both the 17-hydroxyl and the 11-hydroxyl group in determining the ability of steroids to be transported by Pgp as shown by Bourgeois *et al.* (1993). Pgp transports steroids having both these hydroxyl groups (such as prednisolone and dexamethasone). Steroids lacking one of these groups (such as aldosterone, corticosterone and cortexolone) and steroids without any of these groups are minimally transported, if at all. The high affinity MR ligand deoxycorticosterone belongs to the latter group and therefore it should easily be retained in the brain. However, although it readily enters the brain (Kraulis *et al.* 1975), McEwen *et al.* (1976) have shown that deoxycorticosterone is poorly retained by MR in different brain areas and pituitary of ADX rats. This suggests that there are additional factors, e.g. local metabolism, determining the retention of this mineralocorticoid in potential target areas.

In conclusion, we have demonstrated the involvement of Pgp in hampering the access of the synthetic glucocorticoid prednisolone to mouse brain. The ability of the human MDR1 Pgp to transport prednisolone suggests that prednisolone access to human brain is also impeded. The poor penetration of prednisolone into human brain would presumably lead to a discrepancy in the extent of central and peripheral actions of prednisolone. The subsequent imbalance in MR and GR activation may explain the reported changes in cognitive performance and mood in response to prednisone/prednisolone therapy.

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