Glucocorticoid access to the brain

Involvement of the efflux transporter P-glycoprotein

GLUCOCORTICOID ACCESS TO THE BRAIN

INVOLVEMENT OF THE EFFLUX TRANSPORTER P-GLYCOPROTEIN

PROEFSCHRIFT

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Aan Henk

Wetenschap zet onze ervaringen in een prachtig geordend perspectief, maar is ijzingwijkend stil over alles wat ons hart echt raakt.

(Erwin Schrödinger (1887-1961), fysicus, winnaar Nobelprijs 1933)

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ABBREVIATIONS

ABC-transporter	adenosine triphosphate (ATP) binding cassette transporter
ACTH	adrenocorticotropic hormone
ADX	adrenalectomy / adrenalectomised
AVP	arginine vasopressin
BBB	blood-brain barrier
BCB	blood-CSF-barrier
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
CTRL	control
DEX	dexamethasone
DST	dexamethasone suppression test
GFAP	glial fibrillary acidic protein
GR	glucocorticoid receptor
HPA-axis	hypothalamus-pituitary-adrenal-axis
MDR	multidrug resistance
MR	mineralocorticoid receptor
MRP	multidrug resistance-associated protein
Pgp	P-glycoprotein
POMC	pro-opiomelanocortin
PVN	hypothalamic paraventricular nucleus

Chapter 1

Introduction and Objectives

Numerous functions of glucocorticoid hormones have been extensively studied in both clinical and animal research. Such research has particularly focussed on the role of these hormones in the response to stress and in pathogenesis of stress-related disorders. From these studies, glucocorticoids have emerged as important modulators of brain function. To reach their diverse target areas in the brain they have to enter the brain by passing the blood-brain barrier (BBB), a dynamic barrier that protects the brain from peripheral influences. Although the importance of the actions of glucocorticoids in brain is commonly accepted, modulation of glucocorticoid access at the BBB level has hardly been a subject of research as these hormones are considered to readily pass this barrier. Now that it has been demonstrated that transmembrane proteins are able to transport glucocorticoids (Bourgeois *et al.*, 1993; Thompson, 1995; Ueda *et al.*, 1996), this issue becomes an increasingly interesting subject to study. Dysfunction of the central glucocorticoid signalling system might be related to changed efflux of glucocorticoids from the brain.

The research in this thesis is aimed at revealing the importance of glucocorticoid transport at the BBB. It describes the impact of the presence of the efflux transporter P-glycoprotein on the uptake of glucocorticoids and it describes a way of targeting P-glycoprotein to affect the stress system. Knowledge about the role of the BBB in modulating glucocorticoid access to the brain might eventually provide clues for development of new drugs that might be able to restore aberrant corticosteroid functioning in stress-related disorders.

GLUCOCORTICOIDS

Glucocorticoids belong to the class of adrenal corticosteroid hormones. Adrenal corticosteroids are essential for life, as they coordinate the responses of body and brain to changes in both the external and internal environment. They play a crucial role in homeostasis, which comprises the processes of maintaining the internal environment of the body in a condition consistent with survival of the individual (Fink, 2000).
Generally, two subgroups of adrenal corticosteroids are recognised, mineralocorticoids and glucocorticoids. The subgroup of glucocorticoids comprises many structurally related naturally occurring and synthetic hormones (figure 1). The main naturally occurring glucocorticoids are cortisol and corticosterone. The most common synthetic glucocorticoids are dexamethasone and prednisone/prednisolone.

Functions of Corticosteroids

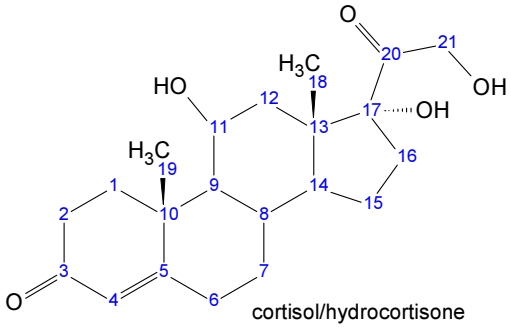
The prime physiological function of mineralocorticoids (mainly aldosterone) is control of electrolyte homeostasis and blood pressure (Agarwal and Mirshahi, 1999). Glucocorticoids have more widespread effects. In fact, virtually every cell in the body is sensitive to their actions. These hormones act on a wide range of physiological functions, including those involved in energy balance and metabolism, immunity, circadian rhythmicity, cardiovascular

regulation, cognitive processing, behavioural adaptation and mood (Murphy, 2000). The actions of synthetic glucocorticoids are generally more potent than those of naturally occurring glucocorticoids. For this reason and because they are often devoid of mineralocorticoid (=salt-retaining) actions, they are commonly used as anti-inflammatory and immunosuppressive drugs.
A major target of glucocorticoids is the brain (McEwen *et al.*, 1986a; Belanoff *et al.*, 2001). Their effects on the brain range from feedback inhibition of their own secretion (Keller-Wood and Dallman, 1984; Dallman *et al.*, 1987a), and modification of neuronal integrity and function (Joëls and De Kloet, 1992; Magariños *et al.*, 1997; Gould and Tanapat, 1999; McEwen, 1999), to modulation of memory and learning processes and behavioural adaptation to stress (Lupien and McEwen, 1997; De Kloet *et al.*, 1998; De Kloet *et al.*, 1999).

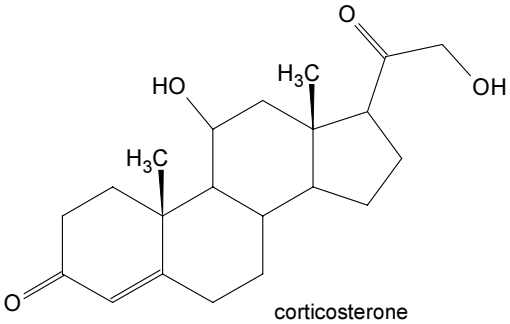
Glucocorticoid secretion

Endogenous glucocorticoids are secreted by the adrenal gland under tight control of the brain. The neuroendocrine system that regulates this secretion is known as the Hypothalamo-Pituitary-Adrenal Axis or HPA-axis (figure 2). This system provides the link between the perception of physical and psychological stress and the regulation of key homeostatic mechanisms in brain and periphery.
Upon activation by a large variety of stimuli, a specific set of neuroendocrine neurons in the paraventricular nucleus of the hypothalamus (PVN) secretes corticotropin-releasing hormone (CRH) into a portal vasculature to the anterior pituitary. In many species these parvocellular neurons, located bilaterally from the third ventricle, co-secrete vasopressin (AVP). Within the anterior pituitary, the peptidergic hormone CRH stimulates cells both to synthesise adrenocorticotropic hormone (ACTH) from its precursor pro-opiomelanocortin (POMC), and to release ACTH into the main circulation, whereas AVP potentiates the effect of CRH. Via the blood circulation ACTH reaches the adrenal cortex to stimulate the synthesis of glucocorticoid hormones from cholesterol. These hormones are immediately secreted into the systemic circulation to act elsewhere in the body.

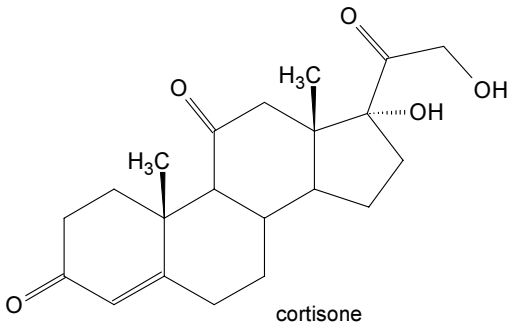
In mice and rats, corticosterone is the only active endogenous glucocorticoid hormone. In other species including humans the principal glucocorticoid is cortisol. However, in these species some corticosterone is also circulating *e.g.* in humans at 10 to 20 times lower levels than cortisol (Underwood and Williams, 1972; Kage *et al.*, 1982). As most research is done in rats and mice, much is known about the actions of corticosterone in the rodent brain. Less is known about the central actions of cortisol in human and other cortisol secreting animals, although it is generally assumed that these actions are similar to -or at least comparable with- those of corticosterone (Lupien and McEwen, 1997). Indeed, no major differences have been described so far between the actions of corticosterone and cortisol.



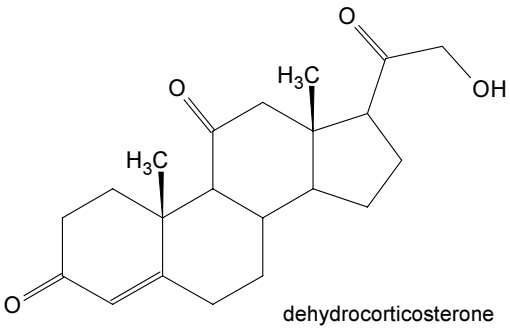
cortisol/hydrocortisone



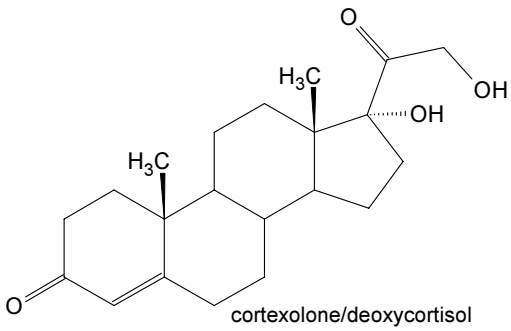
corticosterone



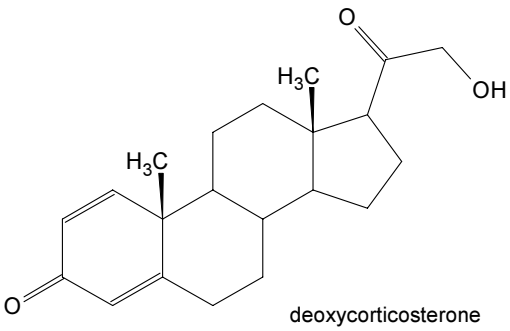
cortisone



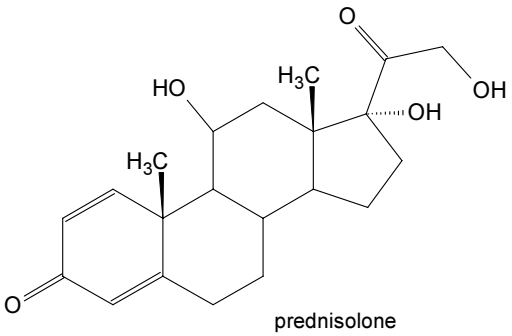
dehydrocorticosterone



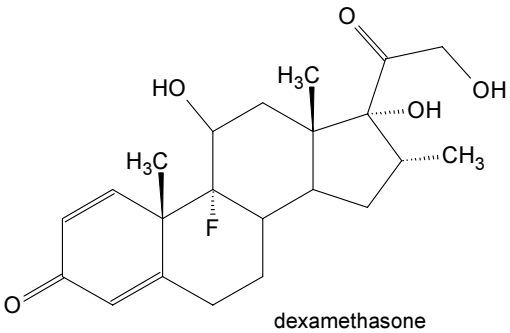
cortexolone/deoxycortisol



deoxycorticosterone



prednisolone



dexamethasone

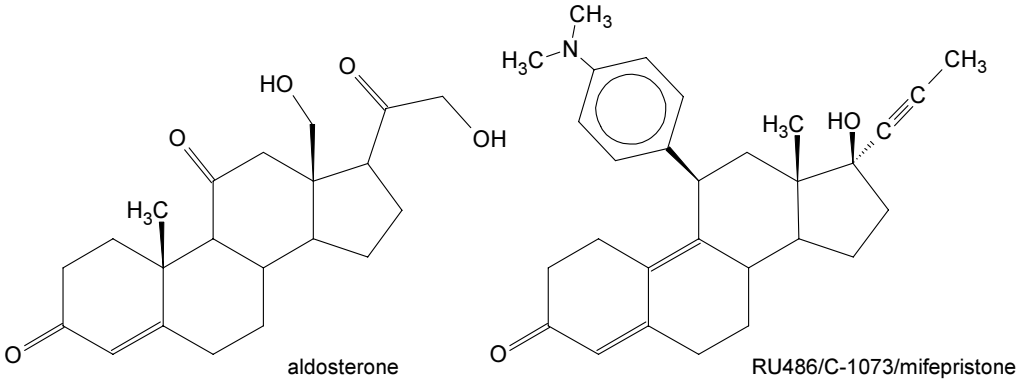


FIGURE 1. Molecular structures of several naturally occurring and synthetic corticosteroids. *Cortisol* and *corticosterone* are the main active glucocorticoid hormones circulating in the plasma of mammals. *Hydrocortisone* is the common name of the synthetic form of cortisol. *Cortisone* and *dehydrocorticosterone* are inactive metabolites, that can be reconverted into cortisol respectively corticosterone in the body. *Cortexolone (also deoxycortisol)* and *deoxycorticosterone* are the immediate precursors of cortisol respectively corticosterone in the biosynthetic pathways of the latter hormones. *Prednisolone* and *dexamethasone* are synthetic hormones, whereas *aldosterone* is the main endogenous mineralocorticoid. Aldosterone mainly circulates in the hemiacetal form with a cyclic 11-18 hemiacetal bridge. *RU486* (also named mifepristone, RU38486 or C-1073) is a synthetic antagonist of both the glucocorticoid and progesterone receptor.

Circadian rhythm

Basal glucocorticoid secretion follows a circadian pattern, with a peak at the start of the active period, which is in early morning for diurnal animals like humans and at onset of darkness for nocturnal animals like mice and rats. There is also an ultradian pattern with a rather constant pulse frequency (Follenius *et al.*, 1987; Windle *et al.*, 1998). The circadian rhythm is driven by the biological clock in the suprachiasmatic nucleus (SCN) which conveys excitatory and inhibitory activity to the PVN (Dallman *et al.*, 1987a; Buijs *et al.*, 1997). Furthermore, the SCN regulates adrenal sensitivity to ACTH and thus corticosteroid secretion directly via sympathetic neural input to the adrenal gland (Buijs *et al.*, 1997; Jasper and Engeland, 1997).

Stress

Secretion of glucocorticoids is also manifold enhanced after stress. Basically any kind of disturbance of homeostasis, either real or perceived, or 'stress', will result in a stress response. This stress response comprises a spectrum of physiological and behavioural adaptations of the individual aimed to restore homeostasis. The stimulus that evokes the stress response is referred to as stressor. Stressors might be divided in systemic stressors, which directly disturb physiological integrity (*e.g.* infections, temperature or blood volume changes), and psychological stressors, which disturb or threaten to disturb mental integrity (*e.g.* fear, social conflict, traumatic life event). The latter require interpretation by higher brain centres and are particularly powerful stressors. Many stressors, however, have both systemic and psychological aspects.

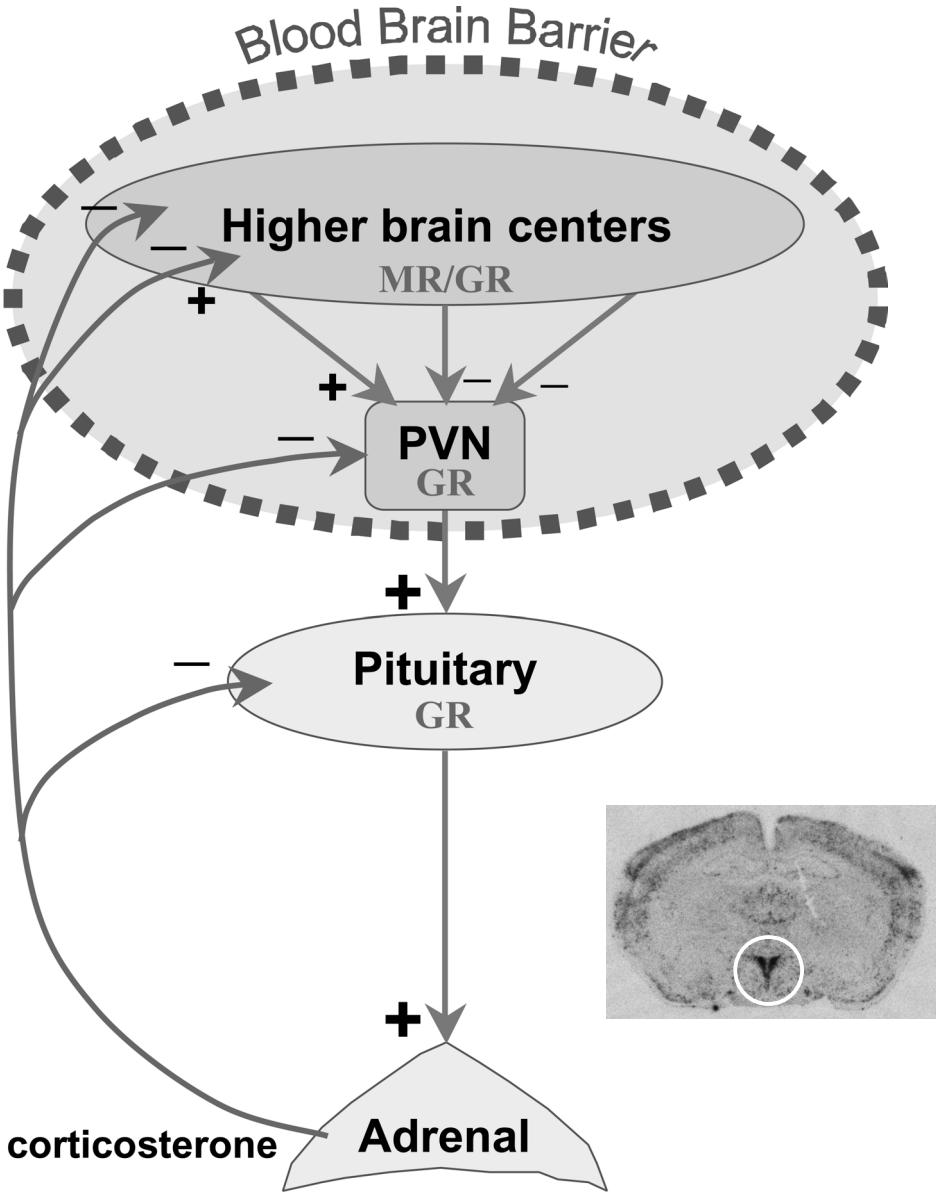


FIGURE 2. The Hypothalamus-Pituitary-Adrenal axis or HPA-axis.
After activation by other brain areas that are stimulated by *e.g.* stress the hypothalamic paraventricular nucleus (PVN) secretes CRH and vasopressin into a portal system to the anterior pituitary. Specific cells in this structure are stimulated to release ACTH, which reaches the adrenal via the main circulation. Adrenal cortical cells are stimulated to secrete corticosterone in rodents or cortisol/corticosterone in humans. These glucocorticoid hormones feedback to the brain and the pituitary to suppress activity of PVN and pituitary. They also have effects on many aspects of brain function. Except for the pituitary most of the feedback sites of glucocorticoids lie behind the blood-brain barrier.
The inset, added for figurative reasons, shows a coronal section of rat brain at the level of the PVN (black area within circle represents parvocellular part of the PVN).

Stressors activate the brain, which via activation of the HPA-axis eventually results in glucocorticoid secretion. The central response is a highly integrated process in which diverse neuronal systems are involved (De Kloet, 1991; Herman and Cullinan, 1997). Distinct types of stressors differentially activate distinct neural and neuroendocrine systems involved in the stress response (Senba and Ueyama, 1997), each affecting neural and neuroendocrine activity in their own specific way (Pacak and Palkovits, 2001). Systemic (aspects of) stressors mainly affect brainstem or hypothalamic areas, while psychological (aspects of) stressors depend on processing in limbic areas (Herman *et al.*, 1996). These different stress-responsive brain areas have direct or indirect projections to the PVN through which they orchestrate the HPA-axis activity (figure 3) (Keller-Wood and Dallman, 1984; Makara, 1985; Herman and Cullinan, 1997). In addition, these neural pathways are subject to input from other regulatory centres, which are not directly involved in the stress response. These regulatory inputs affect the responsiveness of the stress system in general. The magnitude of the glucocorticoid response is thus clearly context-dependent, although the response itself seems to be rather non-specific as essentially any kind of stressor ultimately results in secretion of glucocorticoids.

Although glucocorticoids are intimately linked to stress they do not mediate the rapid primary reaction of the individual. The initial phase of the stress response, occurring within seconds, typically consists of activation of the sympathetic nervous system bringing the individual in a state of arousal ('fright-fight-flight' response). This phase also includes the first part of endocrine cascades (*i.e.* adrenaline, ACTH), which eventually, within minutes, results in the rise of plasma glucocorticoid levels. As it takes minutes to increase glucocorticoids levels in response to stress, glucocorticoids rather play secondary, but critical, roles in concert with the sympathetic nervous system.
Glucocorticoids prevent primary stress responses from overreaction, thus protecting the individual against the potential threat of homeostasis caused by its own defence systems, as was independently postulated by Munck *et al.* (1984) and Tausk (1951). For instance, suppression of the immune system during infection limits the inflammatory response, and stress-induced levels of glucocorticoids facilitate the formation of memory of potentially dangerous events giving an individual the ability to successfully cope with a subsequent stressful event. Thus, generally, glucocorticoids play a beneficial role in the adaptation to stress.

The process of adaptation to stress may also be described as *allostasis*, which means active maintenance of homeostasis through change (McEwen, 2000). In response to stressors, changes in glucocorticoid secretion and in other parts of the stress system help to re-establish stability of body and brain function and to promote adaptation to and coping with the stress. The operational cost of allostasis is termed *allostatic load*, which implies the price to be paid to actively maintain stability when the allostasis response systems are excessively challenged. Repeated or prolonged challenges may lead to vulnerability to stress-related disorders.

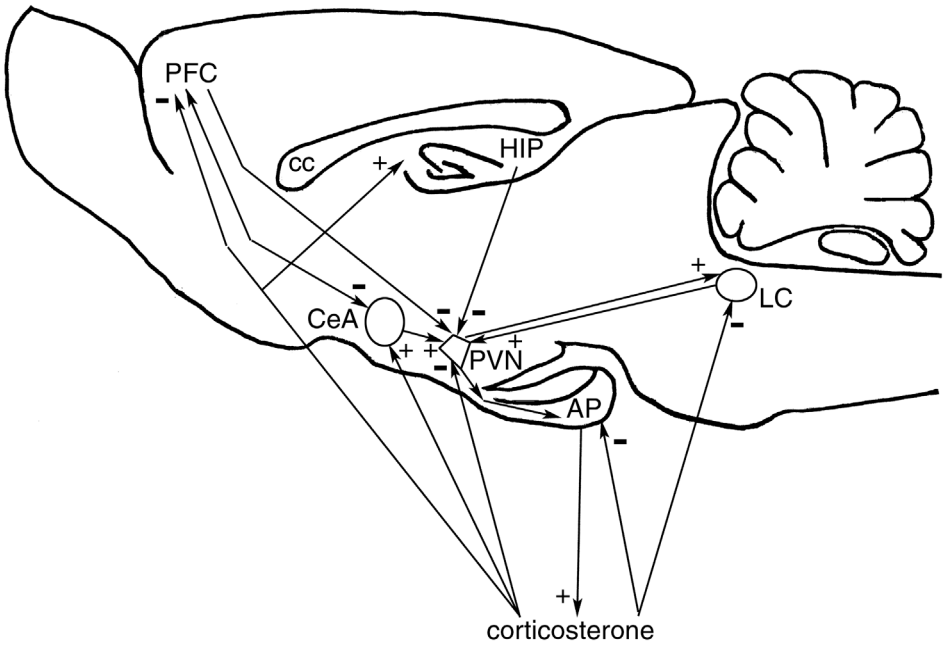


FIGURE 3. Some of the brain areas involved in regulation of HPA-axis activity.
Many different brain areas control the activity of the PVN and thus the activity of the HPA-axis. In turn, the activity of these areas is modulated by glucocorticoids. Each stressor, irrespective of the type, ultimately activates the CRH system in the parvocellular PVN, which results in stimulation of the anterior pituitary (AP) and subsequently in secretion of glucocorticoids from the adrenal. Systemic stressors mainly activate catecholaminergic systems located in the brain stem such as the locus coeruleus (LC), which may directly activate the PVN. Psychological stressors depend on limbic-forebrain circuits (*e.g.* prefrontal cortex (PFC), amygdala, hippocampus (HIP)) which via multisynaptic pathways converge on the PVN. Many of these areas project to GABA-ergic neurons near the PVN, which exert inhibitory influences on the parvocellular neurons.
Glucocorticoids mainly exert negative feedback on the HPA-axis by suppression of CRH in the PVN and POMC in the anterior pituitary, but also by their actions via the hippocampus and prefrontal cortex. In contrast, glucocorticoids activate the CRH system located in the central nucleus of the amygdala (CeA), potentially forming a positive feedback loop. Particularly via the hippocampus and the amygdala, glucocorticoid hormones affect behaviour and cognition.
Abbreviations: AP anterior pituitary; cc corpus callosum; CeA central nucleus of the amygdala; HIP hippocampus; LC locus coeruleus; PFC prefrontral cortex; PVN hypothalamic paraventricular nucleus.

Glucocorticoid actions

Glucocorticoids and stress are often considered to be harmful for the body and particularly for the brain (Sapolsky *et al.*, 1986; Sapolsky, 1996). Indeed, prolonged or out-of-context elevations of glucocorticoids may be maladaptive, enhancing vulnerability to stress, and contributing to human psychopathologies (Holsboer and Barden, 1996; Pariante and Miller,

2001; Belanoff *et al.*, 2001). However, as long as the glucocorticoid actions are confined within certain limits, the glucocorticoid response to stress is in fact crucial for healthy functioning of the brain (De Kloet *et al.*, 1998; De Kloet *et al.*, 1999; Lupien and Lepage, 2001). Without glucocorticoids, the individual likely would not survive many events in daily life.

Context- and time-dependency

The actions of glucocorticoids induced by circadian activity or stress are diverse and complicated and depend on the context in which they are operating. Depending on the physiological endpoint in question, glucocorticoid actions can permit, stimulate or suppress ongoing primary responses to stress or can be preparative for a subsequent stressor (Sapolsky *et al.*, 2000). However, glucocorticoids do not only affect various physiological functions but they also modulate behaviour. They do not cause behaviours, but they induce chemical changes in particular sets of neurons, making certain behavioural outcomes more likely as a result of strengthening or weakening of particular neural pathways. Thus, glucocorticoids can operate in a 'proactive' mode through anticipatory physiological and behavioural activity to prepare the organism for upcoming events in order to maintain homeostasis (De Kloet *et al.*, 1998; De Kloet, 2002). In the 'reactive' mode they operate to restore homeostasis from disturbances by stressors by terminating the primary stress response. The latter mode facilitates storage of behavioural strategies that have appeared to be successful in dealing with stressors and eliminates behaviour of no more relevance.
The permissive actions controlling the sensitivity of the stress system are evident at low levels of glucocorticoids, whereas the suppressive or stimulating actions facilitating the adaptation to stress emerge only when glucocorticoid levels are raised as following a stressor (Sapolsky *et al.*, 2000).

Actions of glucocorticoids are mostly modulatory and conditional, as they can often only be detected in presence of other stimuli (De Kloet *et al.*, 1998). Glucocorticoids change the state of the cell or animal making it more or less receptive for the actions of other subsequent stimuli. Basal neuronal activity of hippocampal cells does not change in presence of glucocorticoids, but glucocorticoids change neuronal excitability as exemplified by the effects of glucocorticoids on voltage-dependent calcium fluxes and aminergic responses of hippocampal neurons (Joëls and De Kloet, 1994). Furthermore, the activity of the affected gene or system at the moment of glucocorticoid action determines the way glucocorticoids effectuate their action. Stress levels of corticosterone immediately after a learning experience promote consolidation of memory, but if corticosterone is administered out-of-context memory storage is impaired (Oitzl and De Kloet, 1992). The repressive actions of glucocorticoids on c-fos expression can only be observed after stress (Imaki *et al.*, 1995), whereas the presence of the apoE gene determines the way mice react on stress in a glucocorticoid dependent learning task (Grootendorst *et al.*, 2001). Previous stress experiences like early life events or chronic stress can change this state affecting the way a cell or animal

will react on subsequent exposure to glucocorticoids (Akana and Dallman, 1997; Workel *et al.*, 2001). Thus, the same hormonal signal can have differential, even opposing effects depending on the circumstances.

Corticosteroid receptors

By far most of the actions of corticosteroids involve altering the expression of target genes. Corticosteroids exert their genomic actions mainly via two types of intracellular receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (De Kloet, 1991). MR and GR are structurally highly homologous. They belong to the protein family of nuclear receptors. Upon binding of hormone, the steroid-receptor complex dissociates from a large protein complex and translocates from the cytosol to the nucleus to function as transcription factors. MR and GR can subsequently enhance (transactivation) or repress (transrepression) gene expression (Truss and Beato, 1993; Beato *et al.*, 1996). Transactivation generally occurs through binding of MR or GR as homodimers to the specific DNA sequences, known as glucocorticoid response elements (GRE) in the promoter regions of target genes. The term transrepression is often used to describe the ability of activated GR as monomer to interact with other transcription factors via protein-protein interactions but may, for both MR and GR, also occur via DNA binding.

Distribution

MR and GR differ in their distribution and in their affinities for corticosteroids (Reul and De Kloet, 1985; De Kloet, 1991). The GR is localised throughout the body, including thymus, anterior pituitary and brain. Within the brain, GR is present in almost every region. Particularly high densities are found in the parvocellular neurons of the PVN, where glucocorticoids regulate the expression of CRH and AVP, and in neurons of hippocampal formation, which is a brain region involved in regulation of the behavioural stress response (Van Eekelen *et al.*, 1988; Chao *et al.*, 1989; Spencer *et al.*, 1990). The MR is mainly localised in the classical peripheral mineralocorticoid target tissues such as the kidney, and in some areas within the brain (Krozowski and Funder, 1983). Central localisation is restricted to limbic regions such as hippocampus, septum and amygdala (Arriza *et al.*, 1988; Van Eekelen *et al.*, 1988; Chao *et al.*, 1989; Spencer *et al.*, 1990; Ahima *et al.*, 1991). In these areas MR is abundantly expressed. MR is also found, although at lower levels, in the anterior hypothalamus and subfornical area (Van Eekelen *et al.*, 1991). Particularly in hippocampus glucocorticoids can activate two signalling pathways via both MR and GR, as high levels of both receptor types are colocalised here (Van Eekelen and De Kloet, 1992).

Affinity

Cortisol, corticosterone and aldosterone can all bind to MR with very high affinity (all Kd values~0.5nM), whereas synthetic glucocorticoids like dexamethasone and prednisolone have a much lower affinity to this receptor (table 1) (Moguilewsky and Raynaud, 1980; Veldhuis *et al.*, 1982; De Kloet *et al.*, 1984a; Reul and De Kloet, 1985; Arriza *et al.*, 1987; Luttge *et al.*,

1989; Spencer *et al.*, 1990; Reul *et al.*, 2000b). On the other hand, whereas cortisol and corticosterone have a ten times lower affinity to GR than to MR, synthetic glucocorticoids have a very high affinity to GR (Coirini *et al.*, 1983; De Kloet *et al.*, 1984a; Reul and De Kloet, 1985; Arriza *et al.*, 1987; Spencer *et al.*, 1990). Aldosterone hardly binds to GR. The best-known effective receptor antagonist of glucocorticoids is mifepristone (RU486; 17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl))-estra-4,9-dien-3-one) (Moguilewsky and Philibert, 1984). This compound, originally developed as a progesterone antagonist, expresses a high affinity for the glucocorticoid receptor, but binding generally does not result in stimulation of transcription. RU486 antagonises glucocorticoid effects *in vitro* and inhibits the effect of dexamethasone on HPA activity *in vivo*, although it may still have agonistic properties in some *in vitro* (Heck *et al.*, 1994) and *in vivo* settings (Bradbury *et al.*, 1991).

Table 1. Some characteristics of common glucocorticoids.

corticosteroid	receptor affinity	plasma binding [a]
cortisol/hydrocortisone	MR/GR [b]	CBG/albumin [f]
corticosterone	MR/GR [b]	CBG/albumin [f]
dexamethasone	GR [c]	-
prednisolone	GR/(MR)	CBG/albumin [f]
cortisone	-	albumine
dehydrocorticosterone	-	n/a
cortexolone/deoxycortisol	(GR) [d]	CBG/albumin [f]
deoxycorticosterone	MR	n/a
aldosterone	MR	albumine [g]
RU486/mifepristone	GR antagonist [e]	AAG [h]

n/a not available

a. Plasma binding in human plasma.
b. Both cortisol and corticosterone have very high affinity to MR and a 10 times lower affinity to GR.
c. *In vitro*, dexamethasone has affinity for MR, but due to the unstable dexamethasone-MR complex dexamethasone does not act via MR *in vivo*.
d. Cortexolone has some affinity for the GR and may act as a partial GR antagonist.
e. RU486 is an effective GR antagonist, but is also a progesterone receptor antagonist.
f. CBG is the main corticosteroid binding protein in plasma, which binds about 70-80% of hormone; albumine binds another 10-20%; 5-10% circulates as unbound fraction.
g. About 40% of aldosterone circulates bound to albumine.
h. In humans (but not in most other species) 95% of RU486 is bound to α-1-acid glycoprotein (AAG).

In spite of the equal affinities of cortisol/corticosterone and aldosterone to MR (Krozowski and Funder, 1983; Coirini *et al.*, 1985), there seems to be aldosterone-selective and glucocorticoid-selective forms of MRs. The predominantly aldosterone-selective MRs are found in classical mineralocorticoid target cells in peripheral tissues like kidney and colon, but also in brain areas involved in central regulation of sodium homeostasis and blood pressure like the anterior hypothalamus and circumventricular organs (McEwen *et al.*, 1986b; Brody *et al.*, 1991; Van Eekelen *et al.*, 1991; Pietranera *et al.*, 2001). Enzymatic conversion by 11ß-hydrosteroid dehydrogenase (11ß-HSD) type 2 into the inactive 11-keto derivatives cortisone respectively 11-dehydrocorticosterone (figure 1), which have little or no affinity for MR or GR, largely excludes cortisol and corticosterone from these MRs explaining the 'MR paradox' (Edwards *et al.*, 1988; Funder *et al.*, 1988; Seckl, 1997). The apparent glucocorticoid selectivity of MR in the limbic brain is presumably mainly explained by competitive predominant occupancy by cortisol/corticosterone, since these hormones circulate in 100-1000 fold excess to aldosterone (Kage *et al.*, 1982; Reul and De Kloet, 1985; Yongue and Roy, 1987).

Occupancy

The difference in affinity of MR and GR for corticosterone/cortisol has important implications for the occupancy of central receptors throughout the day and during stress. At basal trough levels limbic MR is already activated to a considerable extent; even at low plasma levels of glucocorticoids 80-95% of these MRs are still occupied (Reul and De Kloet, 1985; Spencer *et al.*, 1990), although a recent study argued against this high proportion (Kalman and Spencer, 2002). Occupation of GR is low at the nadir of the circadian rhythm, but is progressively increased when glucocorticoids levels rise during the circadian peak or, more pronounced, after stress (Reul *et al.*, 1987a). Due to their differences in affinity for endogenous glucocorticoids and their differential localisation in neuronal structures both types of receptors form a dual receptor system able to mediate glucocorticoid action through different neuronal circuits in face of a wide hormone concentration range.

Glucocorticoid feedback

Glucocorticoids feed back to the brain through both MR and GR to suppress pituitary-adrenal secretion (Dallman *et al.*, 1987a), but also to modulate behaviour (De Kloet *et al.*, 1998). Based on the differential occupancy of MR and GR, it has been postulated that glucocorticoids exert their tonic, permissive influences predominantly via the hippocampal MRs, whereas feedback actions on activated brain areas are mainly mediated by GRs (De Kloet and Reul, 1987; De Kloet, 1991; De Kloet *et al.*, 1999). The MR-mediated *proactive* effects are involved in control of basal activity of the HPA-axis and selection of an appropriate behavioural response. MR activation might function to sensitise the stress system for upcoming challenges and to organise the response to stress. The *reactive* processes of glucocorticoid feedback actions mediated by GR contribute to restoration of homeostasis and facilitation of behavioural adaptation after stress or during the circadian rise.

Through their MR- and GR-mediated feedback on the HPA-axis, the actions of glucocorticoids are constrained within appropriate limits. Direct negative feedback is exerted through GR present in PVN and pituitary (Keller-Wood and Dallman, 1984). Indirect feedback operates through both MR and GR present in diverse brain areas(Dallman *et al.*, 1994) and suppressive as well as facilitatory effects can be exerted through various afferent multisynaptic neural pathways to the PVN (figure 3). Among these are those from limbic areas like hippocampus and amygdala, and different cortical areas but also from serotonergic and catecholaminergic brainstem systems and diverse hypothalamic areas (Dallman *et al.*, 1995; Herman *et al.*, 1996; Herman and Cullinan, 1997; Lopez *et al.*, 1999; Laugero, 2001; Makino *et al.*, 2002). Many of these areas project to GABA-ergic neurons just outside the PVN, which have inhibitory inputs to the parvocellular neurons (Herman *et al.*, 2002). Like the stress-initiating areas, these glucocorticoid feedback circuits operate in a stressor-specific manner (Herman and Cullinan, 1997).

Consistent with the hippocampal MR specificity, intracerebroventricular (icv) administration of MR antagonist elevated basal trough levels of corticosterone (Ratka *et al.*, 1989), whereas a corticosterone but not a dexamethasone implant in the dorsal hippocampus reduced ACTH levels after adrenalectomy (Kovács and Makara, 1988). Icv administration of the GR antagonist RU486 does not interfere with basal trough pituitary adrenal activity, supporting the sole involvement of MR during that period (Ratka *et al.*, 1989; Van Haarst *et al.*, 1996a). However, RU486 increases the basal peak and stress-induced activity (Gaillard *et al.*, 1984; Ratka *et al.*, 1989; Van Haarst *et al.*, 1996a), consistent with a role of GR in feedback regulation at these periods of high circulating corticosterone levels. The GR involved in negative feedback is located at different brain sites including the PVN. After icv injection or local application within the PVN RU486 increased neuroendocrine activity at the circadian peak or after stress only (De Kloet *et al.*, 1988; Van Haarst *et al.*, 1996a; Van Haarst *et al.*, 1997). Only at even higher plasma levels corticosterone also feeds back via the GR in the pituitary (Dallman *et al.*, 1987b; Levin *et al.*, 1988).

Synthetic glucocorticoids acting through GR are particularly potent in suppression of HPA-axis activity. They suppress basal levels of ACTH and corticosterone during the circadian rise, and they reduce stress-induced rises in ACTH and corticosterone plasma levels. It is likely that the pituitary gland is the primary site of action of dexamethasone. In contrast to corticosterone which is preferentially retained by hippocampal neurons (McEwen *et al.*, 1968; McMurry and Hastings, 1972; De Kloet *et al.*, 1975; Coutard *et al.*, 1987), dexamethasone is mainly retained in nuclei of pituitary corticotropes (De Kloet *et al.*, 1974; De Kloet *et al.*, 1975). The exact site of action of other synthetic glucocorticoids like prednisolone is not known, although prednisolone has been presumed to act more similar to cortisol (McEwen, 1997; Pariante *et al.*, 2002).

In presence of endogenous glucocorticoids GR never acts alone. Moreover, both receptor types have to be activated for proper functioning of negative feedback (Bradbury *et al.*, 1994; Spencer *et al.*, 1998) and cognitive processing (Oitzl *et al.*, 1997). Treatment with synthetic glucocorticoids, which mainly bind to GR, may therefore lead to an imbalance in MR and GR-mediated actions, which could have detrimental effects on brain function.

Glucocorticoids do not only exert equivalent actions through MR and GR. Opposing effects of MR and GR activation have also been described, both at the cellular (Joëls and De Kloet, 1994) and at the systemic level. Hippocampal MR blockade results in a disinhibitory effect on CRH containing neurons in the PVN, whereas hippocampal GR blockade results in an inhibition of PVN function (Van Haarst *et al.*, 1997). Although corticosterone-responsive MR is predominantly expressed in the limbic brain, opposing effects are not only restricted to single hippocampal cell types expressing both types of receptors. MR can influence many processes in the brain through the efferent projections of the hippocampus to various brain areas. Therefore, even when expressed in different brain areas, MR and GR can act in a dichotomous co-ordinate action on many neural circuits, ultimately affecting behaviour and HPA-axis activity. This dichotomy may provide the basis of U-shaped or bell-shaped dose response curves often seen in glucocorticoid action (Oitzl *et al.*, 1994; De Kloet and Joëls, 1996; Lupien and Lepage, 2001).

The way many actions of glucocorticoids at the physiological level are ultimately linked to the actions at the levels of gene regulation is hardly understood. Obviously, the type of receptors involved, MR, GR or both, may underlie the differential effects of glucocorticoids on cellular function. Activated MRs and GRs differ in their potency to induce changes in gene transcription with GR generally as the more potent one (Meijer, 2002). Specificity at the cellular level in the way MR and GR influence gene transcription is achieved by receptor-specific efficacy at certain regulatory DNA regions (Rupprecht *et al.*, 1993) and receptor-specific interactions with other proteins like the transcription factor AP1 (Bamberger *et al.*, 1996), or coactivators and corepressors (Meijer, 2002). On the physiological level, however, other mechanisms are involved as well, as apparently simple physiological functions often require interactions among many glucocorticoid regulated target areas, cells and genes, as well as interactions with other hormones and mediators.

Aberrant glucocorticoid action

Because of their differential sometimes even opposing effects, a proper balance between MR and GR mediated actions is critical for homeostatic control and behavioural adaptation to stress. A change in this balance may compromise the ability to maintain homeostasis and may progressively create a condition of disturbed neuroendocrine regulation and impaired behavioural adaptation (De Kloet, 1991; De Kloet *et al.*, 1998; Lupien and Lepage, 2001). This condition may underlie enhanced vulnerability to disease and may eventually lead to stress-related disorders (Holsboer, 2000; Makino *et al.*, 2002). It is therefore not surprising

that stress-related diseases are often associated with abnormalities in glucocorticoid plasma levels. Hypersecretion of these hormones is a consistent finding in various subtypes of major depression (Holsboer and Barden, 1996; Gold and Chrousos, 2002), whereas hyposecretion is found in atypical depression (Gold and Chrousos, 2002) and post traumatic stress disorder (Yehuda, 1998). These abnormalities might be related to malfunctioning of corticosteroid receptor signalling. Inadequate control of glucocorticoid action may severely threaten health and well-being of the individual. Glucocorticoid action, therefore, needs to be tightly regulated.

Ligand availability

Central control of secretion is obviously one of the main regulatory mechanisms of glucocorticoid action, but its effect is rather general. Regulation of expression or function of corticosteroid receptors and/or regulatory proteins provides additional, more locally acting mechanisms. Yet, glucocorticoid action can also be influenced even before glucocorticoids reach their intracellular receptors through modulation of their access to the target cells. Binding of corticosteroids to plasma proteins can affect uptake into tissues and can dampen sharp fluctuations in glucocorticoid plasma levels. Furthermore, at the level of the cell membrane, transport of glucocorticoids can influence uptake into the cell. Finally, within the cell, binding to proteins and enzymatic conversion can affect receptor binding.

Corticosteroid-binding globulin (CBG)

Approximately 90-95% of circulating endogenous glucocorticoid is protein bound mainly to corticosteroid-binding globulin (CBG), a specific low-capacity high-affinity plasma protein, but also to serum albumin, a common carrier protein with low affinity (table 1) (Pardridge, 1981). The synthetic glucocorticoid prednisolone is also bound to both plasma proteins (Jusko and Ludwig, 1992), whereas dexamethasone is not bound to CBG. CBG may regulate bioavailability and metabolic clearance of glucocorticoids (Breuner and Orchinik, 2002). In principle, only free glucocorticoids have ready access to their intracellular receptors (Dallman *et al.*, 1987a; Breuner and Orchinik, 2002), but local mechanisms may locally promote glucocorticoid delivery by releasing corticosteroid from plasma proteins. These include biochemical or physical properties like the ratio between capillary transit time and dissociation rate (Pardridge, 1981), but also biological mechanisms like the presence of plasma membrane binding sites and enzymatic cleavage of CBG (Pemberton *et al.*, 1988; Hammond, 1995). The former may also play a role in brain delivery (Pardridge, 1981; Pardridge *et al.*, 1983), the latter probably not (Hammond, 1995). At the blood brain barrier, the albumin-bound fraction may also be available for transport into the brain probably due to physical properties, increasing the brain uptake to 20-25% of total plasma levels (Pardridge and Mietus, 1979). CBG is also found intracellular in some tissues including pituitary but not in brain (Hammond, 1990) and may affect glucocorticoid actions by sequestering glucocorticoids (De Kloet *et al.*, 1977; De Kloet *et al.*, 1984b). CBG levels can be downregulated by glucocorticoids and stress

due to suppression of hepatic synthesis (Dallman *et al.*, 1987a; Smith and Hammond, 1992) relatively increasing the biologically active levels of glucocorticoids.

11ß-Hydroxysteroid dehydrogenase type 1

In addition to the aforementioned 11ß-HSD type 2, a genetically distinct isoform is ubiquitously present that can also potentially modulate the intracellular levels of glucocorticoids. This 11ß-HSD type 1 (11ß-HSD1) can also mediate the metabolic interconversion of cortisol and corticosterone into inert cortisone and 11-dehydrocorticosterone respectively, although *in vivo* it acts as an exclusive reductase, thus locally reactivating glucocorticoids (Jamieson *et al.*, 2000; Seckl and Walker, 2001). It is expressed in numerous tissues such as liver, lung, and adipose tissue and also in brain and pituitary (Seckl, 1997). The enzyme is often colocalised with GR, thus able to enhance glucocorticoid action by contributing to the intracellular supply of active glucocorticoids available for GR binding (Seckl, 1997). A role of 11ß-HSD type 1 in amplifying glucocorticoid action in the periphery has been demonstrated with 11ß-HSD1 (-/-) knockout mice (Kotelevtsev *et al.*, 1997). These mice are unable to reactivate glucocorticoids. Despite compensatory adrenocortical hyperplasia and increased ACTH/corticosterone secretion, upon starvation they demonstrate diminished activation of glucocorticoid responsive hepatic gluconeogenic enzymes. They are further resistant to hyperglycaemia induced by obesity or stress (Kotelevtsev *et al.*, 1997). This is consistent with impaired intrahepatic regeneration of glucocorticoids.

Within the brain 11ß-HSD1 may also modulate glucocorticoid action, although there is no definitive evidence for this. High expression of 11ß-HSD1 in hippocampus, hypothalamus and pituitary suggests that it may influence negative feedback of the HPA-axis (Moisan *et al.*, 1990). Primary hippocampal cell cultures are able to convert 11-dehydrocorticosterone (DHC) into corticosterone (Rajan *et al.*, 1996). However, the first *in vivo* studies are far from conclusive about the potential role of 11ß-HSD1 in normal hippocampal functioning. In presence of DHC adrenalectomised animals show an exacerbation of kainic acid induced hippocampal damage (Ajilore and Sapolsky, 1999). However, after corticosterone replacement, this effect disappeared, whereas also in intact rats receiving icv carbenoxolone, an inhibitor of 11ß-HSD no effects were seen on kainic acid induced hippocampal damage (Ajilore and Sapolsky, 1999). In another study using adrenalectomised rats carbenoxolone did not affect *in vivo* uptake and retention of ^{3}H-corticosterone in neuronal nuclei of hippocampus (Van Haarst *et al.*, 1996b). This lack of any effect of icv administered 11ß-HSD inhibitor seems to indicate the absence of any role of 11ß-HSD1 in hippocampal functioning in spite of its high brain expression. On the other hand, carbenoxolone is a rather nonselective drug inhibiting 11ß-HSD1 and possibly other enzymes as well.

Recently, the 11ß-HSD1 knockout mouse model was used to further investigate a possible central role of 11ß-HSD1. Although the increased adrenal weight and sensitivity of 11ß-HSD1 deficient mice (Kotelevtsev *et al.*, 1997) may be explained by increased peripheral need for glucocorticoids due to increased metabolic clearance, the raised corticosterone trough plasma

levels would suggest that HPA-axis activity is also relatively increased (Harris *et al.*, 2001). Enhanced corticosterone plasma levels after stress and the inability of a dose of cortisol, which is effective in wild type mice, to suppress the HPA response to a stressor in knockouts has been attributed to a blunted sensitivity to glucocorticoid feedback due to lack of regeneration of active glucocorticoid in the brain (Harris *et al.*, 2001). However, the decreased expression of GR in PVN of 11ß-HSD1 null mice (Harris *et al.*, 2001) may also underlie impaired feedback, and, in addition, the lack of regeneration of active glucocorticoid by hepatic 11ß-HSD1 may result in increased clearance of cortisol.
On the other hand, aged knockout mice do not show the age-related decline in hippocampal-dependent cognitive function, as is shown by wild type mice, even in face of increased corticosterone levels throughout life (Yau *et al.*, 2001). The decreased hippocampal uptake of chronically infused ^{3}H-corticosterone in knockout animals has also been postulated to be in line with their inability to regenerate corticosterone (Yau *et al.*, 2001), although this phenomenon may also be explained by increased clearance of ^{3}H-corticosterone in these mice. The generation of tissue-specific 11ß-HSD1 knockouts will resolve the question whether 11ß-HSD1 is an important amplifier of central glucocorticoid signalling or not.

Brain penetration

Regulating access of corticosteroids at the level of the cell membrane may be another mechanism regulating access to the receptor. Since corticosteroids are commonly believed to cross membranes with relative ease by virtue of their highly lipophilic nature and their small size, information on access of corticosteroids to neurons is still scarce. As all CNS active compounds, corticosteroids have to pass the blood-brain barrier (BBB) before they reach their target cells within the brain. The BBB is a dynamic physical and metabolic barrier consisting of specialised endothelial cells that protects the brain from blood-borne compounds, and plays a role in maintaining brain homeostasis (Bradbury, 1993) (figure 4). Just like the pituitary, some brain areas like the circumventricular organs lie outside this barrier (Gross, 1992), but most of the brain is shielded from the periphery by the tight junctions between brain capillary endothelial cells and other barrier properties of these cells. The latter features comprise the lack of fenestrations and pinocytotic vesicles and the presence of metabolic enzymes and special transporter proteins (Lee *et al.*, 2001b). The BBB can strongly interfere with distribution to the brain of endogenous and exogenous compounds (De Boer and Breimer, 1994). Generally, hydrophilic and large lipophilic compounds are not able to penetrate the brain, as they are not able to pass cell membranes, whereas small lipophilic compounds such as corticosteroids can easily cross the BBB by passive diffusion through the endothelial cells. Any process at the BBB that can influence the endothelial crossing of these hormones would directly affect central corticosteroid receptor occupancy and the magnitude of the central response to corticosteroids.

Consistent with the assumption that small lipophilic compounds can easily cross endothelial barriers, the naturally occurring glucocorticoid, corticosterone, readily gains access to the

brain and accumulates in limbic brain areas expressing MR (figure 5) (McEwen *et al.*, 1968; De Kloet *et al.*, 1975; De Kloet, 1991). In contrast, the synthetic glucocorticoid dexamethasone, when administered in tracer doses to adrenalectomised rats or mice, is poorly retained in GR-containing areas in brain (figure 5) (De Kloet *et al.*, 1975; Rees *et al.*, 1975; McEwen *et al.*, 1976; Coutard *et al.*, 1978). Their diversity in receptor affinity can only partially explain this differential retention pattern (Reul and De Kloet, 1985; Reul *et al.*, 2000b), as the anterior pituitary which also expresses high amounts of GR, retains high amounts of dexamethasone. To explain this puzzling phenomenon the existence of a blood-brain barrier limiting the uptake of dexamethasone into the brain was postulated (De Kloet *et al.*, 1975; Rees *et al.*, 1975; Coutard *et al.*, 1978).

Several transporters have been identified in the brain capillary endothelial cells, which can facilitate the uptake or impede the entry of substrates (Tamai and Tsuji, 2000; Lee *et al.*, 2001b). Besides multiple, mostly specialised influx transporters like the glucose transporter, there are several, more general efflux transporters described of which the multidrug

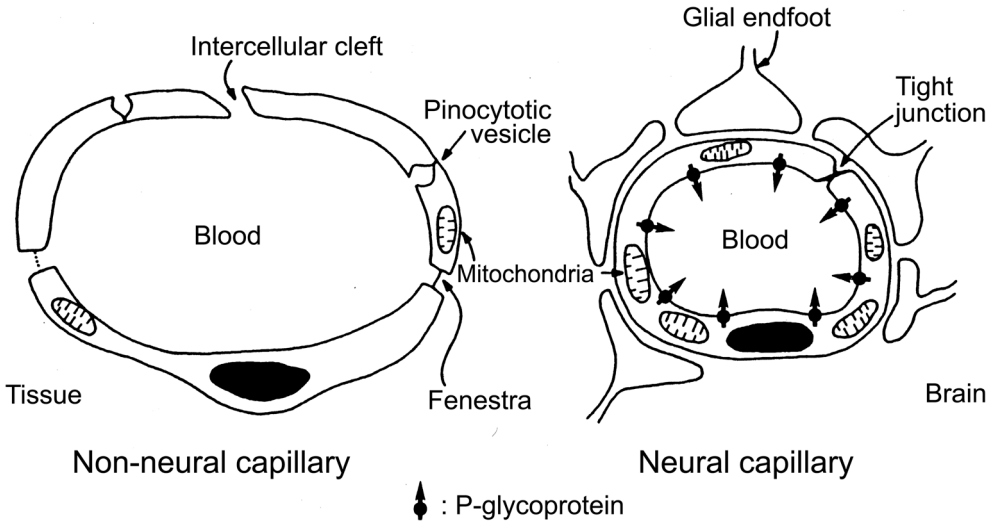


FIGURE 4. Schematic representations of the anatomy of a typical blood vessel in peripheral and brain tissue, respectively.

Unlike peripheral capillary endothelial cells, brain capillary endothelial cells are closely sealed by tight junctions, they display no intercellular clefts and little fenestration or pinocytosis, and they have a relatively high number of mitochrondia. Some of these characteristics are induced and maintained by astrocyte foot processes that are closely attached to and extensively envelop the brain endothelium. For simplicity, the supporting pericytes and the basal lamina, structural connective tissue surrounding the blood capillaries and separating the glial endfoots from the brain endothelial cells are not shown.

The various BBB-specific transporters are not shown except for the efflux transporter P-glycoprotein (indicated by the balls and arrows). This transmembrane protein is localised at the luminal membrane of the endothelial cells and transports its substrates (back) into the blood in an energy-dependent manner. Reprinted from Schinkel (1999) with permission from Elsevier Science.

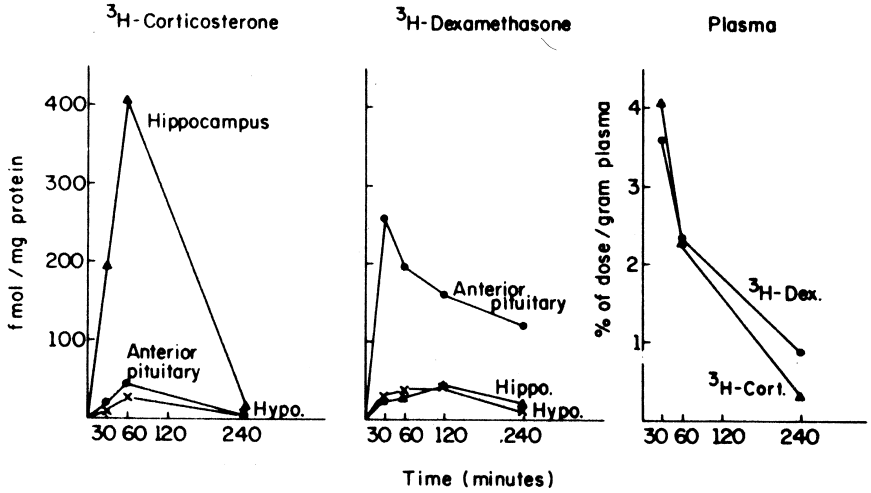


FIGURE 5. Cell nuclear binding of ^{3}H-corticosterone and ^{3}H-dexamethasone in hippocampus, hypothalamus and anterior pituitary, at various time points after intravenous injection of 5 nmol ^{3}H-glucocorticoid to adrenalectomised rats. Binding is expressed as fmol steroid/mg protein in purified nuclei. Radioactivity per ml plasma is expressed as percentage of injected dose. Reprinted from De Kloet *et al.* (1975) with copyright permission from The Endocrine Society.

transporter P-glycoprotein is the best studied (Schinkel, 1999). Pgp is an important functional component of the BBB. It acts like a "gatekeeper" at the BBB keeping a wide variety of drugs out of the brain (Schinkel, 1999). This active drug efflux transporter appeared to be responsible for the apparent low permeation of some compounds including dexamethasone (De Kloet, 1997) that should easily penetrate the BBB as expected based on their size and their sufficiently high lipid solubility. Pgp may protect the brain in two ways, by excluding potentially neurotoxic substances from the brain but also by preventing drugs, which can potentially disrupt the BBB, to enter the endothelial cells (Van der Sandt *et al.*, 2001).

P-GLYCOPROTEIN

P-glycoprotein (Permeability-glycoprotein) plays an important role in multidrug resistance (MDR). This phenomenon is characterised by intrinsic or acquired resistance of cancer cells to a wide variety of structurally and functionally unrelated drugs (Gottesman and Pastan, 1993). MDR is a major problem in the chemotherapeutic treatment of cancer. Many efforts have been put into the characterisation of MDR and into the reversal of MDR. Being the first detected transporter that was implicated in MDR (Juliano and Ling, 1976), Pgp has been extensively characterised.

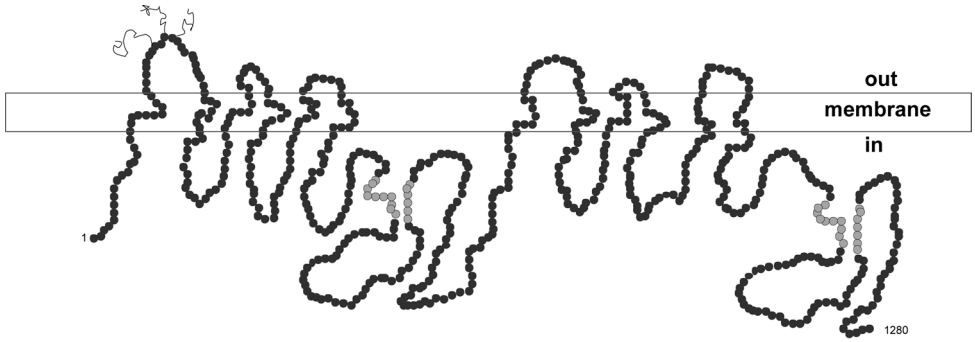


FIGURE 6. Putative two-dimensional topology of the human MDR1 P-glycoprotein based on hydropathy analysis of the amino acid sequence. Two putative ATP-binding sites are shown as grey circles, and N-linked glycosylation sites are represented as squiggly lines.

Structure

The multidrug resistance P-glycoprotein belongs to the subfamily B of the adenosine triphosphate (ATP) binding cassette (ABC) superfamily of transporter proteins. ABC transporters are ubiquitous with over 300 family members identified in all known organisms from bacteria to mammals. They are involved in transport of a great variety of substrates including sugars, amino acids, cholesterol, phospholipids, peptides, proteins, toxins, antibiotics and xenobiotics (Higgins, 1992). Besides Pgp, other members include multidrug resistance related proteins (MRP1-7), MXR/BCRP (mitoxantrone resistance/breast cancer resistance protein) and the cystic fibrosis transmembrane regulator (Dean and Allikmets, 2001). Many of the members of this family are involved in MDR.

Pgp's are N-glycosylated 140-170 kDa proteins of about 1280 amino acids (figure 6). Like a typical ABC transporter Pgp is an integral membrane protein consisting of two homologous halves each containing six putative transmembrane α-helical domains and a large intracytoplasmic loop encoding an energy-coupling ATP-binding site. This topology, however, remains controversial and has been challenged by alternative topologies (Skach *et al.*, 1993; Jones and George, 1998).

Genes

Genes overexpressed in MDR cell lines have been isolated and characterised. Extensive studies have identified three classes of mammalian P-glycoproteins (table 2). Only two classes, I and III, convey the MDR phenotype. Of the two human genes, primarily the MDR1 (Roninson *et al.*, 1986) confers drug resistance (Ueda *et al.*, 1987), whereas out of three rodent gene products two have the MDR phenotype. The murine mdr1b (mdr1, pgp2) cDNA was cloned from a mouse pre-B cell library and confers resistance when transfected into drug sensitive cell lines (Gros *et al.*, 1986), whereas the second MDR conveying gene, mdr1a (mdr3, pgp1), was subsequently cloned based on its high homology (Devault and Gros, 1990).

Table 2. Nomenclature and classification of mammalian multidrug resistance genes.

Species	Class I [a]	Class II [a]	Class III [a]
Human	MDR1/ABCB1		MDR3/ABCB4 [b]
Mouse	mdr1a/Abcb1a [c]	mdr1b/Abcb1b [d]	mdr2/Abcb4
Rat	mdr1a/Abcb1a [e]	mdr1b/Abcb1b [e]	mdr2/Abcb4 [e]
Hamster	Pgp1/Abcb1a	Pgp2/Abcb1b	Pgp3/Abcb4

Nomenclature used in this thesis is the one introduced by Hsu *et al*. (1989). The official nomenclature based on the new system of nomenclature for ATP-binding cassette (ABC) transporter genes is also given. Mdr genes belong to subfamily B of ABC transporters.

a. Class I and II genes convey the multidrug resistance phenotype; class III genes encode a P-glycoprotein functioning as a phosphatidylcholine translocase.
b. MDR3 is also known as MDR2
c. mdr1a is also known as mdr3
d. mdr1b is also known as mdr1
e. Rat mdr genes are designated pgp1, pgp2 and pgp3 in some studies, like the nomenclature of hamster mdr genes.

Both genes have overlapping but distinct transport properties (Devault and Gros, 1990). The mdr1b gene of rats was cloned a decade ago (Silverman *et al.*, 1991), whereas the rat mdr1a gene was only recently cloned (Hooiveld *et al.*, 2000).

As with human MDR3, transfection studies indicated that mouse mdr2 was not capable to confer MDR. Systematic searches have never identified a human class II, suggesting that classes I and II represent a gene duplication occurring after the separation of murine and human speciation (Ng *et al.*, 1989). The mammalian Pgp multigene families are clustered in tandem on a single chromosome, chromosome 7 in humans, chromosome 5 in mouse and chromosome 4 in rat.

The coding sequences of the various MDR-conferring genes show high homology within and between species indicating evolutionary conserved roles (Table 3). The rodent mdr1a and mdr1b genes show 85% identity to each other. Sequence identity of rat mdr1a and human MDR1 genes is 83%, whereas rat and mouse mdr1a are more than 90% homologous. Human MDR1 and rat mdr1b are 80% identical. The homology at the amino acid level is even higher, at least 90% (Table 3).

Biochemistry

The drug transport mediated by Pgp depends on ATP hydrolysis. Interaction of the two halves of Pgp, specifically the proper interaction of two ATP binding sites, seems necessary for the coordinate functioning of the molecule (Sharom, 1997; Ambudkar *et al.*, 1999). Two molecules of ATP are hydrolysed during transport of one molecule of substrate. One is involved directly in a conformational change in the transmembrane domains that results in

translocation of drugs out of the cell, while the second seems to be necessary to restore the transporter to its original high affinity state for substrates (Sauna and Ambudkar, 2000; Sauna *et al.*, 2001). Interaction between ATP sites and the drug binding domains is essential for drug transport. Mutational analysis has shown that the two major drug-binding domains reside in or near transmembrane domains 5, 6 and 11, 12 (Ambudkar *et al.*, 1999). Substrates interact with different overlapping regions of a single drug binding site that is large enough to accommodate more than one compound (Sharom, 1997). Drug binding may occur through a substrate-induced fit mechanism; the packing of the transmembrane segments is changed upon binding of a particular substrate to Pgp (Loo *et al.*, 2003).

Pgp can actively extrude an overwhelmingly wide range of drugs from the cell generating a drug concentration gradient. Many of its substrates are cytotoxic compounds of natural or semisynthetic origin (plants, fungi, bacteria), which are extensively used in the chemotherapy of cancer (Vinca alkaloids, taxanes, anthracyclines,) or for a large variety of other medical purposes like antibiotics (actinomycin D), antiepileptics (phenytoin), hormones (dexamethasone), calcium channel blockers (verapamil), HIV protease inhibitors (indinavir), pesticides (ivermectin), antidepressants (amitriptyline) and immunosuppressants (cyclosporin A, FK506), to name but a few.

Table 3. Genbank and protein entries of various mammalian mdr genes and P-glycoproteins.

Genbank entry	Homology [a]	Gene/protein	Protein entry	Homology [b]
NM_133401 [c]	100	Rat mdr1a	NP_596892 [c]	100
AF286167			AAK83023	99
M81855	85	Rat mdr1b	P43245	84 / >90
NM_011076	90	Mouse mdr1a	NP_035206	95 / 98
NM_011075	83	Mouse mdr1b	NP_035205	83 / >90
XM_02059	83	Human MDR1	XP_02059	87 / >93
NM_000927			NP_000918	

The percentage identity to rat mdr1a gene or protein was calculated using the BLAST-program at the web server of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

a. Percentage homology with rat mdr1a gene entry NM_133401
b. Percentage homology with rat mdr1a Pgp sequence NP_596892; presented is the percentage of identical amino acids and of (chemically) similar amino acids ('positives').
c. Two complete sequences of rat mdr1a are presently available cloned from the Wistar respectively Sprague Dawley strain and differing in only a few nucleotides. The amino acid sequence NP_596892 is derived from NM_133401, whereas the sequence of AAK83023 is derived from Genbank entry AF286167.

Transport models

As these compounds have diverse chemical structures, it is difficult to define common properties of a typical substrate. Physicochemical properties seem to mainly determine whether a particular compound is transported or not. The only features common to Pgp substrates appear to be that they are all hydrophobic and amphipathic, *i.e.* containing spatially separated hydrophilic and hydrophobic moieties, with a molecular mass of 300-2000 Da (Ford and Hait, 1990; Sharom *et al.*, 1999). Due to this wide variety of chemical structures many different models proposing the mechanism used by Pgp to transport substrates have been developed. All models agree that Pgp uses the energy of ATP hydrolysis to translocate substrates from the cell (Gottesman and Pastan, 1988; Sharom, 1997; Stein, 1997), but the precise mechanism by which Pgp transports its substrates is still some matter of debate. The direct-pump models are among the more popular models (Higgins and Gottesman, 1992; Gottesman and Pastan, 1993), although alternative indirect-pump models have been proposed (Roepe, 1995; Zhu, 1999).

One alternative indirect mechanism suggests that Pgp modifies the intracellular pH and membrane potential indirectly altering the transmembrane partitioning or intracellular sequestering of the drugs (altered partitioning model) (Roepe, 1995; Roepe, 2000). Another model hypothesises that Pgp only pumps certain forms of drug conjugates and not the lipophilic parent drugs (Zhu, 1999). According to this model Pgp co-localises with drug metabolising enzymes that conjugate a common moiety to the lipophilic drug. Pgp subsequently excretes the conjugated metabolite.

The majority of experimental data, however, favours the direct transport models (figure 7) (Gottesman and Pastan, 1993; Gottesman *et al.*, 1995; Shapiro and Ling, 1995; Gottesman *et al.*, 1996; Sharom, 1997; Ambudkar *et al.*, 1999; Sauna *et al.*, 2001). According to these hypotheses, pharmacological agents passively diffuse down a concentration gradient through the cell membrane, because they are hydrophobic. Pgp subsequently extrudes drugs directly from the lipid bilayer even before they can enter the cytoplasm (Gottesman and Pastan, 1993; Sharom, 1997; Shapiro and Ling, 1997; Eytan and Kuchel, 1999). Besides the binding affinity for Pgp, also the lipid solubility of the substrate and the rate of partitioning within the bilayer determine whether a substrate is efficiently transported.
It remains to be resolved whether Pgp extracts substrates from the cytoplasmic, extracellular, or both leaflets of the plasma membrane (Chen *et al.*, 2001). According to the 'hydrophobic vacuum cleaner' hypothesis (Raviv *et al.*, 1990; Gottesman and Pastan, 1993) Pgp extracts drugs relative nonselectively from both leaflets of the membrane directly to the extracellular aqueous phase.
The alternative 'flippase model' suggests that Pgp carries its substrate from the inner leaflet to the outer leaflet (Higgins and Gottesman, 1992; Higgins, 1994; van Helvoort *et al.*, 1996; Sharom, 1997; Shapiro *et al.*, 1997) whereupon the substrate is extruded to the extracellular

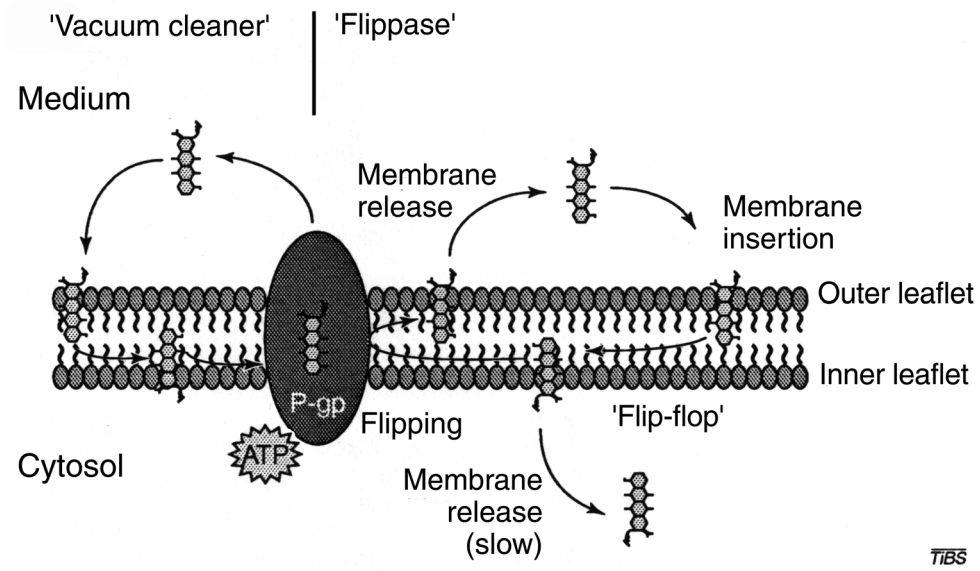


FIGURE 7. Possible mechanisms of action for substrate efflux transport by P-glycoprotein. Substrates enter the membrane before they interact with Pgp. (Left) Pgp might recognise substrates in both leaflets of the membrane and expel them directly to the extracellular medium ('vacuum cleaner model'). (Right) Alternatively, a substrate intercalated into the inner leaflet is flipped into the outer leaflet by Pgp and subsequently released into the extracellular medium ('flippase model'). Membrane release from the inner leaflet to the cytosol should be a slow process for effective functioning of Pgp. Adapted from Johnstone *et al.* (2000) with permission from Elsevier Science.

aqueous phase. This model is based on the analogy between amphipathic drugs and the normal phospholipid constituents of membranes, which do not easily flip between both leaflets. It is supported by the finding that the non-MDR class II Pgp, MDR2/mdr2 Pgp, is a phosphatidylcholine translocase (flippase) essential for extrusion of phosphatidylcholine from the hepatic plasma membrane into the bile (Smit *et al.*, 1993; Ruetz and Gros, 1994). Due to the rapid partitioning of most substrates into the extracellular leaflet, it is very difficult to determine whether Pgp is able to remove substrates from the outer leaflet (Chen *et al.*, 2001). Moreover, as there are many different substrates, it can not be excluded that there will be more than one mechanism with which Pgp transports drugs depending on the particular substrate.

Modulators

The many different inhibitors of Pgp-mediated resistance also interfere with Pgp function through various mechanisms. Pgp-mediated MDR can be reversed by so-called chemosensitizers or modulators resulting in decreased drug efflux and increased cellular drug accumulation (Van Zuylen *et al.*, 2000). There are probably as many Pgp chemosensitizers as there are "true" substrates, in particular because it is not easy to discriminate (Litman *et al.*, 2001). The complexity of interactions of Pgp with its substrates is such that, for instance, substrates can modulate Pgp transport of other substrates, while at the same time they are

transported themselves. Good inhibitors of Pgp usually seem to be substrates as well apparently transported at a low rate. They may have such a high affinity for Pgp that their off-rate is too low to detect. Alternatively, they may enter the membrane faster than Pgp can pump them out occupying binding sites (Barecki-Roach *et al.*, 2003).

Tissue distribution

Besides expression in MDR tumour cells, Pgp is also expressed in various nonmalignant human and rodent tissues including the BBB. Northern blot (Fojo *et al.*, 1987; Croop *et al.*, 1989) and immunohistochemical studies using different antibodies (Thiebaut *et al.*, 1987; Sugawara *et al.*, 1988a; Thiebaut *et al.*, 1989; Cordon-Cardo *et al.*, 1990; Bradley *et al.*, 1990) have shown that Pgp is differentially expressed among tissues in various species. High expression is found in intestine, adrenal, pregnant uterus and placenta. Significant levels of Pgp are further found in brain, spinal cord, liver, kidney, heart, testes, lung and spleen.

Interestingly, very high levels of Pgp are found in the adrenal gland of mice (Croop *et al.*, 1989), hamster (Georges *et al.*, 1990) and human (Thiebaut *et al.*, 1987; Sugawara *et al.*, 1988a), primarily in the cortical regions (Sugawara *et al.*, 1988b; Cordon-Cardo *et al.*, 1990). Pgp was found absent in rat adrenals but this was ascribed to possible preservation problems (Thiebaut *et al.*, 1989). Observations in hamsters indicate that high level of adrenal expression is limited to males, suggesting that Pgp may be involved in transport of sex-specific adrenal hormones (Bradley *et al.*, 1990).

The tissue distribution of murine mdr1a/1b together matches very neatly to that of human MDR1. In conjunction with their overlapping but distinct transport properties (Devault and Gros, 1990), this suggests that they perform together the same set of functions in mouse as MDR1 in man. The distribution of the mdr1a and mdr1b genes is tissue-specific (Croop *et al.*, 1989). In mice the mdr1a gene is predominantly expressed in the intestine, lung, testis and brain. Only in adrenal, kidney and uterus in pregnancy mdr1b is the main isoform in mice. Although the first report showed high mdr1b mRNA levels in placenta (Croop *et al.*, 1989), it is now clear that mdr1a is the major isoform in this tissue (Schinkel *et al.*, 1997; Borst and Elferink, 2002). In other tissues both isoforms are present at similar levels. This distribution pattern was generally similar in hamster (Bradley *et al.*, 1990; Georges *et al.*, 1990) and in rat (Hooiveld *et al.*, 2000).

Pgp frequently appeared to be confined to distinct specialised cells and to have a specific subcellular localisation. With the exception of the homogeneous distribution at adrenal cortical cell membranes, Pgp is mainly expressed at the luminal membrane of epithelial or endothelial cells forming physiological barriers like at the secretory surface of intestinal mucosal and renal proximal tubular cells, at the biliary canalicular surface of hepatocytes, and at pancreatic ductule cells in human (Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1990) as well

as in rat (Thiebaut *et al.*, 1989) and hamster (Bradley *et al.*, 1990). Pgp expression is also observed in hematopoietic stem cells (Chaudhary and Roninson, 1991) and in mature lymphoid cells (Drach *et al.*, 1992; Klimecki *et al.*, 1994).

Importantly, Pgp is highly expressed at blood-tissue barriers of the brain and spinal cord (Cordon-Cardo *et al.*, 1989; Thiebaut *et al.*, 1989; Sugawara, 1990; Cordon-Cardo *et al.*, 1990; Jette *et al.*, 1993; Beaulieu *et al.*, 1995; Lechardeur *et al.*, 1996), and also of the testis (Cordon-Cardo *et al.*, 1989; Thiebaut *et al.*, 1989). In all studied rodents the mdr1a gene has been demonstrated to be the single isoform expressed at the blood-brain barrier (Bradley *et al.*, 1990; Jette *et al.*, 1995; Demeule *et al.*, 2001).

Localisation at the BBB

Most studies support the idea that Pgp is exclusively expressed at the luminal membrane of brain endothelial cells of rat and human, although the exact localisation of Pgp at the BBB is still a matter of debate. By immunoelectron and confocal microscopy of brain sections the presence of Pgp at endothelial cells lining the BBB was detected exclusively at the luminal membrane (Sugawara *et al.*, 1990; Tsuji *et al.*, 1992; Stewart *et al.*, 1996; Virgintino *et al.*, 2002). The most convincing evidence came from a study employing a novel technique in which luminal membranes were isolated and purified from brain microvasculature using a coating of colloidal silica particles and polyacrylate (Beaulieu *et al.*, 1997). This procedure resulted in a luminal membrane preparation strongly enriched of the brain endothelial membrane marker GLUT1 and of Pgp. The strong enrichment of Pgp in the luminal membrane fractions can only be explained by assuming that the luminal membrane is the major site of Pgp expression.

Some studies, however, suggest that Pgp is mainly expressed at the astrocyte foot processes attached to the BBB. Double immunolabelling and confocal light microscopy of isolated human brain capillaries showed that the anti-Pgp antibodies were bound to the microvessels with a similar, overlapping staining pattern as an antibody against the astrocyte marker glial fibrillary acidic protein (GFAP) (Pardridge *et al.*, 1997; Golden and Pardridge, 1999). The punctate pattern contrasted with the continuous staining pattern for the glucose transporter GLUT1 showing only minimal overlap. As astrocyte foot processes are tightly associated with the basement membrane of brain capillaries and remain so after isolation of the capillaries, these results are suggestive for astrocyte specific abluminal expression.

At present, the exact localisation of Pgp is not clear. Most evidence supports the luminal localisation, but this site of expression is inconsistent with some data suggesting presence of Pgp at the astrocyte foot processes. To establish conclusively the site of Pgp expression employment of *in situ* hybridisation detecting mdr1a mRNA may be useful.

Regulation of Pgp expression

It is currently not fully understood how and when Pgp expression might be regulated. Although tumours are known to increase their Pgp expression when treated with

chemotherapeutic drugs, it is not known whether this increase is due to increase of mRNA translation or due to selection of Pgp-expressing cells (Gottesman and Pastan, 1993). Several stressors like oxidative stress (Felix and Barrand, 2002) are known to increase Pgp expression *in vitro* (Sukhai and Piquette, 2000). Furthermore, epileptic insults have been shown to induce Pgp expression in the brain (Rizzi *et al.*, 2002; Seegers *et al.*, 2002a), but the exact mechanism is not known. It is unclear whether Pgp plays a role in adaptational responses to stress. Preliminary results, particularly concerning mdr1b Pgp, suggest that steroids might be able to alter Pgp expression (Arceci *et al.*, 1988; Zhao *et al.*, 1993; Piekarz *et al.*, 1993; Sérée *et al.*, 1998; Demeule *et al.*, 1999). Unlike the mdr1b promoter, the mdr1a and MDR1 promoters do not have a glucocorticoid responsive element (Cohen *et al.*, 1991; Labialle *et al.*, 2002), although regulation of expression through protein-protein interactions of glucocorticoid receptors and other transcription factors might be possible.

Physiological role

The specific tissue distribution and subcellular localisation of Pgp in conjunction with its properties suggests that it is an important factor in limiting absorption and distribution of exogenous toxins and in increasing excretion of these xenobiotics or metabolites. A protective role is also indicated by its expression in specific capillary endothelial cells like those of the BBB. The generation of mice with disrupted mdr1a, mdr1b or both genes has made it possible to investigate the normal physiological role of Pgp in depth (Schinkel *et al.*, 1994; Schinkel *et al.*, 1997). All three types of knockout mice appear to be completely normal as long as they are not challenged with drugs. Each of the deficient mice appears to have normal growth, development, viability, life span and fertility and does not show gross anatomical or histological abnormalities. Except for increased hepatic and renal upregulated expression of mdr1b in mdr1a knockouts, no compensatory enhanced expression of diverse other ABC transporters has been found so far in any other tissue including the brain (Borst and Schinkel, 1997). This lack of any obvious physiological change indicates that Pgp is not essential for normal life.

When treated with drugs that are substrates of Pgp, these knockout mice appear to have severe problems in pharmacological handling of these drugs. The most striking results were obtained with mice with an inactivated mdr1a gene. These mice have no detectable Pgp in gut epithelium and brain capillaries corroborating that this isoform is the single one expressed at these barriers (Schinkel *et al.*, 1994). Increased oral uptake, decreased clearance, shifts in excretion route and enhanced uptake in foetuses of various potentially harmful or therapeutic compounds have been demonstrated in these knockouts (Mayer *et al.*, 1996; Sparreboom *et al.*, 1997; Smit *et al.*, 1999).
Increased accumulation of Pgp substrates in the brain of mdr1a deficient mice has demonstrated the importance of Pgp expressed at the BBB. Brain levels of vinblastine and many other drugs are much higher in mdr1a knockouts than in normal mice while plasma

levels were only slightly increased (Schinkel *et al.*, 1994; Schinkel *et al.*, 1995; Van Asperen *et al.*, 1996; Schinkel *et al.*, 1996). Even more strikingly is the effect of treatment with neurotoxic drugs that are Pgp substrates. The lethal dose of antihelminthic ivermectin is one hundred fold lower in mdr1a knockout mice than in normal mice concomitant with highly increased brain penetration (Schinkel *et al.*, 1994). These studies show that Pgp has no vital function in normal metabolism, but is essential in protecting the body and crucial organs like the brain against exogenous toxic compounds.

In addition to its protective role, various other physiological functions of Pgp have been proposed to provide an explanation for the unusual basal ATP hydrolysis in absence of any known substrate. Recently, Pgp was suggested to actively translocate cholesterol to the outer cell membrane (Garrigues *et al.*, 2002). In addition, Pgp might be involved in transport of cytokines or translocation of phospholipids (Chong *et al.*, 1993) and it may participate in programmed cell death (Johnstone *et al.*, 2000). A specific function of Pgp at the BBB that was recently postulated may be excretion of peripherally acting small peptides, like opioids, out of the brain (King *et al.*, 2001). In addition, Pgp may further be involved in elimination of ß-amyloid from the brain, which is indicative for a possible role in aetiology of Alzheimer's disease (Lam *et al.*, 2001). The most frequently suggested function is, however, a role in steroid transport.

P-GLYCOPROTEIN AND GLUCOCORTICOIDS

Interactions of Pgp and steroids

Relatively little attention has been given to the factors that influence the intracellular concentration of steroids. Glucocorticoids are commonly believed to readily diffuse across plasma membranes by virtue of their highly lipophilic nature and their small size. As early as 1968 there has been evidence that steroids may be transported out of different, but not all, mammalian cells by an energy-dependent mechanism within the membrane resulting in a reduced accumulation (Gross *et al.*, 1968). The extrusion process only affects some steroids such as cortisol, dexamethasone and prednisolone, whereas accumulation of other steroids like deoxycorticosterone, progesterone and cortexolone was not affected (Gross *et al.*, 1969; Gross *et al.*, 1970). The basis for this phenomenon was not understood.
Fifteen years later, another cell line was isolated that showed resistance to dexamethasone unrelated to changes of the GR, and that appeared to have acquired multidrug resistance (Johnson *et al.*, 1984). The phenotype of this dexamethasone resistant variant of a normally glucocorticoid sensitive murine thymona cell line was eventually shown to be associated with expression of mdr1b Pgp (Bourgeois *et al.*, 1993). This cell line turned out to be also resistant to apoptosis induced by various other steroids such as cortisol, prednisolone and, to a lesser extent, corticosterone and aldosterone (Bourgeois *et al.*, 1993).

Data from a substantial number of other studies using different methods have further corroborated the ability of Pgp to transport dexamethasone and several other steroids. Pgp expressed in MDR1 cDNA transfected pig kidney epithelial cell lines can transport dexamethasone, cortisol and aldosterone (Ueda *et al.*, 1992; Schinkel *et al.*, 1995). The Pgp-mediated transport of cortisol was confirmed for hamster Pgp (Van Kalken *et al.*, 1993). Additionally, corticosterone has been reported to be effluxed by mdr1b Pgp expressed at MDR murine macrophage-like cells (Wolf and Horwitz, 1992). Mouse adrenal Y1 cells, in which one copy of the mdr1b gene has been inactivated by insertional mutagenesis, show reduced steroid secretion upon activation with ACTH (Altuvia *et al.*, 1993).

Several other methods have been used to study interactions of various steroids with Pgp, but it is important to keep in mind that photoaffinity labelling, accumulation and inhibition studies all show that a compound that can bind to Pgp, does not necessarily have to be transported by Pgp. Moreover, the more a steroid binds to Pgp, the less effectively it is transported out of the cell.

Several studies have used photoaffinity labelling of Pgp to study interactions of steroids with Pgp. This method uses the ability of ultraviolet irradiation to fix compounds to membrane constituents. Isolated membranes are incubated with a radiolabelled Pgp-substrate with or without unlabelled Pgp-substrates, after which the membranes are immunoprecipitated with a Pgp-specific antibody, followed by gel electrophoresis. Photoaffinity labelling is a measure of the ability of Pgp to bind the particular substrate, rather than a measure of its ability to transport it. Most corticosteroids are able to inhibit ^{125}I-azidopine photoaffinity labelling of Pgp to a greater or less extent depending on their hydrophobicity (Yang *et al.*, 1989; Qian and Beck, 1990). Photoaffinity studies further show that ^{3}H-corticosterone specifically photolabels murine mdr1b Pgp (Wolf and Horwitz, 1992) whereas ^{3}H-cortisol does not label human Pgp (Qian and Beck, 1990). Besides a possible species difference, this may only indicate that cortisol binds less avidly to Pgp than corticosterone.

Studies of accumulation in Pgp expressing cells show reduced accumulation of corticosterone (Wolf and Horwitz, 1992; Barnes *et al.*, 1996), cortisol (Van Kalken *et al.*, 1993; Barnes *et al.*, 1996), dexamethasone (Bourgeois *et al.*, 1993; Barnes *et al.*, 1996) and aldosterone (Barnes *et al.*, 1996), although not all studies agree on this (Fojo *et al.*, 1985; Van Kalken *et al.*, 1993; Gruol *et al.*, 1999). In an extensive study of interactions of Pgp with various steroids using resistant human colon carcinoma cells, it was found that the accumulation of dexamethasone, cortisol, corticosterone, aldosterone and various other steroids was reduced to an extent that correlates well with their respective hydrophilicity (Barnes *et al.*, 1996). An inverse correlation was found when studying steroid induced enhancement of vinblastine or daunorubicin accumulation; increased antagonism of Pgp mediated transport correlates with decreased steroid hydrophilicity (Van Kalken *et al.*, 1993; Barnes *et al.*, 1996). Inhibition of accumulation of other substrates is not a direct measure of transport, but rather a measure of

binding capacity, whereas accumulation studies are less reliable because of nonspecific membrane binding.

Taken together, these studies show that interaction of steroids with Pgp comprises a spectrum with at the one end steroids like dexamethasone and cortisol that are mainly transported and at the other end steroids that mainly inhibit Pgp. The clearest example of the latter is progesterone.

While progesterone binds avidly to Pgp in photoaffinity studies (Qian and Beck, 1990), and is an efficient inhibitor of azidopine photoaffinity labelling (Yang *et al.*, 1989; Qian and Beck, 1990) and Pgp-mediated transport of other substrates (Yang *et al.*, 1989; Ueda *et al.*, 1992; Van Kalken *et al.*, 1993), the hormone itself is not transported out of the cell by Pgp (Yang *et al.*, 1990; Ueda *et al.*, 1992; Bourgeois *et al.*, 1993). This paradoxical phenomenon may be explained by its high lipophilicity, resulting in a fast partitioning into the plasma membrane leaflets (Eytan *et al.*, 1996). This high rate of back diffusion fully counteracts the Pgp-mediated outward flipping of progesterone and no drug gradient can be established (Eytan *et al.*, 1996; Sharom, 1997). Progesterone is a strong MDR reversal agent, because like some other highly lipophilic compounds functioning as MDR modulator, it strongly competes for Pgp mediated transport (Eytan *et al.*, 1996). Due to its continuous rapid insertion into the plasma membrane progesterone overwhelms the Pgp transport machinery, which leads to the inability of Pgp to transport other substrates.

Possible role in steroid transport

The above-mentioned *in vitro* studies in conjunction with the presence of Pgp in adrenal cells has lead to suggestions that Pgp has a physiological role in steroid secretion. Steroid efflux by drug transporters may be a more generally occurring phenomenon than is currently appreciated (Thompson, 1995; Kralli and Yamamoto, 1996), as a yeast homologue of Pgp, LEM1, has also been shown to transport several steroids (Kralli *et al.*, 1995).

The importance of Pgp as a steroid transporter is questioned, however, by the lack of Pgp in rat adrenal and the generation of the mdr1b knockout mice and the mdr1a/b double knockout mice. These mice show no gross disturbances in corticosteroid handling, although mdr1a/b double knockouts have been reported to have consistently lower ACTH and corticosterone plasma levels than their wild type littermates under various conditions (Müller *et al.*, 2003) suggesting an altered HPA-axis regulation. The lack of gross changes in glucocorticoid targets as reported thus far suggests that both mdr1a and mdr1b Pgp have no essential function in the normal steroid metabolism of the adrenal (Schinkel *et al.*, 1997). Pgp might be still involved in steroid transport in a more subtle way by protecting the plasma membranes of steroid-secreting cells from the toxic effects of high steroid concentrations (Van Kalken *et al.*, 1993; Ambudkar *et al.*, 1999) under conditions of *e.g.* stress-induced HPA-axis activity. A protective role of Pgp would also be more consistent with its property to transport drugs out of the plasma membrane. Alternatively, Pgp may play a role in regulation of glucocorticoid exposure of glucocorticoid responsive cells like neurons.

Possible role in steroid transport at the BBB

Evidence that Pgp may be able to protect the brain against potentially harmful glucocorticoid action was derived from studies with mdr1a knockout mice receiving dexamethasone. Pgp was demonstrated to be responsible for the impaired access of low to moderate doses of dexamethasone in brain, as it excludes this synthetic glucocorticoid from brain. Uptake of dexamethasone to the brain was enhanced in mdr1a (-/-) mice compared to wild type mice (Schinkel *et al.*, 1995) increasing the access to the glucocorticoid receptor (Meijer *et al.*, 1998) (figure 8). A role of Pgp at the BBB in protecting the brain against steroids has therefore been postulated. Its presence at the BBB and ability to transport several glucocorticoids suggests that Pgp may play an important role in modulation of glucocorticoid access to the brain corticosteroid receptors, thus affecting central glucocorticoid action. This suggestion formed the basis of the research described in this thesis.

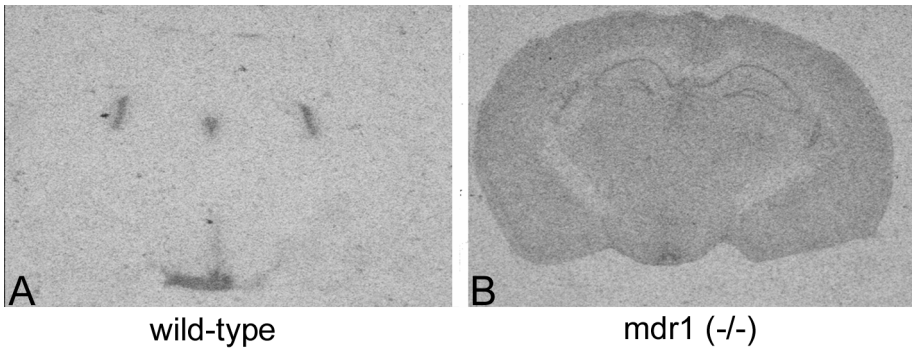


FIGURE 8. Representative autoradiograms of 10-µm coronal sections of the brain of wild type (A) and mdr1a knockout mice (B) at hippocampus level.
Autoradiograms show radioactive labelling at 1 hour after systemic treatment with a tracer dose of ^{3}H-dexamethasone. The dark spots in (A) represent transverse sectioning of the chorioid plexus and adjacent cerebroventricular space. Adapted from Meijer *et al.* (1998) with copyright permission from The Endocrine Society.

SCOPE OF THE THESIS

Rationale and objectives

The aim of the studies described in this thesis is to examine the interaction of glucocorticoids and the efflux transporter P-glycoprotein expressed at the BBB as a possibly new level at which access to the brain and thus central corticosteroid receptor function may be controlled. Modulation of access of glucocorticoids to the brain may provide a new way to restore aberrant corticosteroid signalling associated with hypercortisolemia, glucocorticoid feedback resistance or MR/GR imbalance. In this thesis, I focus on the following objectives.

First, the expression of Pgp in the brain at the level of mRNA was examined to determine which particular cell types express Pgp and whether glucocorticoid treatment would affect expression levels.
The second phase of the project was aimed to investigate whether various synthetic as well as naturally occurring glucocorticoids are substrates of Pgp. Since previous studies have shown that mdr1a Pgp hampers dexamethasone to enter the brain, the question arose whether Pgp may also exclude other glucocorticoids from the brain. We hypothesised that endogenous glucocorticoids would rather easily reach the central glucocorticoid target areas, whereas Pgp would protect the brain against exogenous synthetic glucocorticoids.

Further research was based on the hypothesis that treatment with moderate amounts of dexamethasone provides a strategy to correct aberrant corticosteroid levels and receptor dysfunction in brain. Dexamethasone is well known as potent suppressor of HPA-activity. It acts at the level of the pituitary to suppress ACTH and consequently corticosterone secretion (De Kloet *et al.*, 1974). As dexamethasone poorly penetrates into the brain, this implies that administration of low to moderate amounts of dexamethasone depletes the brain from endogenous glucocorticoids, for which dexamethasone does not appropriately substitute (figure 9). Peripherally, glucocorticoid receptors are still activated by dexamethasone. The resulting condition is a brain-selective state of adrenalectomy. Neural functions would suffer from underexposure to corticosteroids, whereas peripheral functions would still be directly influenced by dexamethasone.

Experimental approach and outline

To examine the expression of mdr1a mRNA in brain *in situ* mRNA hybridisation was employed, using both radioactive and non-radioactive RNA-probes (**chapter 2**). To determine the exact localisation of cells expressing mdr1a mRNA a digoxigenin-labelled RNA probe was applied on brain sections of naive animals. To reveal whether expression might be regulated under some conditions, expression levels were measured in adrenalectomised rats and in intact rats treated with dexamethasone and kainic acid, a seizures-inducing agent.

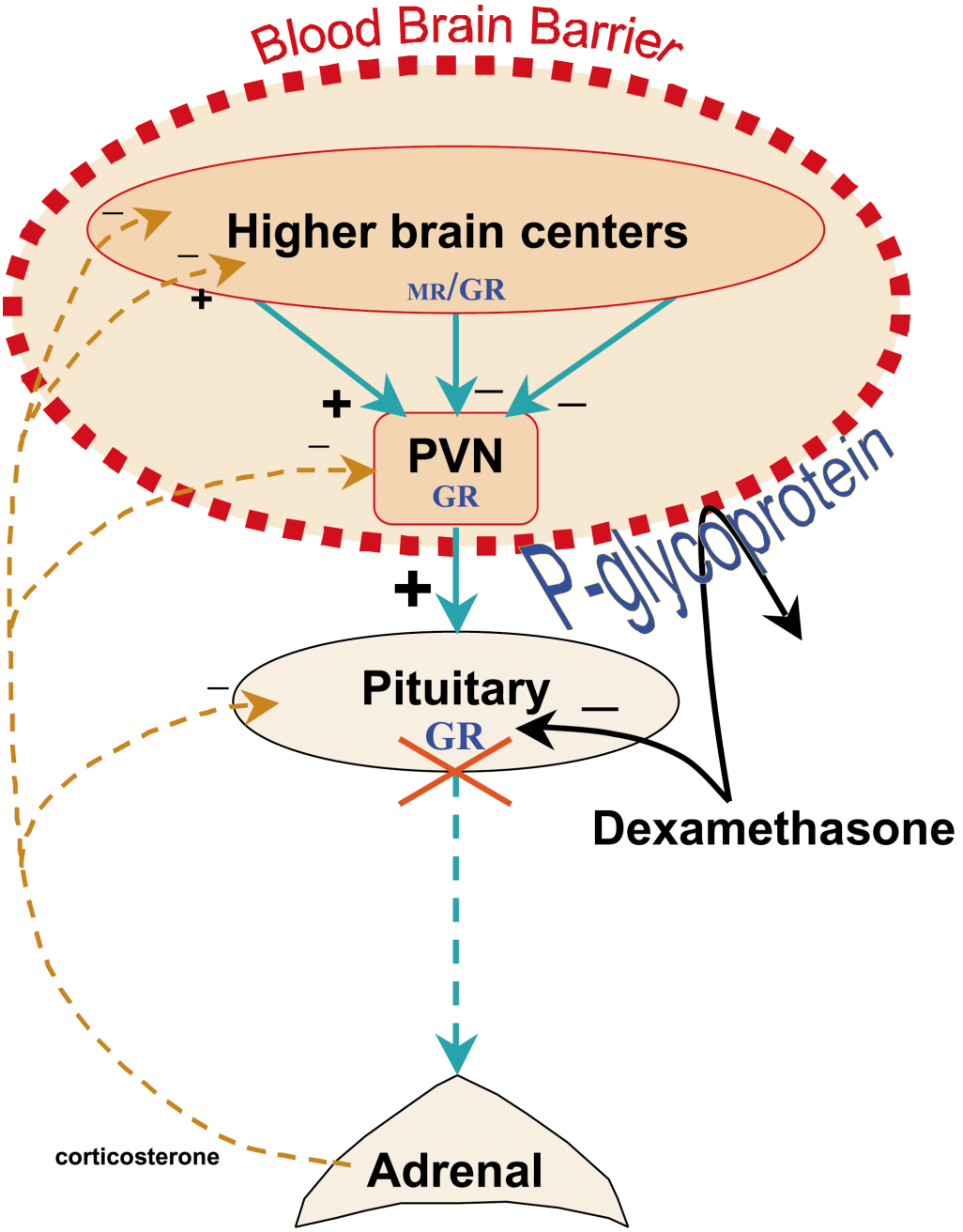


FIGURE 9. Hypothesised creation of brain-selective low-corticosteroid condition by low-dose dexamethasone. Low plasma concentrations of dexamethasone primarily act on the anterior pituitary to suppress pituitary-adrenal secretion. Dexamethasone replaces corticosterone at peripheral glucocorticoid targets. However, due to the presence of P-glycoprotein at the BBB dexamethasone can not replace corticosterone in the brain. The ensuing low-corticosteroid state in the brain is reminiscent of a brain-selective adrenalectomy condition, and will likely affect MR/GR balance and glucocorticoid actions on brain function.

In order to study the uptake of various glucocorticoids in brain we used mice with a disrupted mdr1a gene lacking Pgp at the BBB. After adrenalectomy, which removes the endogenous source of corticosterone, wild type and mdr1a (-/-) knockout mice were systemically injected with radiolabelled corticosteroids. Uptake in brain and retention in central corticosteroid receptor expressing areas was measured with autoradiography both in brain homogenates and in brain sections apposed to sensitive films. Uptake of corticosterone and cortisol (**chapter 3**) and prednisolone (**chapter 4**) was determined this way.

The role of Pgp in uptake of glucocorticoids in human brain was examined in two different albeit indirect ways. Pig kidney epithelial (LLC-PK1) cells stably transfected with human MDR1 cDNA were used to study the Pgp mediated transport of radiolabelled glucocorticoids (figure 10). These cells form monolayers when seeded on filters and express Pgp at their apical membrane (Florea *et al.*, 2001). These monolayers are a suitable model for Pgp-mediated transport at the BBB as they express Pgp at high levels, presumably comparable to brain endothelial cells and unlike many *in vitro* models of the BBB (Lechardeur and Scherman, 1995; Barrand *et al.*, 1995; Seetharaman *et al.*, 1998; Gaillard *et al.*, 2000). Polar transport of glucocorticoids indicates Pgp-mediated transport. The involvement of Pgp was confirmed by comparison with monolayers of untransfected cells and by the abolishing effect of a potent and selective Pgp blocker (**chapter 3 and 4**). Further, cortisol and corticosterone levels were measured in extracts of human post-mortem brain samples and plasma using LC-MS to determine the ratio of corticosterone over cortisol (**chapter 3**).

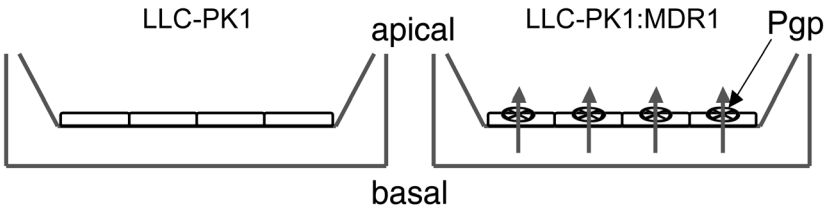


FIGURE 10. Pgp-mediated transepithelial transport in monolayers of pig kidney epithelial (LLC-PK1) cells stably transfected with human MDR1 cDNA. Pgp is localised at the apical side, transporting its substrates from the basal compartment to the apical compartment, or from the apical compartment back into the apical compartment. Monolayers of non-transfected host cells are used as a control. Pgp-mediated polar transport will be demonstrated as increased transport from basal to apical and decreased diffusion from apical to basal as compared with host cells.

To test the working hypothesis about the brain-selective depletion of glucocorticoids provided by exclusion of low amounts of dexamethasone from the brain, rats were treated with low amounts of dexamethasone and different central as well as peripheral markers of glucocorticoid action were measured (**chapter 5**). Dexamethasone was administered chronically in two different ways, via subcutaneous injection or through the drinking water. The focus was on the stress system as endogenous glucocorticoids play an important role in

the stress response. Since central effects of glucocorticoids are often only seen after stimulation of the stress system the animals were stressed at the end of the treatment. The brain was examined for diverse molecular markers of the stress system. Various peripheral markers of glucocorticoid action were used to determine the effects of dexamethasone in the periphery.

All data are discussed in a broader perspective (**chapter 6**) and general conclusions are drawn (**chapter 7**). Finally, in an **addendum** I summarise the latest results regarding brain uptake of the antiglucocorticoid C-1073/RU486, a novel fast-acting powerful antidepressant.

Chapter 2

mRNA EXPRESSION OF MULTIDRUG RESISTANCE P-GLYCOPROTEIN IN BRAIN

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D. Pons
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Abstract

The multidrug resistance (mdr) P-glycoprotein (Pgp) is an energy dependent efflux transporter that protects the brain against a wide variety of neurotoxic compounds including glucocorticoids. This transmembrane protein is a well-known functional component of the blood-brain barrier and might be present in brain parenchyma cells as well, although there is some controversy about the particular cell types expressing Pgp.

Since *in situ* mRNA hybridisation might be helpful in determining the exact localisation of Pgp expression, we have developed a riboprobe against the murine multidrug resistance 1 mRNA recognising both subtypes of the rodent mdr1 gene. We have also studied the effects of treatment with known inducers of Pgp expression, the chemical convulsant kainic acid and the synthetic glucocorticoid dexamethasone.

The data demonstrate that mdr1 mRNA is present in the endothelial cells of brain capillaries throughout the rat brain indicating that Pgp is expressed at the endothelial cells forming the blood-brain barrier.

Surprisingly, specific mdr1 mRNA expression was also found in neuronal layers of hippocampal fields, particularly in the granule cells of the dentate gyrus. Three-week high-dose dexamethasone treatment did not affect mRNA expression in granule cells. However, kainic acid treatment surprisingly decreased the expression levels of mdr1a mRNA in the dentate gyrus 6 and 24 hours after treatment. Our methodology did not permit to detect whether any treatment altered the expression at the BBB.

Our data indicate that under normal, healthy conditions Pgp is likely only expressed by endothelial cells and possibly dentate gyrus neurons, although it can not be excluded that certain pathological conditions may induce Pgp expression in astrocytes.

Introduction

The multidrug resistance (mdr) P-glycoprotein (Pgp) is a transmembrane protein that functions as an energy dependent efflux transporter of numerous substrates (Gottesman and Pastan, 1993). Due to its presence at the blood-brain barrier (BBB) (Cordon-Cardo *et al.*, 1989; Thiebaut *et al.*, 1989; Jette *et al.*, 1993) Pgp plays a vital role in the defence of the brain against a wide variety of excitotoxic compounds, particularly hydrophobic, amphipathic agents (Schinkel, 1999). Pgp-mediated exclusion from the brain is of particular importance for compounds that act centrally including antiepileptic drugs (Loscher and Potschka, 2002) and glucocorticoids (Meijer *et al.*, 1998).

A small highly homologous gene family, the multidrug resistance genes, encodes Pgp. The human MDR1 is the only gene conveying multidrug resistance present in human (Van de Vrie *et al.*, 1998). In rodents the mdr1a gene is the single isoform expressed at the BBB (Bradley *et al.*, 1990; Jette *et al.*, 1995; Demeule *et al.*, 2001). The second isoform mdr1b may be present in brain at low levels (Croop *et al.*, 1989; Schinkel *et al.*, 1994), most likely in glial cells (Lee *et al.*, 2001a) although the exact localisation is not yet known.

Although a vast amount of studies support the idea that Pgp is exclusively expressed at the luminal membrane of brain endothelial cells (Sugawara *et al.*, 1990; Tsuji *et al.*, 1992; Stewart *et al.*, 1996; Beaulieu *et al.*, 1997; Virgintino *et al.*, 2002), the exact localisation of Pgp at the BBB is still a matter of debate. Some studies have provided data suggesting that Pgp is primarily expressed at astrocyte foot processes tightly attached to the capillaries (Pardridge *et al.*, 1997; Golden and Pardridge, 1999). Although other studies have never reported Pgp expression in healthy brain glial cells, nor in neurons, Pgp was found overexpressed in glial cells within the epileptogenic lesion of brains of patients with different pathologies causing refractory epilepsy (Tishler *et al.*, 1995; Sisodiya *et al.*, 1999; Sisodiya *et al.*, 2002) and in brains of rats treated with the chemical convulsant kainic acid (Zhang *et al.*, 1999; Rizzi *et al.*, 2002; Seegers *et al.*, 2002b).

To establish whether Pgp is synthesised by astrocytes, endothelial cells or both, in normal and epileptogenic brain *in situ* mRNA hybridisation was applied using a riboprobe against both mdr1a and mdr1b. Radioactive and DIG-labelled probes were used in order to visualise mdr1 mRNA in rat and mouse brain tissue. The effect of kainate treatment was examined in rats, because of its potency to induce Pgp expression. In addition, the effect of glucocorticoid treatment on expression in rat brain was studied, since glucocorticoids may induce expression (Aquilante *et al.*, 2000).

Materials and methods

Animals

Young adult male Wistar rats (Charles River, Sulzfeld, Germany) were housed under a 12-12 hour light dark cycle with lights on at 7:00hr in a temperature (21°C) and humidity controlled room. Food and drinking water were available *ad libitum*. Before and during experiments rats were handled daily. 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 (The Netherlands).

Treatments

Twelve rats weighing around 230g received an injection of the potent non-NMDA glutamate receptor agonist kainate (8 mg/kg body weight) in the morning of the experiment. Another twelve rats received an injection of vehicle (0.9% saline). After injection animals were watched and epileptic behaviour was recorded based on the five stadia described by Sperk (Sperk, 1994). At six hours and 24 hours after injection rats were decapitated. Thus, all groups consisted of 6 animals. Trunk blood was collected in EDTA-coated tubes and centrifuged for determination of corticosterone plasma levels using a ^{125}I-corticosterone radioimmunoassay kit (ICN Biomedicals, Costa Mesa, USA). Brains were rapidly removed from the skull and quickly frozen in isopentane precooled on dry ice/ethanol. All tissues were stored at –80°C until further use. Brains were used for *in situ* hybridisation of mdr1a mRNA.

To detect changes of mdr1a mRNA expression after glucocorticoid treatment *in situ* hybridisation was employed on brains of rats that have been treated with 10 µg/ml dexamethasone 21-phosphate through their drinking water for three weeks (see chapter 5). This dose is known to enter the brain at sufficient concentrations to act in glucocorticoid target areas. Control animals not treated with dexamethasone were also included.

Probe

From the pJ3Ω vector containing the full-length mouse mdr1a cDNA (courtesy of A.H. Schinkel and P. Gros) an 890 basepair fragment (1348-2237) was excised using BglII and EcoRI and cloned into the BamHI/EcoRI site of pBluescript KS vector. The identity of the probe was confirmed by partial DNA sequence analysis. Specificity of the probe was confirmed by blasting it to known genes in GenBank. The homology of the probe sequence was 100% with mouse mdr1a, 91% with mouse mdr1b, 93% with rat mdr1a, 88% with rat mdr1b and less than 80% with any other mdr gene.

Non-radioactive *in situ* hybridisation

To qualitatively determine the cellular localisation of mdr1 mRNA non-radioactive *in situ* hybridisation was applied. The protocol used for *in situ* hybridisation with digoxigenin (DIG)-labelled RNA probes has been described previously (Asbreuk *et al.*, 2002). The linearised

vector was *in vitro* transcribed using a DIG RNA labelling mix (Roche Diagnostics, Mannheim, Germany) and T3 polymerase (Roche) to obtain a DIG-labelled anti-sense probe.
Twenty-micrometer coronal sections of brain were cut on a cryostat and thaw-mounted on poly-L-lysine (Sigma Chemical, St Louis, USA) coated microscopic slides. The sections were postfixed in a freshly prepared 4% paraformaldehyde solution (pH 7.2) for 10 minutes at room temperature, rinsed three times in phosphate buffered saline (PBS) for 3 minutes at room temperature. Subsequently the sections were acetylated (0.25% acetic anhydride in triethanolamine (TEA)) for 10 minutes at room temperature and rinsed three times in PBS. Thereafter the sections were prehybridised for 2 hours at room temperature: each slide was loaded with 300 µl hybridisation mix containing 50% deionised formamide, 5x SSC (SSC=0.15M NaCl and 0.015M sodium citrate), 5x Denhardt's, 250 µg/ml bakers yeast tRNA, 500 µg/ml sonificated salmon sperm DNA. After prehybridisation the mix was removed and the slides were loaded with 150 µl hybridisation mix containing the digoxigenin-labelled RNA probe (890 ng/ml). The slides were covered with NESCO-film and hybridisation was performed overnight in a moist chamber at 72°C. The next day, NESCOfilm was removed in 2xSSC (72°C) and the slides were rinsed in 0.2xSSC for 2 hours at 72°C. The slides were then transferred to 0.2xSSC at room temperature.
For immunohistochemical detection slides were incubated in 100 mM Tris/150 mM NaCl (pH 7.4) for 5 minutes at room temperature. Subsequently, heat-inactivated fetal calf serum (HI-FCS) was added to this buffer (0.2%) shortly before preincubation with 1.5 ml 10% HI-FCS/Tris/NaCl for 1 hour at room temperature. Finally, overnight incubation with an alkaline phosphatase-labelled antibody raised against DIG (Roche, 1:5000 in 10% HI-FCS/Tris/NaCl) was performed at 4°C. The next day the slides were rinsed three times for 5 minutes in Tris/NaCl (pH 7.4) and 5 minutes in 50 mM $MgCl_2$ in Tris/NaCl (pH 9.5) at room temperature. The DIG-probe labelled sites were visualised using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate *p*-toluidine (NT/BCIP, Boehringer Mannheim) as a substrate. The enzymatic reaction was terminated in 10 mM Tris/5 mM EDTA (pH 8.0).

Radioactive *in situ* hybridisation

The vector was linearised with NotI respectively EcoRI and *in vitro* transcribed using ^{33}P-labelled UTP (ICN Biomedicals, Isoblue stabilised, S.A. 3000 Ci/mmol) and T3 respectively T7 polymerase to obtain anti-sense or sense probe, respectively.
Cryostate sections were postfixed in a freshly prepared 4% paraformaldehyde solution (pH 7.2) for 60 minutes at room temperature, rinsed twice in PBS for 5 minutes at room temperature, and treated with proteinase K (1 µg/ml in 0.1M Tris, pH 8.0) at 37°C for 10 minutes. After a brief rinse in diethyl pyrocarbonate (DEPC) treated water, they were treated with 0.25% acetic anhydride in 0.1M TEA (pH 8.0) for 10 minutes at room temperature and finally rinsed in 2xSSC for 10 minutes at room temperature. Subsequently the sections were dehydrated trough a graded series of ethanol and air-dried. Each slide, containing six sections, was loaded with a 100µl mix containing 70% deionised formamide, 10% dextran sulphate, 3xSSC, 1xDenhardt's solution, 0.1 mg/ml yeast tRNA, 0.1 mg/ml sheared herring sperm DNA

and $3x10^6$ dpm of the probe, and covered with microscopic coverslips. Overnight hybridisation was performed in a moist chamber at 55°C. As a control, a few slides were hybridised with sense probe. The next day, coverslips were removed and the slides were washed in 2xSSC at room temperature for 10 minutes, treated with RNAse A (2 mg/100 ml in 0.5M NaCl, pH 7.5) at 37°C for 10 minutes and 3 times washed in 2xSSC/50% formamide at 60°C for 15 minutes. After a short wash with 2xSSC, sections were dehydrated in an ethanol series and air-dried. Finally, the slides were put in an X-ray exposure holder and apposed to X-OMAT AR film (Kodak, Rochester, NY) for 12 days.

Quantification and statistics

Optical density was quantified with an Olympus image analysis system with the appropriate software (Paes Nederland B.V., the Netherlands) equipped with a Cue CCD camera or using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). After shading correction, the hippocampal images were corrected for film background. Densities were determined by outlining the granule cell layer of the dentate gyrus, the pyramidal cell layers of CA1 and CA3 areas and cortex. The optical density of the CA2/CA3 stratum radiatum was used for tissue background. Generally, 6-9 sections were measured per animal and averages were used for subsequent statistical analysis of treatment groups. Data were analysed using ANOVA and, when appropriate, post-hoc comparisons were performed using Tukey's Honestly Significant Difference test. Statistical difference was accepted at $p < 0.05$.

Immunohistochemistry

Pgp expression was detected with the primary monoclonal mouse antibody C219 (Calbiochem; Bad Soden, Germany). The antibody C219 reacts against the cytoplasmic VVQEALD epitope of human, mouse and rat Pgp (Vore *et al.*, 1996) and has been used to detect Pgp in rat brain (Zhang *et al.*, 1999; Seegers *et al.*, 2002b). It does not discriminate between the mdr1a and mdr1b isoforms of Pgp.

Cryostate sections thaw-mounted on poly-L-lysine slides were fixated for 30 minutes in 4% paraformaldehyde on ice, washed two times with PBS, dehydrated, and delipidated for 1 minute in chloroform. After each incubation sections were washed several times in 0.1M Tris buffered saline (TBS). The sections were treated with 1% hydrogen peroxide (H_2O_2) in TBS for 15 min to eliminate endogenous peroxidase activity. To minimise nonspecific binding, sections were immersed in 10% normal goat serum in TBS containing 2% NS and 0.2% Triton X-100 (TBS-T) for 30 minutes. The slides were incubated overnight at 4ºC with the primary antibody in a 1:40 dilution in TBS-T/NS. The antibody was detected using a biotinylated goat anti-mouse secondary antibody (Vector Laboratories, USA) in a 1:200 dilution in TBS-T/NS at 37ºC for 1 hour. Sections were then incubated with an avidin-biotine horseradish peroxidase complex (ABC) in 0.1 M TBS-T at room temperature for 45 minutes followed by two washes in 0.01M Tris buffer (pH 7.5-7.6) for 10 min each. The staining was visualised by incubation with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.01M Tris containing

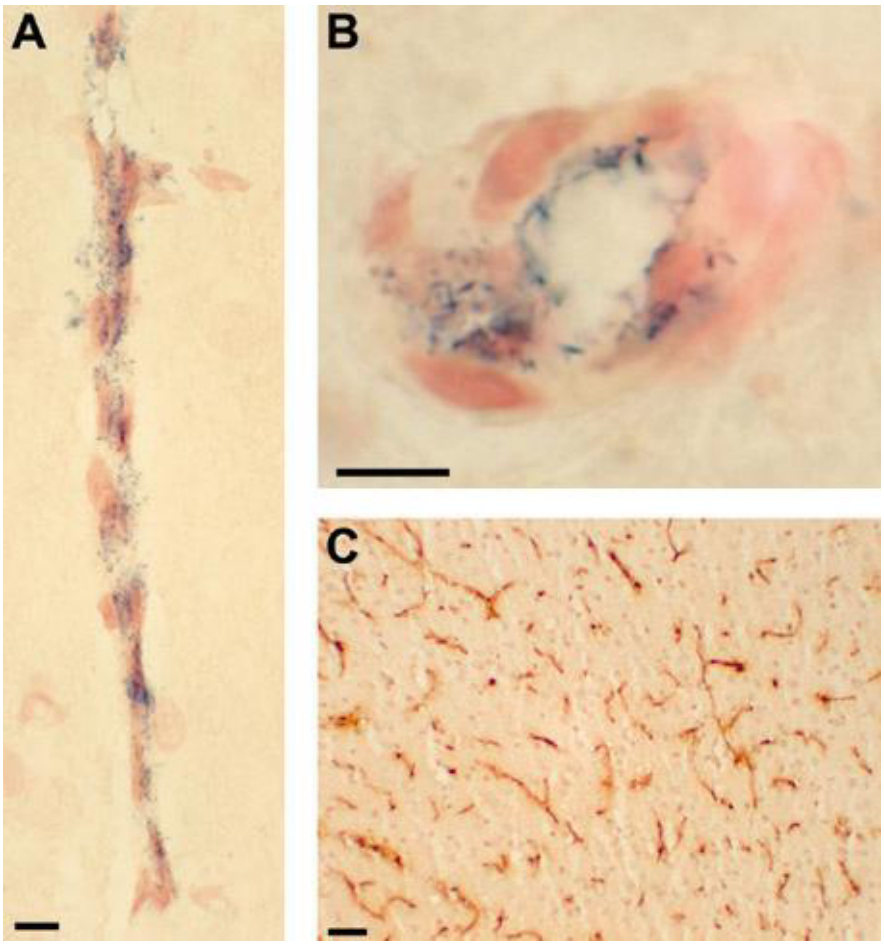


FIGURE 1. Expression of mdr1 mRNA at brain capillaries.
DIG-labelled *in situ* hybridisation showing grains overlaying larger brain capillaries reveals expression of mdr1 mRNA in endothelial cells (A,B). Grains are located at the luminal side of endothelial cells (B). Bars indicate 10 µm.
C219 immunohistochemical staining shows presence of P-glycoprotein throughout the brain at both small and larger brain capillary. Bar indicates 50 µm.

0.033% H_2O_2 for ten minutes. After dehydration, the slides were air-dried and lightly counterstained with haematoxylin before coverslipping with Permount. Control sections incubated with preimmune NS instead of primary antibody showed absence of staining.

Results

Expression mdr1 Pgp at BBB

Using a DIG-labelled RNA probe against mouse mdr1a/mdr1b, which has high homology with rat mdr1a/mdr1b, we demonstrated that mdr1a/mdr1b mRNA is present in the endothelial cells of brain capillaries throughout the rat brain (figures 1A and B). Grains were found

mainly in larger vessels close to nuclei. Immunohistochemistry using the C219 antibody demonstrated a more widespread presence of Pgp at both small and larger brain capillaries (figure 1C). A high density of C219 labelled vessels was found in the hypothalamic paraventricular nucleus (Figure 2).

Expression mdr1 mRNA in hippocampus

Surprisingly, we also found mdr1a/mdr1b mRNA in neuronal layers of hippocampal subfields in both rat and mouse brain, either using radioactive labelling (figure 3A) or DIG-labelling (figure 4B). Highest expression levels were found in dentate gyrus (DG) with lower levels in CA3. Expression in CA1 was as low as in cortex. Hybridisation with a sense probe did not result in labelling of hippocampal cells (figure 3C). The staining overlaying granule cells is suggestive of neuronal localisation of the mdr1 mRNA (figure 4B). Within the hippocampus, C219 labelling was relatively weak and mainly localised in the stratum lacunosum moleculare (Figure 4A). The C219 antibody did not visualise Pgp in the dentate gyrus granule cell layer.

Regulation expression mdr1a/b by kainate or dexamethasone

Kainate treatment resulted in a significant effect on mdr1 mRNA expression levels in rat hippocampus as determined by *in situ* hybridisation (ANOVA, $F(3,19)=11.326$, $p<0.001$). At both time points examined, 6 hours and 24 hours after s.c. injection of kainate, mdr1 mRNA expression was strongly reduced in DG, but not in other areas (figures 3A, 3B and 5A), although the reduction in CA3 tended to be significant ($p<0.07$). We did not find apoptotic cells or any other indication for massive cell loss at both time points. There was a significant correlation between severity of the epileptic convulsions and the magnitude of the reduction of expression (t-value= -5.08, $p<0.0005$) (figure 5B). Corticosterone plasma levels were not significantly elevated at both time points.

Treatment with dexamethasone 21-phosphate administered through drinking water at a dose of 10 µg/ml for three weeks did not significantly affect mdr1 mRNA expression levels in any hippocampal subfield (data not shown).

Discussion

The present study demonstrates the localisation of mdr1a/mdr1b mRNA encoding Pgp around brain capillaries, suggesting that Pgp is expressed at the endothelial cells forming the blood-brain barrier. This finding is consistent with previous studies demonstrating expression of Pgp in endothelial cells at the protein level (Thiebaut *et al.*, 1989; Sugawara, 1990; Cordon-Cardo *et al.*, 1990; Beaulieu *et al.*, 1995; Lechardeur *et al.*, 1996). By immunoelectron and confocal microscopy of brain sections the presence of Pgp at endothelial cells lining the BBB appeared to be exclusively present at the luminal membrane (Sugawara *et al.*, 1990; Tsuji *et al.*, 1992; Stewart *et al.*, 1996; Virgintino *et al.*, 2002). Convincing evidence also came from a study using luminal membrane preparations from rat brain microvasculature isolated by density modification (Beaulieu *et al.*, 1997). These preparations were strongly enriched of the brain

endothelial membrane marker, the glucose transporter GLUT1, and of Pgp, whereas the presence of the abluminal membrane marker integrin αv was reduced. The strong enrichment of Pgp in the luminal membrane fractions can only be explained by assuming that the luminal membrane of brain capillaries is the major site of Pgp expression.
Contradictory results from studies of Pardridge *et al*., however, suggest that Pgp is mainly expressed at the astrocyte foot processes (Pardridge *et al.*, 1997; Golden and Pardridge, 1999). They observed that the anti-Pgp antibody MRK16 bound to isolated human brain microvessels with a similar and overlapping punctuated staining pattern as an antibody against the astrocyte marker glial fibrillary acidic protein (GFAP). In contrast, staining for GLUT1 was continuous and showed only minimal overlap. As astrocyte foot processes are tightly associated with the basement membrane of brain capillaries and remain so after isolation of the capillaries, these results are suggestive for astrocyte specific expression (Golden and Pardridge, 2000).
Although Pgp present at astrocytes may affect total brain uptake of Pgp substrates, astrocyte localisation is not likely to affect uptake into neurons. Hence, it would not explain the reduction of neuronal retention of dexamethasone in its neuronal targets like hippocampal and paraventricular neurons due to presence of Pgp (Meijer *et al.*, 1998). Another study examining colocalisation of GFAP and Pgp did not find both proteins colocalised in brain sections of adult rats (Matsuoka *et al.*, 1999). Our data show that mRNA encoding for Pgp is expressed in endothelial cells of adult rat brain suggesting that Pgp is localised at brain endothelial cells. This finding is consistent with a previous study performing *in situ* hybridisation histochemistry on brain sections of mice at embryonic stages (Qin and Sato, 1995), during which expression of Pgp is very low (Matsuoka *et al.*, 1999; Tsai *et al.*, 2002).
Although it might be possible, though unlikely, that mRNA is expressed in astrocyte foot processes closely attached to the endothelial cells, the discontinuous staining pattern of antibodies against Pgp may satisfactorily be explained by local induction of Pgp due to unknown astrocyte-derived factors. It is known that in *in vitro* cultures astrocytes induce various BBB properties including expression of Pgp (Tatsuta *et al.*, 1994; Gaillard *et al.*, 2000). Thus, it appears from this and other studies that under normal, healthy conditions Pgp is only expressed by endothelial cells, although it can not be excluded that under certain pathological conditions astrocytes may express Pgp as well.

A remarkable finding of the present study is the mRNA expression found in hippocampal neurons, particularly in the granule cells of the dentate gyrus. No other studies have reported neuronal expression of Pgp. The differential expression of various hippocampal neuronal cell layers and the fact that the sense probe did not give any signal indicate that labelling was specific and not due to the high cell density in the granule cell layer. It is at present unknown whether mdr1a, mdr1b or both are expressed in these neurons. Recently, using reverse transcriptase PCR mdr1a and mdr1b were both found to be expressed in hippocampus, whereas only mdr1a was found in other brain regions (Kwan *et al.*, 2003).

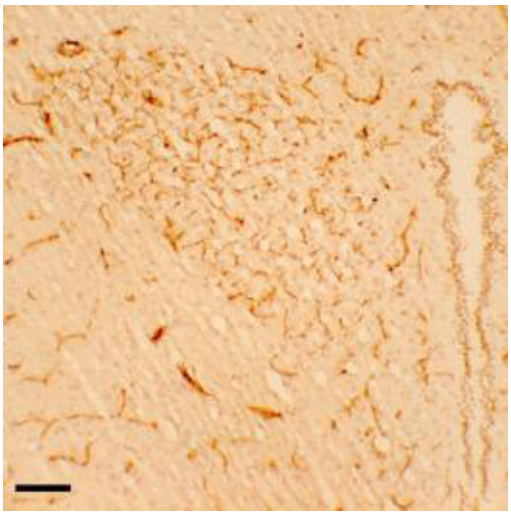


FIGURE 2. Anti-Pgp (C219) immunohistochemical staining of hypothalamic paraventricular nucleus illustrating the high vascularisation of this brain area. Bar indicates 100 μm.

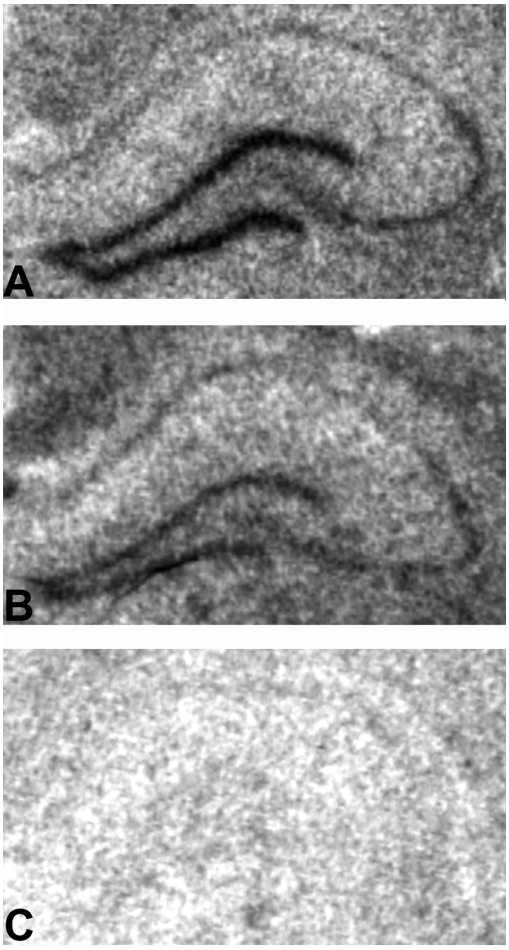
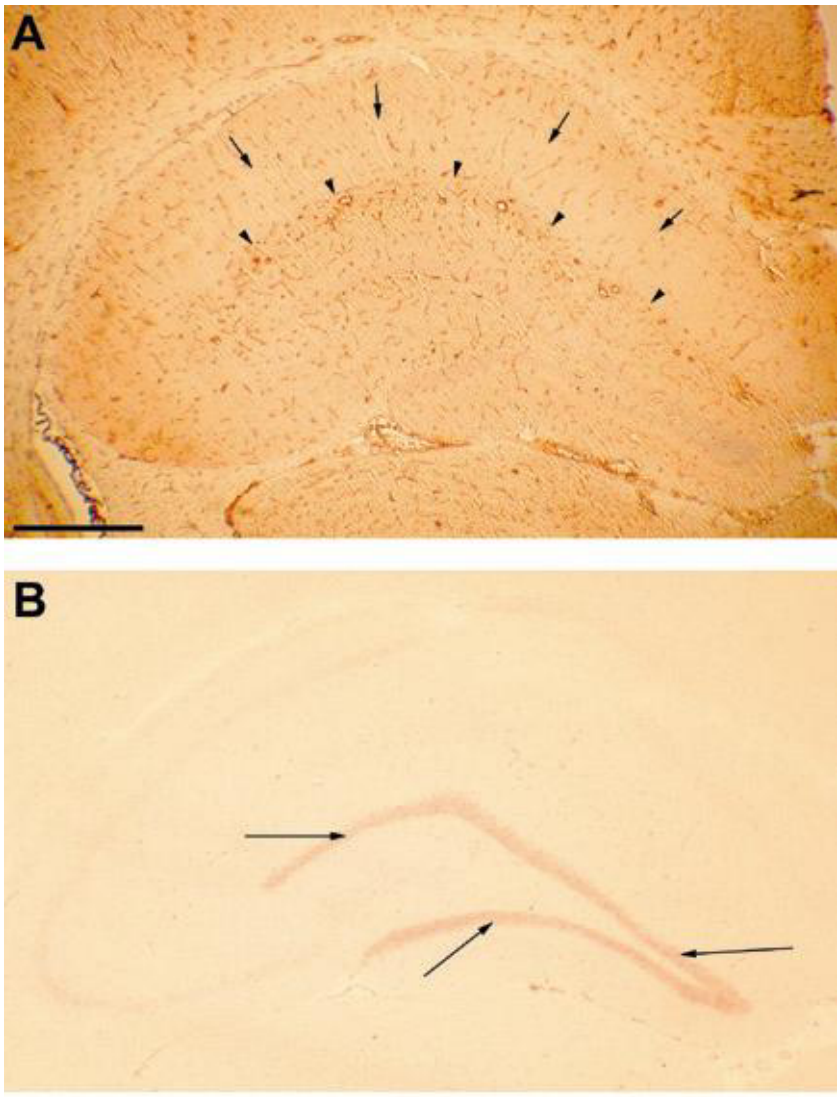


FIGURE 3. Reduced expression of mdr1 mRNA in dentate gyrus after kainic acid. Representative autoradiographs of coronal brain sections showing mdr1 mRNA expression in hippocampus of a rat treated with saline (A) or a rat treated with kainic acid 6 hours before decapitation (B). Sense probe did not result in signal confirming specificity of the mdr1 probe (C). *In situ* hybridisation was performed with a radioactive-labelled probe.



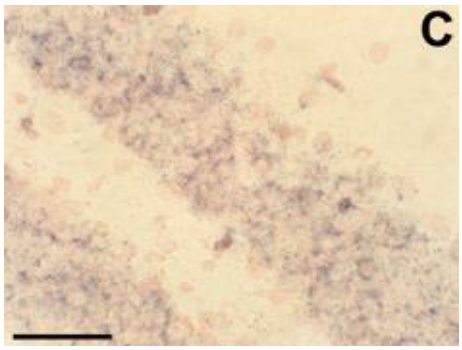


FIGURE 4. Presence of P-glycoprotein and mdr mRNA encoding Pgp in rat hippocampus. (A) Immunohistochemical staining shows highest staining in stratum granulosum moleculare (arrowheads), while staining is relatively weak in CA1 (arrows). (B) DIG-labelled *in situ* hybridisation of mdr1 mRNA in hippocampus demonstrates that mdr1 mRNA is mainly present in the dentate gyrus (arrows). (C) Detail of medial dentate gyrus shows granule cells expressing mdr1 mRNA. Bar in A/B indicates 500 µm, while bar in C indicates 50 µm.

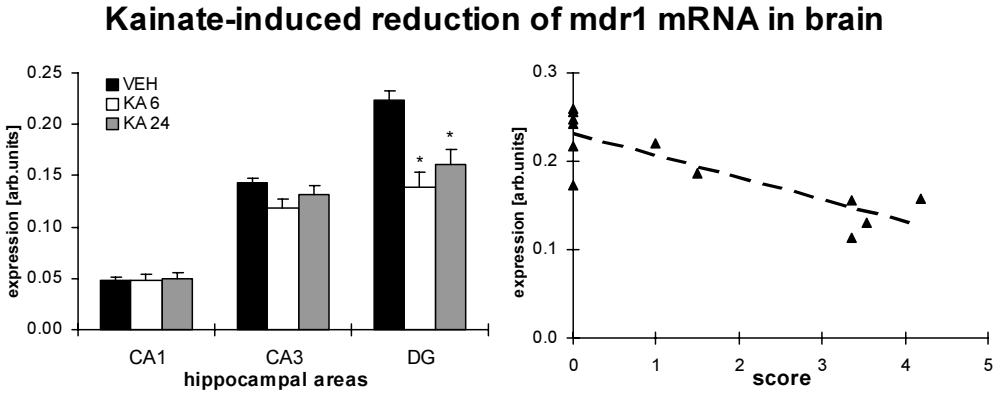


FIGURE 5. Optical density measurements showing reduced expression of mdr1 mRNA in dentate gyrus after kainic acid. Kainate treatment resulted in a reduction of mdr1 mRNA expression specifically in the dentate gyrus (A). The decrease in DG was 35% or 30% at 6 hours respectively 24 hours after injection. Expression tended to be reduced in CA3 6 hours after injection ($p<0.07$). Half of the saline treated animals was killed after 6 hours, the other half was killed after 24 hours.
There was a correlation ($r^2= 0.72$; $p<0.05$) between the average epileptic scores and mdr1 mRNA expression in DG, as illustrated by the data of the 24 hour group (B).

Unfortunately, C219 immunohistochemistry did not reveal protein expression nor in the DG itself nor in its projection area CA3, even though the C219 epitope is encoded by the part of the gene covered by the probe sequence. Protein expression may be low and/or diluted and, thus, below threshold of detection of C219 immunohistochemistry. With respect to this, it is interesting that a recent report using RT-PCR showed presence of mdr1a/1b mRNA in LMCAT fibroblast cells (Webster *et al.*, 2002). These cells are known to express a functional Pgp-like transporter (Kralli and Yamamoto, 1996; Medh *et al.*, 1998; Pariante *et al.*, 2001), that can not be detected at Western blot with the C219 antibody (Kralli and Yamamoto, 1996). Alternatively, our mRNA probe may detect mRNA that encodes a hippocampal protein closely related to Pgp, although blasting the sequence against known sequences in Genbank did not result in any significant hits of other than mdr proteins. Whether granule cells express functional protein, remains therefore unresolved for now.

Dexamethasone did not affect mRNA expression in granule cells, while our methodology did not permit to detect whether dexamethasone has altered the expression at the BBB. The resolution of radioactive *in situ* hybridisation is not high enough to detect the widespread and scattered distribution throughout the brain of mdr mRNA expression at the BBB. Non-radioactive *in situ* hybridisation is not suitable to detect group differences due to nonlinearity of the visualisation technique. Several studies have evaluated the effects of dexamethasone on Pgp expression at protein as well as mRNA expression level in various settings (Zhao *et al.*, 1993; Chieli *et al.*, 1994; Sérée *et al.*, 1998; Salphati and Benet, 1998; Demeule *et al.*, 1999; Aquilante *et al.*, 2000). However, contradictory results were obtained which suggests that

modulation of mdr expression is both gene-specific and cell-type-dependent. With regard to the brain, dexamethasone may possibly induce Pgp (Aquilante *et al.*, 2000), perhaps by induction of mdr1b mRNA expression (Sérée *et al.*, 1998), but these studies are far from conclusive.

Kainate treatment surprisingly decreased the expression levels of mdr1a mRNA in the dentate gyrus. As cell density was not decreased, this downregulation was not due to cell death. Kainate is known to cause apoptosis or necrosis, but this is confined to CA3 respectively CA1 (Pollard *et al.*, 1994; Nishiyama *et al.*, 1996), areas that are not affected in this study. Furthermore, both processes take generally more than 24 hours before their effects can be detected (Sperk *et al.*, 1983).
Using Western blots and immunohistochemistry, two studies described induction of hippocampal Pgp expression at the protein level 24 hour after intracerebroventricular (Zhang *et al.*, 1999) respectively intraperitoneal (Seegers *et al.*, 2002b) injection of kainate. The former study reported that induction of Pgp primarily occurred in cell bodies and processes of astrocytes in the CA1 field, whereas the latter study observed induction of Pgp primarily in microvascular endothelial cells of dentate gyrus. Both studies used the C219 and the mdr1 (Ab-1) antibodies, which both recognise a similar epitope, without proper controls of specificity. As the high incidence of cross-reactivity of various Pgp antibodies including C219 (Thiebaut *et al.*, 1989; Beaulieu *et al.*, 1995; Liu *et al.*, 1997) is a serious caveat of Pgp immunostaining studies, the necessity of the use of more than two distinct antibodies in a single study is highly recommended (Beck *et al.*, 1996). On the other hand, a study using RT-PCR showed induction of mdr mRNA in mouse hippocampus after kainate-induced limbic seizures (Rizzi *et al.*, 2002). Kainate-induced regulation of mdr1 mRNA expression might be cell-type specific, explaining the discrepancy between our study and others. At least, it is clear that regulation of Pgp expression is complex.

In conclusion, using *in situ* hybridisation we demonstrated the presence of mdr1 mRNA encoding Pgp in endothelial cells forming the BBB and surprisingly in the neuronal layer of the dentate gyrus. Although Pgp in the granule cells at the protein level was below the detection limit, the mRNA signal appears to be specific. Kainate treatment of rats resulted in a reduction of mdr1 mRNA expression levels.

Acknowledgements

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Chapter 3

THE MULTIDRUG EFFLUX TRANSPORTER P-GLYCOPROTEIN HAMPERS THE ACCESS OF CORTISOL BUT NOT OF CORTICOSTERONE TO MOUSE AND HUMAN BRAIN

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Abstract

In the present study, we investigated the role of the multidrug resistance P-glycoprotein (Pgp) at the blood-brain barrier in the control of access of cortisol and corticosterone to the mouse and human brain.

^{3}H-cortisol poorly penetrated the brain of adrenalectomised wild type mice, but its uptake was 3.5 fold enhanced after disruption of Pgp expression in mdr1a (-/-) mice. In sharp contrast, treatment with ^{3}H-corticosterone revealed high labelling of brain tissue without difference between both genotypes.

Interestingly, human MDR1 P-glycoprotein also differentially transported cortisol and corticosterone. LLC-PK1 monolayers stably transfected with MDR1 cDNA showed polar transport of ^{3}H-cortisol that was blocked by a specific Pgp blocker, whereas ^{3}H-corticosterone transport did not differ between transfected and host cells.

Determination of the concentration of both steroids in extracts of human post mortem brain tissue using liquid chromatography mass spectrometry revealed that the ratio of corticosterone over cortisol in brain was significantly increased relative to plasma.

In conclusion, the data demonstrate that in both mouse and human brain the penetration of cortisol is less than that of corticosterone, because of the differential Pgp-mediated efflux transport of both hormones. This finding suggests a more prominent role for corticosterone in control of human brain function than hitherto recognised.

Introduction

The naturally occurring glucocorticoid in rodents, corticosterone, readily gains access to the brain and accumulates particularly in cell nuclei of limbic brain areas such as hippocampus, septum and amygdala (McEwen *et al.*, 1968; McMurry and Hastings, 1972; De Kloet *et al.*, 1975; Coutard *et al.*, 1987). In these brain areas corticosterone is retained by mineralocorticoid receptors (MR) that bind corticosterone with a ten fold higher affinity than glucocorticoid receptors (GR) (Reul and De Kloet, 1985). In contrast, the synthetic glucocorticoid dexamethasone, when administered in tracer doses to adrenalectomised rats or mice, is poorly retained in glucocorticoid target areas in brain (De Kloet *et al.*, 1974; De Kloet *et al.*, 1975; Rees *et al.*, 1975; McEwen *et al.*, 1976; Coutard *et al.*, 1978). Uptake and retention in the anterior pituitary is very high, although both brain and pituitary express similar amounts of GR.

These observations raised the possibility that the blood brain barrier (BBB) limits the access of dexamethasone to the brain (De Kloet *et al.*, 1975; Rees *et al.*, 1975; Coutard *et al.*, 1978). Recently, it was indeed demonstrated that the penetration of dexamethasone into brain is hampered because the multidrug resistance 1a (mdr1a) P-glycoprotein (Pgp) excludes this exogenous compound from mouse 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 (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).

Thus, Pgp may explain why moderate amounts of dexamethasone primarily act at the anterior pituitary level to suppress ACTH release (De Kloet *et al.*, 1975). In contrast, in rodents the endogenous glucocorticoid corticosterone primarily acts in the brain on functions underlying the activity of the hypothalamic-pituitary-adrenal (HPA) axis and behavioural adaptation (De Kloet, 1991; Dallman *et al.*, 1992; De Kloet *et al.*, 1998). In many other species cortisol is the principal endogenous glucocorticoid; *e.g.*, in human blood cortisol circulates in 10- to 20-fold higher levels than corticosterone (Underwood and Williams, 1972; West *et al.*, 1973; Nishida *et al.*, 1977; Kage *et al.*, 1982). As a naturally occurring glucocorticoid, cortisol is commonly accepted to exert similar actions in human brain as corticosterone does in rat and mouse brain. However, although it has a high affinity for MR, a tracer dose of cortisol has been reported to be poorly retained in cell nuclei of rat hippocampi (McEwen *et al.*, 1976). This may not be surprising, because rat and mouse lack adrenal 17α-hydroxylase needed for synthesis of cortisol, which therefore makes this steroid exogenous in these species and hence, given that Pgp substrates are predominantly exogenous compounds (Van de Vrie *et al.*, 1998), a potential target for Pgp-mediated export from the brain,

In the present study, we have first tested the hypothesis that mdr1a Pgp at the mouse BBB limits *in vivo* brain penetration of cortisol. For this purpose we have used adrenalectomised mdr1a null and wild type mice injected with a tracer dose of ^{3}H-cortisol or ^{3}H-corticosterone. The latter glucocorticoid freely crosses the BBB (Meijer *et al.*, 1998). In addition, we have investigated whether a species difference exists between the multidrug resistance P-glycoproteins of mouse and man, which would allow free entrance of cortisol into the human brain. To explore this possibility, we have examined the corticosteroid transport capabilities of monolayers of human MDR1 transfected porcine LLC-PK1 cells compared to nontransfected LLC-PK1 cells. In order to examine the *in vivo* effect of MDR1 Pgp we have extracted both corticosteroids from human plasma as well as from post mortem human brain material to simultaneously determine cortisol and corticosterone concentrations using liquid chromatography-mass spectrometry.

Materials and methods

In vivo distribution and autoradiography

The *in vivo* distribution experiments were carried out as described previously (Meijer *et al.*, 1998) with some modifications. Male mdr1a (-/-) and wild-type FVB (for Friend's virus B-type susceptible) mice were bred under SPF conditions at TNO (Leiden, The Netherlands). Male mice at the age of 15-20 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 at a 12/12 hour lighting schedule (lights on at 7, lights out at 19 hr) 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 weighted 27 ± 2.7 gr. (mean ±SD).

Two days after ADX, the animals were subcutaneously injected with tritiated steroids (dissolved in 2% ethanol/0.9% saline) for *in vivo* autoradiography. Wild type (n=4) and mutant mice (n=6) were injected with 13 µCi/10 gr (1,2,6,7)-^{3}H-cortisol (Amersham Pharmacia Biotech, Little Chalfont, UK; specific activity 63 Ci/mmol). As a control for non-specific retention, one mouse of each genotype was pretreated with a 100-fold excess of unlabelled cortisol 30 minutes before treatment. In a separate but similar experiment, mice (n=7-8) were treated with 2.5 µCi/10 gr (2,4,6,7)-^{3}H-corticosterone (Amersham Pharmacia Biotech; specific activity 70 Ci/mmol). 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, Costa Mesa, USA). Liver, testis, intestine and

cerebellum were dissected and frozen on dry ice. The pituitary was dissected and mounted on top of the brain (without cerebellum), which were then frozen together in isopentane precooled on dry ice/ethanol. 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 ß-counter (Packard Instruments, Meriden, USA). Twelve-micrometer coronal sections of brain were cut on a cryostat and thaw-mounted on poly-L-lysine (Sigma Chemical, St Louis, USA) coated microscopic slides. The slides were put in an X-ray exposure holder (Kodak) and apposed to Ultrofilm (Leica, Heerbrugg, Switzerland) for 8 weeks. Optical density of radiolabelled steroid retained in pituitary and different brain areas was quantified after subtraction of film background using an computerised Olympus image analysis system (Paes, The Netherlands) equipped with a Cue CCD camera. From each brain, 3-5 sections were measured by outlining the different brain regions. The means were used for further analysis of group differences.

Transepithelial transport and inhibition studies

In order to examine the interactions of cortisol and corticosterone 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 originally obtained from the American Type Culture Collection (Manassas, USA) were kindly provided by the Netherlands 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; Florea *et al.*, 2001). 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 at the BBB in excluding drugs from the brain.
Cells were cultured at 37°C in the presence of 5% CO_2 in complete medium, which consisted of DMEM (BioWhittaker, Verviers, Belgium) supplied with HEPES (25 mM) and glucose (4.5 g/l) and supplemented with penicillin (100.000 U/l), streptomycin (100 mg/l), L-glutamine (2 mM) and 10% (v/v) foetal calf serum. The LLC-PK1 and LLC-PK1:MDR1 cell lines were subcultured by trypsinisation 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 µM pore size, 12 mm diameter, Transwell^{TM}; Costar, Cambridge, USA) at a density of $120*10^3$ cells/cm^2. The cells were grown for 5-6 days in complete medium with one 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 µM; kindly provided by Eli Lilly, USA) or water was added at the basal side. To measure the transepithelial transport from

the apical to the basal side or from basal to the apical side 8 µl of a 100x stock of tritiated steroid (^{3}H-cortisol, ^{3}H-corticosterone or (1,2(n))-^{3}H-cortisone (Amersham Pharmacia Biotech; specific activity 50 Ci/mmol)) in ethanol was added in triplicate at the apical or basal side respectively, at the start of the experiment (t=0). The starting concentrations for each experiment are mentioned in the legends of the appropriate figures. In the dose-response experiment, different concentrations of unlabelled cortisol were used, supplemented with ^{3}H-cortisol. Over the four hours of study 75 µl aliquots were taken once every hour from both compartments. Eight µl samples of the 100x stock, and samples from the compartment opposite that to which activity was added, were counted in a Tricarb ß-counter after adding 3 ml Emulsifier Safe (Packard Bioscience). Basal to apical and apical to basal transport is presented as percentage of total activity 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 and De Boer, 2000).

Corticosteroid determination in post mortem human brain

Human brain material was collected through the rapid autopsy program of the Netherlands Brain Bank (NBB) (Amsterdam, The Netherlands; Coordinator: Dr. R. Ravid). The NBB abides to all local ethical legislation. All tissue has been obtained with informed consent of the donor or next of kin to perform brain autopsy and the subsequent use of brain tissue for scientific purposes, that is requested in advance together with the permission to use the medical records. Patient tissue was carefully selected; none of the subjects was reported to suffer at the moment of death or to have suffered before from a known neurological or psychiatric disease, or from conditions that might have affected BBB integrity, like transient ischaemic attacks (TIA), (suspected) prolonged arterial blood pressure changes, prolonged fever or the presence of multiple brain infarcts. Moreover, none of the subjects had been treated with synthetic steroids or antidepressants at time of death or at any time during life. From every subject, a standard set of brain areas has been carefully investigated (Ravid *et al.*, 1995) by neuropathologists Prof. Dr. D. Troost (Academic Medical Centre, Amsterdam), Prof. Dr. F.C. Stamand and Dr. W. Kamphorst, (Free University, Amsterdam). The final diagnosis was established by relating this neuropathological examination to the outcome of the clinical diagnosis. Following this careful examination, all present subjects were confirmed to be true controls as the tissue was free of any such changes. Post mortem delay was kept as short as possible and was on average 6:45 hr. Further clinicopathological details are presented in Table 1. All 11 brain tissue samples used in this study were dissected from superior parietal cortex of male control subjects (mean age 65 ± 5.1) and rapidly frozen in liquid nitrogen and then stored at –80 °C until use.
Plasma samples were obtained from 11 male volunteers (mean age 57 ± 6.3).

Samples were prepared for assay by dichloromethane/ethanol extraction. The brain samples (weighing about 350 mg) were homogenised in 2 ml 0.1 M perchloric acid with a Potter-Elvehjem tissue homogeniser (10 times up and down, 1000 rpm). To check for differences in

TABLE 1. Clinicopathological data of the male subjects.

case	NBB #	Autopsy #	age	PMD	pH	BW	cause of death
1	90-090	90/234.3	59	4:25	7.23	1409	Myocardial infarction and cardial decompensation
2	94-125	S94/340	51	6:00	6.50	1518	Progressive liposarcoma and ileus
3	95-007	S95/019	54	9:10	6.89	1335	Bleeding from right A.carotis communicans
4	97-162	S97/387	38	10:45	6.71	1618	Wegener's disease, aluminium intoxication
5	98-006	S98/014	50	8:30	6.65	1436	Cardiac arrest
6	98-127	S98/235	56	5:25	6.55	1522	Cardiac infarction
7	96-085	S96/251	84	9:00	6.20	1367	Heartfailure, uremia
8	97-157	S97/368	69	5:55	6.41	1475	Serious prostate cancer with metastasis
9	98-062	S98/142	85	4:35	6.95	1332	Respiratory insufficiency secondary to a metastasised adenocarcinoma
10	98-157	S98/280	85	5:13	6.23	1394	Cardiac tamponade
11	98-189	S98/326	81	5:20	6.64	1276	Respiratory insufficiency

All tissue was taken from the superior parietal gyrus.
Abbreviations used: NBB#: Netherlands Brain Bank identification number; PMD: post mortem delay (hrs); pH: pH of the cerebrospinal fluid; BW: brain weight (g)

recovery 100 ng of dexamethasone was added to each sample. The homogenates were transferred with a 4 ml wash of dichloromethane (DCM) to screw-capped glass tubes. After adding an extra 4 ml DCM the tubes were shaken on a horizontal reciprocating shaker for 30 minutes and subsequently centrifuged at 1000x g at 4°C for 10 minutes. The DCM layer was transferred to a clean coned tube and rinsed with 1 ml water, centrifuged at 700x g for 10 minutes. Then, the DCM-layer was transferred to a long tube and evaporated to dryness in a SpeedVac. To maximise the amount transferred, the extracts were redissolved in 750 µl ethanol and after transferring to an eppendorf, evaporated again. The final extracts were resuspended in 100 µl 25% methanol and centrifuged at 13000 rpm for 5 minutes. To avoid potential dissimilarities between different extraction methods, the 250 µl plasma samples were extracted in the same way.

Liquid chromatography-mass spectrometry (LC-MS) was the method of choice to measure the levels of cortisol and corticosterone in the supernatants of the extracts as it allowed the simultaneous measurements of both hormones in small samples with dexamethasone as

internal standard. The assays were performed on a Triple Stage Quadrupole mass spectrometer (Finnigan MAT TSQ-700, San Jose, USA) with a custom-made atmospheric pressure chemical ionisation interface. A modification of the method of Van der Hoeven *et al.* (1997) was used. The analysis was performed in negative ionisation mode using selective ion monitoring of $[M+CH_3COO]^-$ of cortisol, corticosterone and dexamethasone, alternately scanning *m/z* 421, 406 and 452. The ion-source temperature and the nebulisation heater were kept at 200°C and 400°C, respectively. The voltages on the corona needle and on the electron-multiplier were set at -3200 and –1800 V, respectively. Each experiment, a new calibration series was made in 25% methanol with eight concentrations ranging from 5-500 ng/ml of both cortisol and corticosterone. Dexamethasone (1µg/ml) was used as an internal standard. An ADS C_{18} column was used to separate the steroids. After injection of 20 µl of the calibration or extraction samples, the column was washed with acetonitrile-water (40/60%, v/v) containing 1 g/l acetic acid, at a flow rate of 0.5 ml/min. The detection limit of this assay was 5 ng/ml. Corticosteroid concentrations were calculated from a standard plot of area under the curve versus concentration. Presented data are corrected for recovery of dexamethasone, which was in the order of 20-40%.

Statistical analysis

Human and 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 and retention of ^{3}H-corticosterone and -cortisol

At 1 hour after administration of ^{3}H-cortisol to ADX mice, the uptake of radioactivity in brain showed a clear difference between mdr1a (-/-) mutant mice and wild type mice (table 2). The

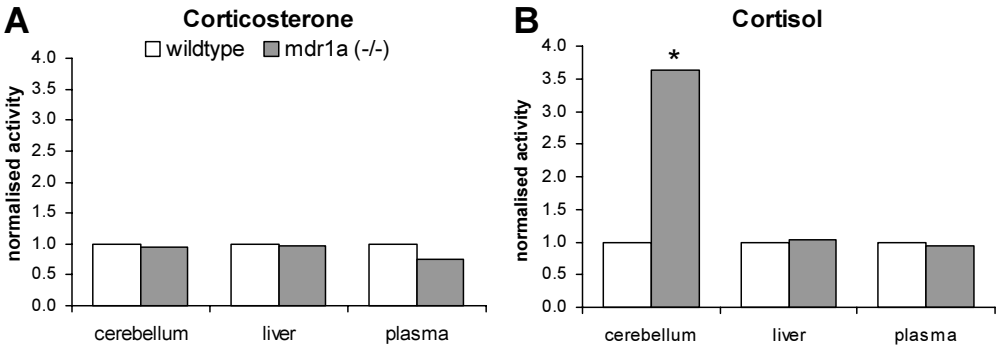

FIGURE 1. Radioactivity of ^{3}H-corticosterone (A) and ^{3}H-cortisol (B) in cerebellum and liver homogenates and plasma of wild type and mdr1a (-/-) mice (n=3-7). Data are presented relative to wild type set at 1.0. * $p< 0.01$, t-test on untransformed data.

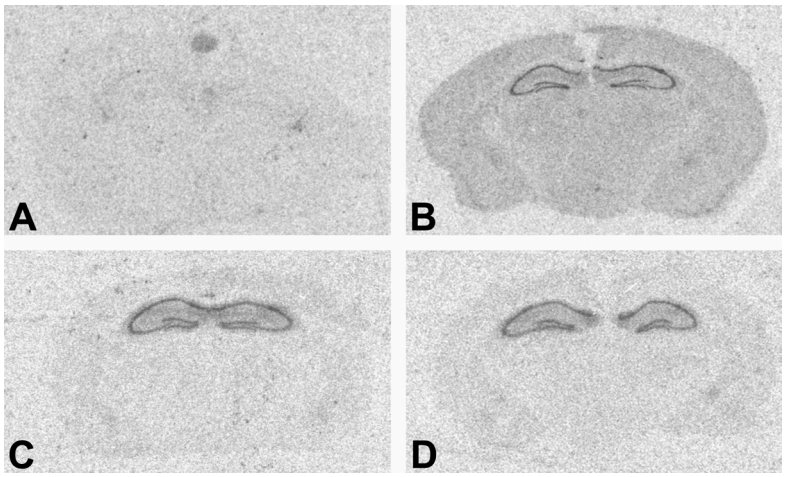

FIGURE 2. Representative autoradiograms of 12-µm coronal sections of the brain of wild type (A,C) and mdr1a (-/-) mice (B,D) at hippocampus level. Autoradiograms show labelling with ^{3}H-cortisol (A,B) or ^{3}H-corticosterone (C,D). Note the pituitary mounted on top of the brain. The *dark spots* in (A) represent transverse sectioning of choroid plexus and adjacent cerebroventricular space.

TABLE 2. Uptake of radioactivity in tissue homogenates and blood 1 hr after administration of ^{3}H-cortisol without or with pretreatment with 100-fold excess unlabelled cortisol.

	wild type	**mdr1a (-/-)**
^{3}H-Cortisol	dose 8 µg/kg	
N	3	5
cerebellum [nCi/mg]	0.093 ± 0.001	0.335 ± 0.034 *
plasma [nCi/µl]	0.441 ± 0.038	0.422 ± 0.056
liver [nCi/mg]	8.390 ± 0.380	8.479 ± 0.657
testis [nCi/mg]	0.224 ± 0.005	0.217 ± 0.027
intestine [nCi/mg]	2.245 ± 1.232	2.399 ± 0.938
brain/blood ratio	0.215 ± 0.020	0.809 ± 0.034 *
Pretreatment with 0.8 mg/kg unlabelled cortisol		
N	1	1
cerebellum [nCi/mg]	0.088	0.321
plasma [nCi/µl]	0.590	0.563
liver [nCi/mg]	6.852	7.200
brain/blood ratio	0.148	0.571

* $p < 0.01$ compared to wild type

amount of cortisol in cerebellum homogenates was 3.5-fold higher in mdr1a knockouts than in wild type mice (figure 1). In contrast, the amount of ^{3}H-corticosterone in cerebellum did not differ between the two genotypes (figure 1 and table 3). For both corticosteroids the presence or absence of the mdr1a gene did not affect their concentration in plasma, liver, testis and intestine (figure 1; tables 2 and 3).

The autoradiograms (figures 2 and 3) extend these results to the regional distribution of the ^{3}H-steroids in the brain. The mdr1a (+/+) animals showed hardly any labelling of brain tissue after administration of ^{3}H-cortisol (figure 2A). Labelling in brain sections was restricted to the cerebral ventricles. The mdr1a (-/-) mutants, however, showed increased labelling of whole brain (figure 2B). In particular, radioactivity was retained in hippocampal cell fields and, to a lesser extent, the amygdala. In contrast, after treatment with ^{3}H-corticosterone the mutant and wild type ADX mice did not differ in their strong labelling of hippocampal neurons or of any other part of the brain (figures 2C and D). In both mutants and wild types, ^{3}H-cortisol labelling of the pituitary, which lies outside the BBB, was not affected by the absence of mdr1a Pgp.

TABLE 3. Uptake of radioactivity in tissue homogenates and blood 1 hr after administration of ^{3}H-corticosterone without or with pretreatment with 100-fold excess unlabelled corticosterone.

	wild type	**mdr1a (-/-)**
^{3}H-Corticosterone	dose 1.5 µg/kg	
N	5	7
cerebellum [nCi/mg]	0.111 ± 0.016	0.102 ± 0.015
plasma [nCi/µl]	0.264 ± 0.026	0.194 ± 0.027
liver [nCi/mg]	1.801 ± 0.050	1.718 ± 0.085
testis [nCi/mg]	0.110 ± 0.021	0.130 ± 0.009
intestine [nCi/mg]	1.227 ± 0.411	1.968 ± 1.000
brain/blood ratio	0.415 ± 0.023	0.546 ± 0.048
Pretreatment with 0.15 mg/kg unlabelled corticosterone		
N	1	1
cerebellum [nCi/mg]	0.075	0.086
plasma [nCi/µl]	0.260	0.242
liver [nCi/mg]	1.641	1.612
brain/blood ratio	0.290	0.355

Pretreatment with excess unlabelled cognate steroid to block specific labelling to receptors resulted in loss of labelling of hippocampal neuronal fields and amygdala, but not of the rest of the brain (data not shown). Moreover, hippocampal optical density showed an inverse correlation with residual levels of endogenous corticosterone, illustrating that the signal represents specific mineralocorticoid receptor bound steroid (data not shown). The cortex and the pituitary lack this correlation, but cortex labelling showed a clear effect of disruption of the mdr1a gene (figure 3). In accordance, uptake of radioactivity in cerebellum was hardly affected by pretreatment (tables 2 and 3). These data evidently demonstrate that the presence of mdr1a Pgp in the BBB hampers the access of cortisol to the mouse brain, but does not have any effect on the access of the endogenous glucocorticoid corticosterone.

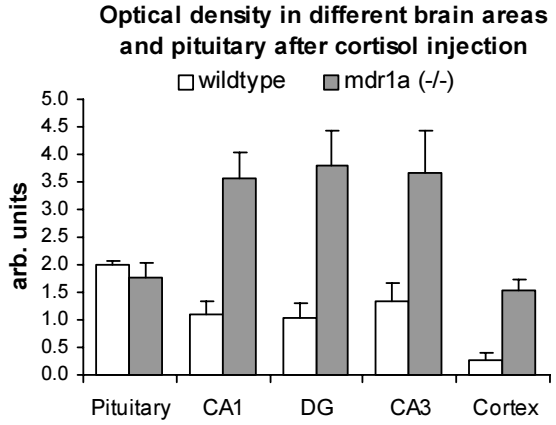

FIGURE 3. Quantification of the autoradiograms of ^{3}H-cortisol in wild type (n=3) and mdr1a (-/-) (n=5) mice. Presented is the mean ± SEM of both genotypes for pituitary, cortex and hippocampal areas. There are no differences between wild type and mutants for pituitary. Differences in cortex and hippocampal areas CA1 and DG are significant at $p < 0.05$. The values for CA3 are not significantly different ($p = 0.07$). Three sections per animal were measured.

Transepithelial transport of steroids in LLC-PK1- and MDR1-monolayers

Corticosterone transport in the monolayers of LLC-PK1 cells stably transfected with the human MDR1 gene was not different from transport in monolayers of its control cell line (figure 4B), although some polar transport was observed in both cell lines in all our experiments (figures 4B and 5B). Nonetheless, this demonstrates the absence of human MDR1 Pgp mediated transport of corticosterone. In contrast, cortisol was transported in a polarised fashion in the MDR1 transfected monolayers, but not in the host cells (figure 4A). Polarised transport in MDR1 monolayers of cortisol was abolished in presence of LY335979, a potent and selective Pgp blocker (Starling *et al.*, 1997; Dantzig *et al.*, 1999), resulting in similar fractions transported as in monolayers of untransfected cells (figure 5A). This confirms that cortisol transport is mediated by human P-glycoprotein. LY335979 did not change the fraction of corticosterone translocated through the membrane (figure 5B).

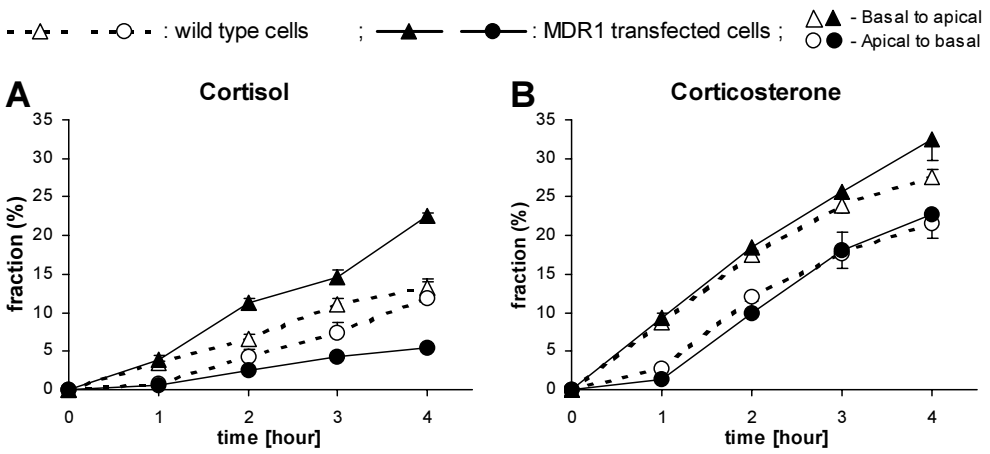

FIGURE 4. Activity of ^{3}H-cortisol (A) and ^{3}H-corticosterone (B) present in medium at different time points after adding ^{3}H-steroid to the opposite compartment at t=0. Transepithelial transport from basal to apical (Δ,▲) and from apical to basal (○,●) compartment was measured in wild type LLC-PK1 (broken line) or MDR1 transfected LLC-PK1 (solid line) monolayers. Presented is the fraction of the dose of radioactivity, which is 9 nM for both steroids, added to the respective compartment. Each point represents the mean of three monolayers ± SEM. Repeated measures ANOVA showed a significant interaction of time*celltype*transport for cortisol ($p< 0.0005$), but not for corticosterone.

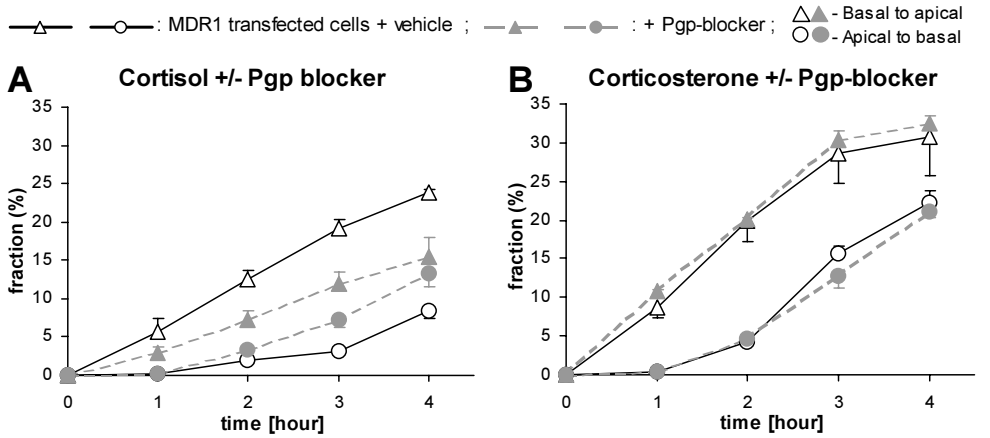

FIGURE 5. Activity of ^{3}H-cortisol (A) and ^{3}H-corticosterone (B) present in medium at different time points after adding ^{3}H-steroid to the opposite compartment at t=0 and 1 μM LY335979 (broken line) or water (solid line) one hour before. Transepithelial transport from basal to apical (Δ,▲) and from apical to basal (○,●) compartment was measured in MDR1 transfected LLC-PK1 monolayers. Presented is the fraction of the dose of radioactivity, which is 8 nM for cortisol and 28 nM for corticosterone, added to the respective compartment. Each point represents the mean of three monolayers ± SEM. Repeated measures ANOVA showed a significant interaction of time*cell type*transport for cortisol ($p< 0.0005$), but not for corticosterone.

We also examined transepithelial transport of different concentrations of cortisol, ranging from 5 to 625 nM, but did not demonstrate any saturation at higher dose. At all concentrations tested, about 3.5 times more ^{3}H-cortisol had been transported from basal to apical sides than from apical to basal sides after four hours (data not shown). Interestingly, our data show that human MDR1 Pgp is also able to transport cortisol, while corticosterone passage remains unchanged.

A potential limitation of our assay is the use of radiolabelled glucocorticoids and, consequently, the possibility that the transport of metabolites has been measured rather than the unmetabolised compounds. Because of the presence of 11ß-HSD type 2 in LLC-PK1 cells (Leckie *et al.*, 1995), the main probable metabolites are the inactive forms of the glucocorticoids, *i.e.* cortisone in case of cortisol and 11-dehydrocorticosterone in case of corticosterone. Therefore, we first tested cortisone transport in our monolayers. Cortisone showed polarised transport in LLC-PK1:MDR1 monolayers, which could be blocked by LY335979 (figure 6). Thus, transport of metabolites may potentially have interfered. However, cotreatment with the 11ß-HSD inhibitor carbenoxolone (10^{-6} and 10^{-5} M) did not

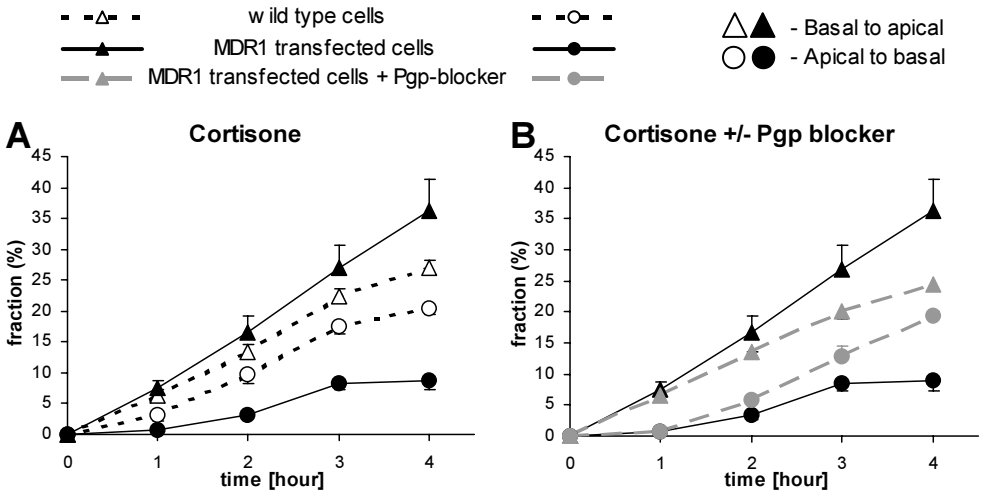

FIGURE 6A. Activity of ^{3}H-cortisone present in medium at different time points after adding ^{3}H-cortisone to the opposite compartment at t=0. Transepithelial transport from basal to apical (Δ,▲) and from apical to basal (○,●) was measured in wild type LLC-PK1 (broken line) or MDR1 transfected LLC-PK1 (solid line) monolayers.

B. Transepithelial transport of ^{3}H-cortisone was measured in MDR1 transfected LLC-PK1 monolayers after adding 1 µM LY335979 (broken line) or water (solid line) one hour before start of the experiment.

Presented is the fraction of the dose of radioactivity, which is 9 nM, added to the respective compartment. Each point represents the mean of three monolayers ± SEM. Repeated measures ANOVA showed a significant interaction of time*celltype*transport for cortisone in both (A) and (B) ($p < 0.0005$).

change the transport capabilities of LLC-PK1 or LLC-PK1:MDR cells in any way (data not shown) excluding the possibility that we have measured the metabolites.
These monolayer data suggest that P-glycoprotein in human BBB like in mice limits the access of cortisol to the brain, but does not affect the penetration of corticosterone. Accordingly, we expected more corticosterone relative to cortisol in human brain than in human plasma.

TABLE 4. Corticosterone and cortisol levels and the corticosterone/cortisol ratio in extracts of human brain tissue and plasma

Brain	NBB #	corticosterone [ng/mg]	cortisol [ng/mg]	ratio
case 1	90-090	81.28	291.48	0.28
case 2	94-125	32.20	201.62	0.16
case 3	95-007	35.07	121.88	0.29
case 4	97-162	11.23	33.08	0.34
case 5	98-006	14.18	46.49	0.31
case 6	98-127	55.83	176.90	0.32
case 7	96-085	83.41	507.46	0.16
case 8	97-157	122.76	442.02	0.28
case 9	98-062	84.17	265.42	0.32
case 10	98-157	280.57	443.14	0.63
case 11	98-189	24.10	70.25	0.34
	AVG	**74.98**	**236.34**	**0.31**
	SEM	23.14	50.92	0.04
Plasma	**age**	**[ng/ml]**	**[ng/ml]**	
case 1	27	5.77	306.49	0.02
case 2	20	8.14	286.48	0.03
case 3	32	4.87	224.65	0.02
case 4	62	12.57	78.50	0.16
case 5	83	3.89	57.70	0.07
case 6	70	11.42	126.29	0.09
case 7	76	4.18	66.68	0.06
case 8	69	4.38	84.18	0.05
case 9	63	2.21	71.03	0.03
case 10	68	4.11	54.22	0.08
case 11	56	0.95	39.22	0.02
	AVG	**5.68**	**126.86**	**0.06**
	SEM	1.09	29.53	0.01

Corticosteroid levels in human brain

In order to test whether MDR1 Pgp in human BBB increases the ratio of corticosterone over cortisol in brain, we measured the concentrations of both glucocorticoids in human brain samples (table 4). Thus, we were able to determine the brain corticosterone/cortisol ratio of 11 subjects, which was 0.31 ± 0.04 (mean ± SEM) (figure 7). In contrast, we measured a corticosterone/cortisol ratio in plasma samples of age-matched males of 0.06 ± 0.01 (figure 7). Statistical analysis showed that the difference between the brain and plasma ratios was significant ($t(1,20) = 6.444$, $p < 0.01$). Thus, corticosterone appears to penetrate more easily than cortisol in the human brain, resulting in a higher ratio of corticosterone over cortisol present in brain as compared to plasma.

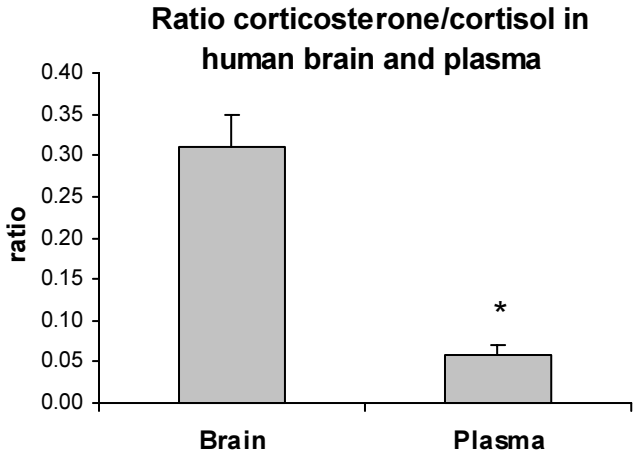

FIGURE 7. Ratio of corticosterone over cortisol in extracts of human brain and plasma. The ratio is significantly higher in brain compared to plasma indicating preferential uptake into brain of corticosterone compared to cortisol. Data are presented as mean ± SEM. * $p < 0.01$.

Discussion

The present study indicates that Pgp at the level of the BBB is of importance with respect to the degree of brain exposure to the naturally occurring glucocorticoids cortisol and corticosterone. Our data show that the mdr1a Pgp present at the murine BBB hampers the penetration of cortisol into the mouse brain, whereas corticosterone uptake is not affected. Interestingly, our results with monolayers of human MDR1 transfected LLC-PK1 cells suggest that Pgp exports cortisol and not corticosterone from human brain as well. This is, at least, consistent with the accumulation of corticosterone over cortisol in the samples of human post mortem brain relative to plasma, as determined in our LC-MS experiments.

Previous studies had already established *in vivo* the low cell nuclear retention of cortisol in rat brain (McEwen *et al.*, 1976). In rats, the first-pass uptake in brain after a carotid injection of ^{3}H-cortisol appeared to be negligible in contrast to uptake of ^{3}H-corticosterone, while uptake of the labelled steroids in liver after portal injection was not different (Pardridge and Mietus, 1979). A tracer dose of corticosterone is known to label only the high affinity hippocampal MR leaving the lower affinity GR undetectable. The uptake of this steroid is not affected by disruption of the mdr1a gene (Meijer *et al.*, 1998, this study). Cortisol also binds with a rather high affinity to MR (De Kloet, 1991). In fact, our autoradiography study revealed a pattern of cortisol labelling in the mdr1a (-/-) mouse hippocampus reminiscent of that of corticosterone. In the present study, the effect of mdr1a ablation on specific binding of cortisol to the low capacity MR in the hippocampus is less pronounced than its effect on whole brain uptake in *e.g.* the cortex. This is probably due to the lower affinity of cortisol for MR in rodent brain (De Kloet *et al.*, 1984a; Myles and Funder, 1994). Anyhow, our data convincingly demonstrate that the mouse mdr1a Pgp hampers the brain uptake of cortisol but not of corticosterone.

As a model for Pgp function in human BBB, we have used monolayers of pig kidney epithelial LLC-PK1 cells stably transfected with the MDR1 gene to measure transport of steroids by human Pgp. Such monolayers of epithelial cells are a suitable model for Pgp-mediated transport at the BBB, given the apical localisation of Pgp forming a barrier between the two compartments. A confounding factor is that LLC-PK1 host cells contain low levels of porcine Pgp (Horio *et al.*, 1990; Ueda *et al.*, 1992; Decorti *et al.*, 1998). Thus, in theory, porcine Pgp might be responsible for polar transport of corticosterone seen in both cell lines, although any effect after application of the potent and selective Pgp-blocker was absent. LLC-PK1 cells also have endogenous 11ß-hydroxysteroid dehydrogenase type 2 activity, able to inactivate cortisol and corticosterone (Leckie *et al.*, 1995). Because we used radiolabelled glucocorticoids it is possible that we have actually measured transport of labelled metabolites rather than the parent hormone. However, the transport of ^{3}H-cortisol and ^{3}H-corticosterone did not change in the presence of the 11ß-HSD inhibitor carbenoxolone, indicating that 11ß-HSD activity did not interfere. We conclude therefore that our monolayers are a suitable model of Pgp function at human BBB.

Our data corroborate several studies on transport of cortisol and corticosterone by Pgp (Yang *et al.*, 1989; Wolf and Horwitz, 1992; Ueda *et al.*, 1992; Van Kalken *et al.*, 1993; Gruol and Bourgeois, 1994; Orlowski *et al.*, 1996; Barnes *et al.*, 1996). Differential transport of these two steroids by murine Pgp has been observed in several drug-resistant cell lines, using steroid induced apoptosis (Bourgeois *et al.*, 1993; Gruol and Bourgeois, 1994) or steroid accumulation (Wolf and Horwitz, 1992; Barnes *et al.*, 1996) as read-outs. The murine mdr1b Pgp has some capacity to transport corticosterone (Wolf and Horwitz, 1992), but this second murine multidrug resistance Pgp is not expressed at the BBB. In view of the lack of

corticosterone transport that we have observed in cells stably transfected with the human MDR1 gene, the corticosteroid transport capabilities of the human MDR1 Pgp apparently correspond to that of murine mdr1a rather than to that of mdr1b Pgp. Using comparable monolayers to those in this study, Ueda *et al.* (1992) have already demonstrated that cortisol is transported by the human MDR1 Pgp, but corticosterone was not included in their assay. In human colon carcinoma cells the amount of accumulated ^{3}H-cortisol is lower than of corticosterone (Barnes *et al.*, 1996), while both steroids equally increase accumulation of the Pgp substrate ^{3}H-vinblastine – exemplifying a difference between actual transport by and binding of steroids to the pump.

The difference in interaction of Pgp with cortisol and corticosterone is remarkable considering their large similarity in molecular structure. Pgp is an efflux transporter with a surprisingly broad substrate spectrum (Schinkel *et al.*, 1994), but corticosterone only differs from cortisol in the lack of the 17-hydroxyl group. However, a detailed study of Bourgeois *et al.* (1993) provided indications that both the 17-hydroxyl and the 11-hydroxyl group determine the ability of steroids to be transported by Pgp. Pgp transports steroids having both these hydroxyl-groups while steroids lacking one of these groups are probably minimally if at all transported. A caveat is that these indications are based on the extent of glucocorticoid resistance, which also depends on GR affinity. It is difficult to assess the influence of the 11-hydroxyl group because steroids lacking this group do also have a low affinity for GR. Therefore, cortisone could not be identified as a substrate of Pgp in the previous study; using LLC-PK1:MDR1 monolayers, however, we demonstrated that cortisone is also transported by Pgp.

Our study with the mdr1a null mice is the first to directly show the involvement of Pgp in excluding a naturally occurring glucocorticoid from the brain. Previous studies have demonstrated that access of the synthetic glucocorticoid dexamethasone to the brain was also enhanced in the mdr1a (-/-) mouse (Schinkel *et al.*, 1995; Meijer *et al.*, 1998). *In vitro* studies have confirmed that dexamethasone is a Pgp substrate (Ueda *et al.*, 1992; Bourgeois *et al.*, 1993; Gruol *et al.*, 1999). In fact, in our stably MDR1 transfected LLC-PK1 monolayers dexamethasone behaved very similar to cortisol (Karssen *et al.*, 2003). Thus, human MDR1 Pgp, like mouse mdr1a Pgp, transports both cortisol and dexamethasone, but not corticosterone.

Our *in vitro* results using monolayers of stably MDR1 transfected LLC-PK1 cells show that the endogenous presence in a species of a naturally occurring glucocorticoid is not a prerequisite to exclude transport by Pgp. We have clearly demonstrated that human MDR1 Pgp is able to discriminate between cortisol and corticosterone. Both glucocorticoids are present in human plasma, although cortisol circulates in about 10 to 20 times higher levels than corticosterone (Underwood and Williams, 1972; West *et al.*, 1973; Nishida *et al.*, 1977;

Kage *et al.*, 1982; Seckl *et al.*, 1990). The data strongly suggest that corticosterone rather than cortisol can freely gain access to the human brain.

An *in vivo* cell nuclear retention study in ADX rhesus monkeys, which have cortisol as their main glucocorticoid, showed a similar regional pattern for both corticosteroids, but the amount of cortisol radioactivity was lower than that of corticosterone (Gerlach *et al.*, 1976). This observation substantiates that even in an animal that normally produces cortisol, this glucocorticoid penetrates less efficiently into the brain than corticosterone.
Alternatively, BBB passage of cortisone and subsequent conversion of cortisone to cortisol by 11ß-hydroxysteroid dehydrogenase type 1 (11ß-HSD1) present in brain (Seckl, 1997), might regenerate cortisol in brain. The fact that MDR1 Pgp also transports cortisone, argues against the possibility that cortisol would be able to circumvent Pgp in the BBB through this route, as less cortisone would be available for 11ß-HSD1 conversion in brain as well. Therefore, the limited access of cortisol and cortisone is likely to result in overall lower brain levels of glucocorticoids.

Since corticosterone is not transported by Pgp, reduction of cortisol brain levels would lead to an increase of corticosterone levels relative to cortisol levels in human brain when compared to plasma. Indeed, we demonstrated a shift in the corticosterone/cortisol ratio in favour of corticosterone in human autopsy brain samples as compared to plasma samples. These results support data reported by Brooksbank *et al.* (1973), who also demonstrated that corticosterone is accumulated in brain to a substantially greater extent than cortisol. They found a ratio of corticosterone to cortisol of about 0.4. Earlier, Fazekas & Fazekas (1967) also determined corticosteroid levels in human brain using paper chromatography and similarly reported high levels of corticosterone relative to cortisol.

The privileged uptake of corticosterone in brain is also expected to promote its receptor occupancy relative to cortisol. There are indications that corticosterone might have a higher affinity for the MR than cortisol. At least this is the case for the rat MR (De Kloet *et al.*, 1984a; Myles and Funder, 1994), but data presented by Arriza *et al.* (1987) also suggests that corticosterone is the more potent competitor at human MR. Furthermore, transactivation of human MR in response to cortisol and corticosterone indicates that corticosterone is more effective than cortisol (Lombes *et al.*, 1994). Thus, besides the hampered uptake in human brain, cortisol might also less effectively mediate the human brain MR response. Should it indeed be confirmed that levels of GR are relatively low in the human hippocampus, as was recently claimed for the rhesus monkey (Sanchez *et al.*, 2000), glucocorticoid mediated effects on hippocampal functioning might then mainly reflect corticosterone acting through MR rather than cortisol. At least, our data suggest that the human glucocorticoid feedback system might be more complex than the rodent system in view of the potentially different roles for cortisol and corticosterone.

The influence of cortisol on brain functioning and its role as main corticosteroid in glucocorticoid feedback to the human brain is commonly accepted. However, in contrast to rodents where corticosterone readily enters the brain, the main glucocorticoid in human appears to be partially excluded from the brain. It would be interesting to know how much either corticosterone and cortisol contributes to stabilisation of neuronal excitability (Joëls and De Kloet, 1994), maintenance of neuronal integrity (McEwen *et al.*, 1993), suppression of HPA activity (Dallman *et al.*, 1992) and facilitation of behavioural adaptation (Oitzl *et al.*, 1997). The preferential uptake of corticosterone in human brain may further be used as a lead towards the development of novel selective steroids for treatment of stress-related brain disorders.

In conclusion, we have demonstrated the involvement of Pgp in hampering the access of the naturally occurring glucocorticoid cortisol rather than corticosterone to both mouse and human brain. The data, therefore, suggest that corticosterone may play a more prominent role in the modulation of human brain function than hitherto recognised.

Acknowledgements

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Chapter 4

THE ROLE OF THE EFFLUX TRANSPORTER P-GLYCOPROTEIN IN BRAIN PENETRATION OF PREDNISOLONE

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Abstract

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

In vivo, a tracer dose of ^{3}H-prednisolone poorly penetrated the brain of adrenalectomised wild type mice. In contrast, the uptake was more than three fold enhanced in 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. This polar transport of ^{3}H- prednisolone was blocked by a specific Pgp blocker. Human Pgp does not transport all steroids, as cortexolone was not at all transported and aldosterone was only weakly transported.

The ability of Pgp to export the synthetic glucocorticoid prednisolone suggests that uptake of prednisolone in 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.

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, where they play important roles in mediating glucocorticoid actions on brain function. Treatment with synthetic glucocorticoids, therefore, has major effects on cognitive function (Belanoff *et al.*, 2001), whereas blockade of GR action seems to be a promising anti-depression strategy (Belanoff *et al.*, 2002). 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 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 a substrate of this efflux transporter as well 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 each steroid. 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 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 brain in two ways. First, we tested whether mdr1a Pgp at the mouse BBB limits *in vivo* brain penetration and retention of prednisolone. For this purpose we have injected adrenalectomised mdr1a (-/-) and wild type mice with ^{3}H-prednisolone with or without pretreatment with excess of unlabelled prednisolone. Secondly, we investigated *in vitro* whether the human homologue, MDR1 Pgp, is also able to transport prednisolone.

Therefore, we have measured polar transport of prednisolone in monolayers of pig kidney epithelial cells stably transfected with human MDR1 cDNA in comparison 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 SPF 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 at a 12/12 hour 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 gr. (mean ±SD). 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 µCi/10 gr (2,4,6,7)-^{3}H-prednisolone (Amersham Pharmacia Biotech, UK, specific activity (S.A.) 48 Ci/mmol). For *in vivo* autoradiography mice (n=2) were treated with 13.5 µCi/10 gr ^{3}H-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, Costa Mesa, 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 ß-counter (Packard Instruments, Meriden, U.S.A.). Twelve-micrometer coronal sections of brain were cut on a cryostat and thaw-mounted on poly-L-lysine (Sigma Chemical Co., St Louis, USA) coated microscopic slides. The slides were put in an X-ray exposure holder (Amersham Pharmacia Biotech) and apposed to Ultrofilm (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 partial 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 originally obtained from the American Type Culture Collection (Manassas, 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; Florea *et al.*, 2001). 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 at 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, 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 trypsinisation 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 µM pore size, 12 mm diameter, Transwell^{TM}; Costar, Cambridge, USA) 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 µM in water; kindly provided by Eli Lilly, 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 µl of a 100x stock of tritiated steroid in ethanol was 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, S.A. 64 Ci/mmol), (1,2(n))-^{3}H-deoxycortisol (=cortexolone; NEN Life Science Products, Boston, USA, S.A. 57 Ci/mmol) and, as a positive control, (1,2,4,6,7)-^{3}H-dexamethasone (Amersham Pharmacia Biotech, S.A. 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 µM unlabelled prednisolone. The starting concentrations for each experiment are mentioned in the legends of the corresponding figures. Over the four hours of study 75 µl aliquots were taken once every hour from both compartments. Eight µl samples of the 100x stock, and samples from the compartment opposite the one to which activity was added, were counted in a Tricarb ß-counter after adding 3 ml Emulsifier Safe (Packard). Basal to apical and apical to basal transport is presented as 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 and 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 $p < 0.05$.

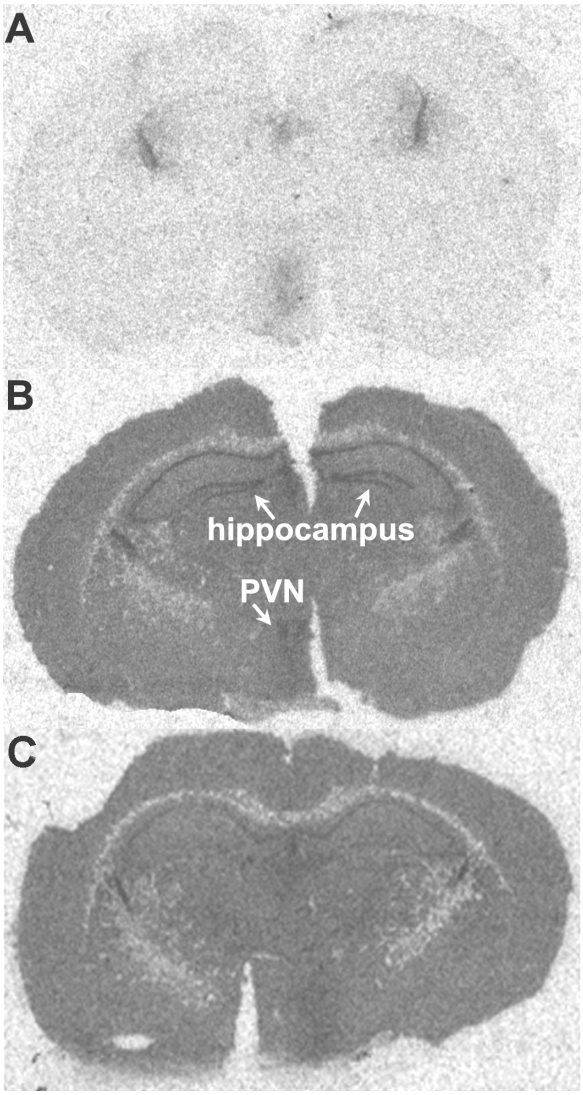

FIGURE. 1. Representative auto-radiograms of 12-µm coronal sections of the brain demonstrate the large difference in uptake of ^{3}H-prednisolone in wild type (A) and mdr1a (-/-) mouse (B) brains one hour after administration of 13.5 µCi/10 gr. body weight. The dark spots in (A) represent transverse sectioning of the cerebroventricular space and adjacent ventricular walls. Pretreatment with unlabelled prednisolone abolishes specific hippocampal labelling in mdr1a (-/-) mouse brain but does not affect the labelling of the rest of the brain (C).

Results

Difference in ^{3}H-prednisolone brain uptake 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 µg/kg radiolabelled prednisolone the amount of radioactivity in cerebellum homogenates was 3.2-fold higher in mutants than in wild types (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 both genotypes (table 1). Comparable results were obtained after administration of 10 µg/kg ^{3}H-prednisolone (table 2). Although absolute levels in all tissues and blood were obviously higher after this higher dose, the brain-to-blood ratios were not significantly different from the ratios obtained after administration of the lower dose. Remarkably, pretreatment with unlabelled prednisolone does not lead to any change in uptake of radioactivity in cerebellum (tables 1 and 2).

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 (figure 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 to the amount taken up by the

TABLE 1. Uptake of radioactivity in tissue homogenates and blood 1 hr after administration of 2.5 µg/kg ^{3}H-prednisolone without or with pretreatment with 100-fold excess unlabelled prednisolone

	wild type	mdr1a (-/-)
Prednisolone	dose 2.5 µg/kg	
N	7	5
cerebellum [nCi/mg]	0.010 ± 0.001	0.030 ± 0.001 *
plasma [nCi/µl]	0.046 ± 0.004	0.044 ± 0.001
liver [nCi/mg]	1.500 ± 0.086	1.631 ± 0.160
testis [nCi/mg]	0.031 ± 0.001	0.034 ± 0.004
intestine [nCi/mg]	0.779 ± 0.198	0.937 ± 0.305
brain/blood ratio	0.226 ± 0.027	0.708 ± 0.023 *
Pretreatment with 0.25 mg/kg unlabelled prednisolone		
N	2	1
cerebellum [nCi/mg]	0.009 ± 0.001	0.031
plasma [nCi/µl]	0.040 ± 0.003	0.038
liver [nCi/mg]	1.951 ± 0.533	2.191
brain/blood ratio	0.222 ± 0.002	0.796

* $p < 0.01$ compared to wild type

TABLE 2. Uptake of radioactivity in tissue homogenates and blood 1 hr after administration of 10 µg/kg ^{3}H-prednisolone without (-) or with (+) pretreatment with 100-fold excess unlabelled prednisolone

	wild type	mdr1a (-/-)
Pretreatment 1mg/kg	- / +	- / +
Prednisolone	dose 10 µg/kg	
N	1 / 1	1 / 1
cerebellum [nCi/mg]	0.040 / 0.061	0.246 / 0.204
plasma [nCi/µl]	0.212 / 0.282	0.371 / 0.303
liver [nCi/mg]	6.277 / 8.647	11.22 / 7.657
brain/blood ratio	0.189 / 0.216	0.664 / 0.673

brain of mdr1a (-/-) mice. These mutant mice showed increased labelling of whole brain (figure 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 (figure 1C). Remarkably, this pretreatment does not affect the labelling of the rest of the brain as was also shown in the cerebellum homogenates. Thus, disruption of the mdr1a gene leads to enhanced uptake of prednisolone into the brain. High-dose pretreatment did not abolish this effect. 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.

Transepithelial transport of prednisolone in LLC-PK1 and MDR1 monolayers

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 human MDR1 cDNA. For comparison, we also examined prednisolone transport in monolayers of untransfected cells. Unlabelled prednisolone (1µM) 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 (figure 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 presence of LY335979, a potent and selective Pgp blocker (Starling *et al.*, 1997; Dantzig *et al.*, 1999), resulting in similar fractions transported as in monolayers of untransfected cells (figure 2B). This confirms that prednisolone transport is largely mediated by human P-glycoprotein.

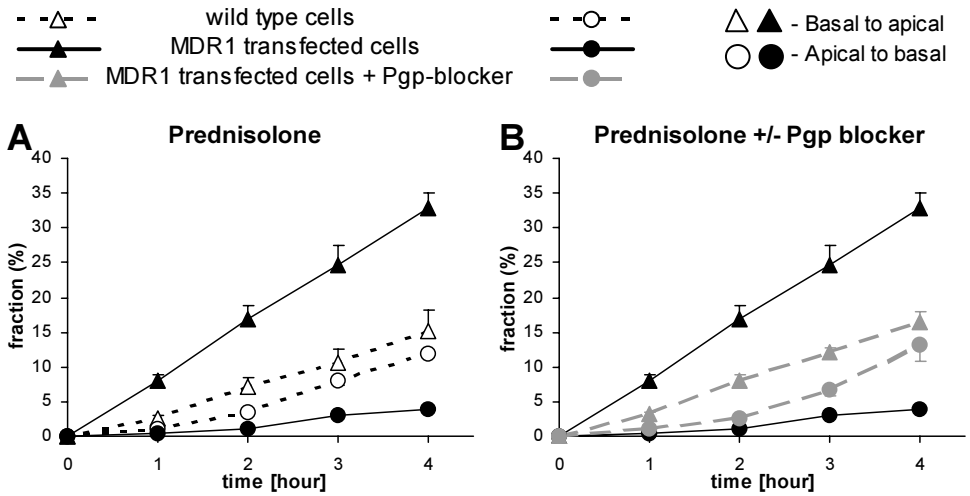

FIGURE 2. (A) Activity of ^{3}H-prednisolone present in medium at different time points after adding 1 µM prednisolone supplemented with ^{3}H-prednisolone to the opposite compartment at t=0. Transepithelial transport from basal to apical (Δ,▲) and from apical to basal (○,●) was measured in wild type 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 µM LY335979 (broken line) or vehicle (water, solid line) one hour before start of the experiment.
Presented is the fraction of the dose of radioactivity, which is 7 nM, added to the respective compartment. Each point represents the mean of three monolayers ± SEM. Repeated measures ANOVA showed a significant interaction of time*cell type*transport for prednisolone in both (A) and (B) ($p < 0.0001$).

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).

Transepithelial transport of naturally occurring steroids

We further examined Pgp-mediated transport of corticosteroids that are naturally occurring in humans. Previously we published the marked difference between Pgp-mediated cortisol and corticosterone transport (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 (figure 3A), indicating the absence of human MDR1 Pgp mediated transport of cortexolone. Although polarised transport was observed in both cell lines, this is likely caused by other renal transporters. Administration of LY335979 did not change the fraction of cortexolone translocated through the membrane (figure 3B), confirming the lack of Pgp contribution to the transport of cortexolone.

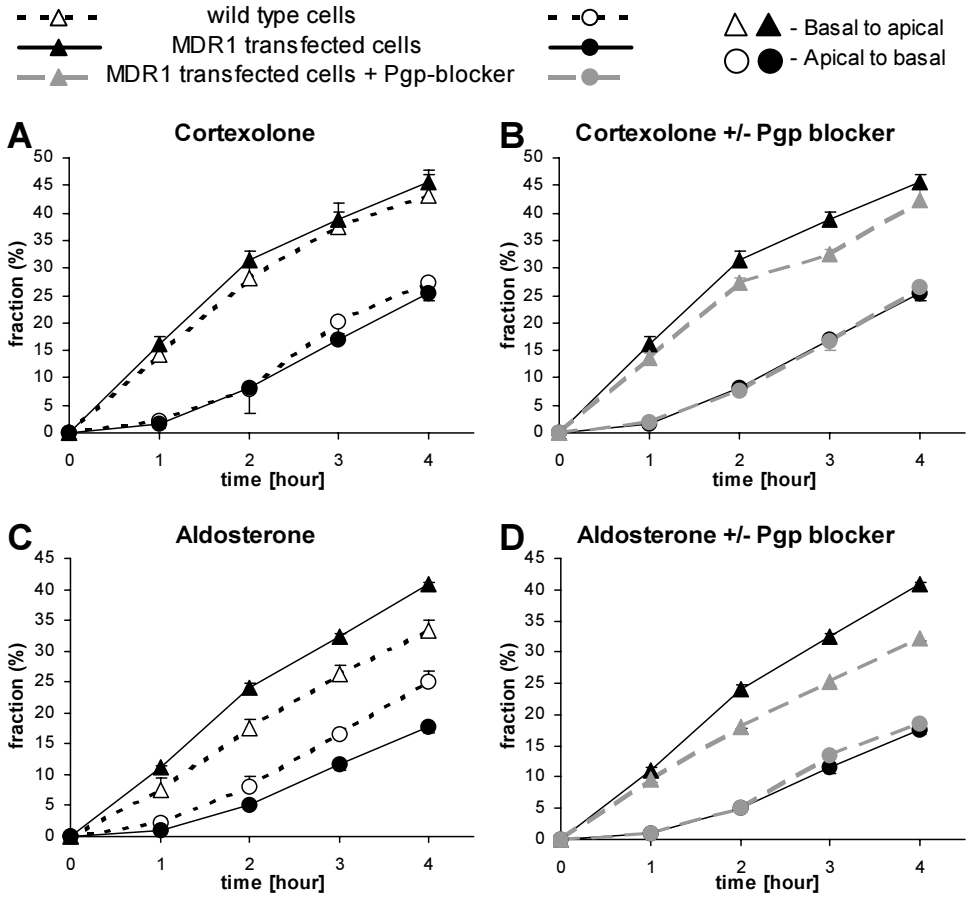

FIGURE 3. Activity of ^{3}H-cortexolone (A,B) and ^{3}H-aldosterone (C,D) present in medium at different time points after adding ^{3}H-steroid to the opposite compartment at t=0. (A,C) Transepithelial transport from basal to apical (Δ,▲) and from apical to basal (○,●) compartment was measured in wild type LLC-PK1 (dotted line) or MDR1 transfected LLC-PK1 (solid line) monolayers. (B,D) Transepithelial transport from basal to apical (Δ,▲) and from apical to basal (○,●) compartment was measured in MDR1 transfected LLC-PK1 monolayers after adding 1 μM LY335979 (broken line) or water (solid line) one hour before start.
Presented is 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 ± SEM. In 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.

Examination of the transport of aldosterone shows that, although aldosterone displayed polarised transport in both transfected and parental monolayers, there was a small but significant MDR1 Pgp contribution (figure 3C). This Pgp mediated transport could be partly blocked by LY335979 (figure 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 at the 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 may export prednisolone from human brain as well. We further demonstrated that aldosterone is transported by Pgp to a much smaller extent, while cortexolone is not at all transported.

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 cDNA (Ueda *et al.*, 1992; Schinkel *et al.*, 1995,this study; Karssen *et al.*, 2003). Furthermore, penetration of dexamethasone into mdr1a (-/-) mouse brain is enhanced compared to wild type brain (Schinkel *et al.*, 1995) increasing its 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 implicates that any GR expressing cell type that also express the efflux transporter Pgp is resistant to these glucocorticoids, which is in line with recent results of Pariante *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 (LC-MS) (Karssen *et al.*, 2001). We now demonstrate that Pgp is also unable to transport cortexolone, which is a weak partial agonist/antagonist at the GR *in vitro* (Kaiser and Mayer, 1980; Schmidt and Davidson, 1987) and *in vivo* (Acs and Stark, 1975; Duncan and Duncan, 1979; Kaiser and Mayer, 1980). In addition, the high affinity MR ligand aldosterone

is only weakly transported by Pgp in our MDR1-monolayers. 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 cortexolone was not and aldosterone was only weakly transported by mdr1b Pgp.

Although prednisolone is among the most common clinically used glucocorticoids, this study is one of the first that has examined the fate of prednisolone in brain, whereas other major glucocorticoids have been extensively studied before (for review see (McEwen *et al.*, 1986a)). In contrast to uptake into the brain of both corticosterone and aldosterone (McEwen *et al.*, 1968; De Kloet *et al.*, 1975; McEwen *et al.*, 1976; De Nicola *et al.*, 1981; Coutard *et al.*, 1987), access of dexamethasone and cortisol to brain is impaired (De Kloet *et al.*, 1974; De Kloet *et al.*, 1975; Rees *et al.*, 1975; McEwen *et al.*, 1976), because of the presence of Pgp at the BBB (Meijer *et al.*, 1998; Karssen *et al.*, 2001). We now demonstrate that prednisolone is also hampered 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 immunohistochemistry and *in situ* hybridisation (Van Eekelen *et al.*, 1987; Van Eekelen *et al.*, 1988). Pretreatment of mice with 100-fold excess of unlabeled prednisolone abolishes this selective retention in hippocampal cells and 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 many parts of the brain.

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; Lan *et al.*, 1982; De Kloet *et al.*, 1984a). 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 and 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 a too low affinity for this steroid to be detectable.

The lack of effect of pretreatment of unlabeled 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 µM prednisolone also suggest that even at high concentrations Pgp is able to reduce prednisolone concentrations in the brain. Interestingly, a recent study was not able to detect prednisolone in guinea pig brain, in contrast to liver and plasma, at various time points after administration of a high dose of 100 mg/kg (Tobita *et al.*, 2002). Therapeutically prednisolone is used in high doses in the treatment of diverse medical conditions, including pulmonary, rheumatologic, 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 and 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 and Lepage, 2001). Although mainly based on animal studies, De Kloet *et al.* (1991; 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 (Wu *et al.*, 1999; Shibamoto *et al.*, 1999; Maipang and 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 prednisolone and other drugs, that are all Pgp substrates, may mutually increase their active brain levels by saturating Pgp.

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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Chapter 5

EXCLUSION OF DEXAMETHASONE FROM THE BRAIN LEADS TO A SELECTIVE CENTRAL HYPOCORTICOSTEROID STATE

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E.R. de Kloet

Abstract

At the blood-brain barrier the efflux transporter P-glycoprotein hampers the access into the brain of the potent glucocorticoid receptor agonist dexamethasone, but does not affect the penetration of the endogenous glucocorticoid corticosterone. We hypothesised that the poor access of dexamethasone to the brain provides a subtle way to deplete glucocorticoids selectively from the brain. At low levels, dexamethasone will strongly block secretion of ACTH/corticosterone at the pituitary level and replace corticosterone at peripheral glucocorticoid targets, while in the brain dexamethasone would poorly substitute corticosterone. The ensuing central adrenalectomy-like condition would result in a relative lack of central glucocorticoid actions.

To test our hypothesis, rats were treated with various amounts of dexamethasone-21-phosphate either for five days by subcutaneous injection or for three weeks through their drinking water. At the last day animals were restrained for 30 minutes and then immediately decapitated. Our results show that basal and stress-induced corticosterone secretion, body weight gain and thymus weight were reduced even at low concentrations of dexamethasone demonstrating the potent peripheral glucocorticoid action of dexamethasone. In contrast, central markers like stress-induced responses of c-fos mRNA and CRH hnRNA in the paraventricular nucleus were not altered, while paraventricular CRH mRNA expression was increased after treatment with low concentrations of dexamethasone particularly after a longer period of treatment. Adrenalectomy affected these markers in a similar way.

It is concluded that the poor central access of low circulating dexamethasone concentrations has functional consequences and that treatment with small amounts of dexamethasone may provide a subtle model to selectively study central effects of corticosterone.

Introduction

The synthetic glucocorticoid dexamethasone is a potent and rather selective glucocorticoid receptor (GR) ligand *in vivo* (Reul *et al.*, 2000b). It has profound effects on energy metabolism, the immune system and Hypothalamus-Pituitary-Adrenal (HPA)-axis activity. In the clinic, it is commonly used for diagnostic purposes to test HPA-axis function in affective disorders. Several studies have demonstrated a pituitary rather than a brain site of action of moderate amounts of dexamethasone in suppression of HPA-axis activity (De Kloet *et al.*, 1974; De Kloet *et al.*, 1975; Rees *et al.*, 1975; Miller *et al.*, 1992; Cole *et al.*, 2000). In support of these findings, we have demonstrated that the efflux transporter P-glycoprotein, expressed at the luminal side of the blood-brain barrier (BBB) (Cordon-Cardo *et al.*, 1989), hampers the penetration of dexamethasone into the brain (Schinkel *et al.*, 1995; Meijer *et al.*, 1998). Therefore, we hypothesised that dexamethasone exclusion from the rat brain by P-glycoprotein provides a subtle way to create a brain-selective adrenalectomy-like state without affecting the exposure of peripheral corticosteroid targets to glucocorticoids. This state may arise because treatment with small amounts of dexamethasone results in suppression of endogenous corticosterone secretion and, at the same time, dexamethasone may poorly substitute corticosterone in the brain. As a consequence, a reduced feedback of glucocorticoids to the central glucocorticoid targets will ensue, which is reminiscent of the adrenalectomised state.

To test our hypothesis we treated rats with different doses of dexamethasone for either five days or three weeks. We compared rats treated with small amounts of dexamethasone with various control groups. Adrenalectomised (ADX) rats were used as a control for central and peripheral hypocorticoid state, and rats treated with a high dose of dexamethasone were used to demonstrate central and peripheral hypercorticoid effects, whereas untreated control rats formed the 'normo-corticoid' control group. As effects of central glucocorticoid negative feedback are most obvious under stress circumstances, the animals were exposed to a restraint stressor for 30 minutes at the last day and decapitated immediately thereafter. We used the psychological stressor of acute restraint stress to activate the central stress responsive pathways to minimise possible confounding peripheral effects of systemic stressors.

We chose a number of well-described glucocorticoid responsive markers in both brain and periphery to determine the effects of dexamethasone. Its action at the pituitary level results in suppression of markers of HPA-axis activity like proopiomelanocortin (POMC) mRNA expression in the anterior pituitary (Lundblad and Roberts, 1988) and corticosterone plasma levels (De Kloet *et al.*, 1974). Adrenal weight is decreased as the dexamethasone-induced reduction of adrenocorticotropin (ACTH) levels results in atrophy of adrenal cortical cells (Lesniewska *et al.*, 1992). Thymus weight is dramatically reduced after treatment with dexamethasone due to glucocorticoid induced apoptosis of thymocytes (Wyllie, 1980; Cohen,

1992), while body weight gain is suppressed or reversed due to GR mediated catabolic actions of dexamethasone (Simpson *et al.*, 1974; Konagaya *et al.*, 1986). Central effects were measured in the parvocellular part of the hypothalamic paraventricular nucleus (PVN), as this area is highly responsive to both stress and glucocorticoids (Dallman *et al.*, 1992). Glucocorticoids are known to reduce the expression of various genes in the PVN including c-fos and corticotropin-releasing hormone (CRH) hnRNA and mRNA levels (Swanson and Simmons, 1989; Herman *et al.*, 1992; Imaki *et al.*, 1995). Whereas we expected to observe clear effects in the periphery, we expected to see an escape from glucocorticoid suppression in the response of central (stress) markers.

Materials and methods

Animals

Young adult male Wistar rats (Charles River, Sulzfeld, Germany), weighing around 185-265 g at the time of arrival, were used. Animals were group-housed except for the drinking water experiment during which they were solitary housed, under a 12/12 hour light-dark cycle with lights off at 20hr in a temperature (21°C) and humidity controlled room. They had free access to food and drinking water. The experiments took place two weeks after arrival at the lab. During this period rats were handled daily. 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 (The Netherlands).

Experiment 1: Five day treatment with dexamethasone subcutaneously

At the start of experiment the rats weighed 303±15 gr (mean±SD). They were divided into four groups each consisting of 6-10 animals (table 1). One group (ADX) was adrenalectomised under gas anaesthesia (isoflurane) by dorsal approach at the start of the experiment. After ADX animals had free access to 0.9 % saline and normal drinking water. All other animals were sham-operated. For 5 days one group (DEXlow) was subcutaneously injected with a low dose of 50 µg/kg dexamethasone-21-phosphate (Sigma Aldrich; dissolved in 0.9% saline) twice daily at 9:00 and 21:00 hour. A second group (DEXhigh) was treated with a high dose of dexamethasone (500 µg/kg twice daily). Sham-operated (VEH) and ADX groups were treated with vehicle. During the whole experimental period body weight was monitored. At day 4 blood was sampled one hour after lights off to determine corticosterone plasma levels at the circadian peak. At day six animals received one last injection in the morning. Six hours later at 15:00 hour half of each group (3-5 rats per group) was exposed to restraint stress in a wire mesh/plastic cylinder for 30 minutes. At the start and after 15 minutes blood samples were taken using the tail incision method (Fluttert *et al.*, 2000). After 30 minutes animals were removed from the restrainers and immediately decapitated. Nonstressed animals (the second half of each group) were decapitated directly after removal out of their home cage. Trunk blood was collected in EDTA coated tubes and centrifuged. Plasma was kept at –20°C until determination of corticosterone plasma levels. Brains were rapidly

TABLE 1. Treatments of experimental groups of experiment 1 and 2

Groups	Label	Operation	Drug	Stress
Experiment 1				
1A	**VEH** nonstress	SHAM	VEH	nonstress
1B	**VEH** stress	SHAM	VEH	stress
2A	**DEXlow** nonstress	SHAM	DEX 100 µg/kg day^{-1}	nonstress
2B	**DEXlow** stress	SHAM	DEX 100 µg/kg day^{-1}	stress
3A	**DEXhigh** nonstress	SHAM	DEX 1 mg/kg day^{-1}	nonstress
3B	**DEXhigh** stress	SHAM	DEX 1 mg/kg day^{-1}	stress
4A	**ADX** nonstress	ADX	VEH	nonstress
4B	**ADX** stress	ADX	VEH	stress
Experiment 2				
1	**CTRL** nonstress	SHAM	none	nonstress
2	**CTRL** stress	SHAM	none	stress
3	**DEX0.5**	SHAM	DEX 0.5 µg/ml	stress
4	**DEX1.0**	SHAM	DEX 1.0 µg/ml	stress
5	**DEX10**	SHAM	DEX 10 µg/ml	stress
6	**ADX**	ADX	none	stress

removed from the skull and quickly frozen in isopentane precooled on dry ice/ethanol. Thymus, pituitary and adrenal glands were dissected and frozen on dry ice. All tissues were stored at –80°C until further use. Thymus and adrenals were weighed.

Experiment 2: three weeks treatment with dexamethasone in drinking water

At the start of the drinking water experiment rats weighed 225 ± 9 gr (mean±SD). They were divided into six treatment groups consisting of 5-8 animals (table 1). Three groups were treated with different concentrations (0.5, 1.0, 10 µg/ml) of dexamethasone 21-phosphate in their drinking water for three weeks (DEX0.5, DEX1.0 and DEX10 groups). One group was adrenalectomised (ADX) at the start of the experiment. After adrenalectomy animals had free access to 0.9 % saline and normal drinking water. All other animals were SHAM operated including control animals (CTRL) that received normal drinking water during the whole experiment. Each day animals and bottles were weighed to determine the body weight gain and volume of drinking solution that each animal had drunk over 24 hours. One animal of the highest concentration group died before the end of the experiment.

After three weeks rats were stressed by restraint in a wire mesh/plastic cylinder and decapitated after 30 minutes as described for experiment 1. A control nonstress, nontreated group (CTRL nonstress) was decapitated immediately after removal from the home cage.

In situ hybridisation

Coronal sections of 14 µm through the paraventricular nucleus of the hypothalamus and hippocampus were cut in a cryostat. Pituitaries were sectioned at 12 µm. Sections were thaw-mounted on poly-L-lysine coated microscopic slides. These slides were stored at –80 °C until hybridisation. The sections were postfixed in a freshly prepared 4% paraformaldehyde solution (pH 7.2) for 60 minutes at room temperature, rinsed twice in phosphate buffered saline (PBS) for 5 minutes at room temperature. In case of *in situ* mRNA hybridisations, sections were permeabilised with proteinase K (1 µg/ml in 0.1M Tris, pH 8.0) at 37°C for 10 minutes. After a brief rinse in diethyl pyrocarbonate (DEPC) treated water, they were treated with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 minutes at room temperature and finally rinsed in 2xSSC (SSC=0.15M NaCl and 0.015M sodium citrate) for 10 minutes at room temperature. Subsequently the sections were dehydrated through a graded series of ethanol and air-dried.

Preparation of probes

To visualize mRNAs, *in situ* mRNA and oligonucleotide hybridisations were performed. Different 33P- and 35S-labelled cRNA antisense probes were employed to hybridise with complementary brain tissue c-fos and CRH mRNA, and CRH heteronuclear (hn)RNA. The c-fos mRNA probe was transcribed from a pBluescript (pBS) KS plasmid containing a 2.1 kB full-length rat c-fos cDNA sequence (courtesy of T. Curran, USA) in presence of 33P-UTP (ICN Biomedicals, Costa Mesa, CA; Isoblue stabilized, S.A. 3000 Ci/mmol). This probe was hydrolysed by incubation in 90 mM 0.2 M Na2CO3 and 60 mM 0.2 M NaHCO3 at 60°C for 10 minutes to facilitate the tissue penetration. The CRH hnRNA 33P-UTP labelled probe was transcribed from a 687 bp fragment (courtesy of P. Sawchenko, USA) covering the single intron of the rat CRH gene subcloned into a pBS vector. A full length probe for CRH mRNA (1.2 kB subcloned into pBS; courtesy of Dr. K. Mayo, USA) was synthesised in presence of 35S-UTP. Incorporation of labelled UTP was at least 75%.

A 42-nucleotides mouse POMC oligonucleotide (GGT-TTT-CAG-TCA-GGG-GCT-GTT-CAT-CTC-CGT-TGC-CAG-GAA-ACA; 90% homology with rat POMC; Eurogentec, Belgium) was end-labelled with 33P-dATP (NEN Life Science Products, Hoofddorp, The Netherlands, 2000 Ci/mmol, 10 mCi/ml) using terminal transferase with the manufacturer's protocol (Roche Molecular Biochemicals, Almere, The Netherlands). A 0.33 pmol oligonucleotide was labelled at molar ratio of 1:20 (oligo:label). Incorporation was typically between 50 and 75%, resulting in a tail of 5 to 7.5 A-residues per oligonucleotide.

Hybridisation procedure

When using 33P-labelled probes, each slide, containing four sections, was loaded with a 100µl mix containing 70% deionised formamide, 10% dextran sulphate, 3xSSC, 50 mM dithiothreitol, 1xDenhardt's solution, 0.1 mg/ml yeast tRNA, 0.1 mg/ml sheared herring sperm DNA (ssDNA) and 1-3x106 dpm of the probe, and covered with microscopic coverslips. Overnight hybridisation was performed in a moist chamber at 55°C. As a control, a few slides

were hybridised with sense probe. The next day, coverslips were removed and the slides were washed in 2xSSC at room temperature for 10 minutes, treated with RNAse A (2 mg/100 ml in 0.5M NaCl, pH 7.5) at 37°C for 10 minutes and 3 times washed in 2xSSC/50% formamide at 60°C for 15 minutes. After a short wash with 2xSSC, sections were dehydrated in an ethanol series and air dried. Finally, the slides were put in an X-ray exposure holder and apposed to Biomax MR film (Kodak) for 3-12 days.
When using 35S-labelled probes, hybridisation procedures were used as previously described (Sibug *et al.*, 1998).

Oligonucleotide *in situ* hybridisation

In situ hybridisation using oligonucleotides was performed essentially as described (Meijer *et al.*, 2000). 0.5-0.8x106 dpm of labelled oligonucleotide per 100 µl hybridisation mix was applied to each slide. Hybridisation mix consisted of 50% formamide, 10% dextran sulfate, 4xSSC, 25mM sodium phosphate (pH 7.0), 1 mM sodium pyrophosphate, 20 mM DTT, 5x Denhardt's, 100 µg/ml poly-A, 100 µg/ml ssDNA. Sections were coverslipped and hybridised overnight in a moist chamber at 42°C . The next morning, coverslips were removed, rinsed in 1xSSC at room temperature, washed twice for 30 min in 1xSSC at 50°C, washed for 5 min in 1xSSC at room temperature, dehydrated in an ethanol series, air dried. Then, sections were apposed to Kodak (Rochester, NY) Biomax MR film and films were developed after 17-20 hours.

Densitometric quantification

Optical density was quantified with analysis performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image)
Densities were determined by outlining the parvocellular part of the paraventricular nucleus (PVN). Nissl-staining and light microscopy was used to confirm presence of PVN. The optical density of the area dorsolateral from the PVN was used to correct for tissue background. Optical density measurements of 3-5 sections were averaged per animal with the mean value from each animal used in subsequent statistical analysis.

Corticosterone plasma levels

Total plasma corticosterone was determined using a standard in-house radioimmunoassay procedure. The antiserum raised in sheep against corticosterone-21-hemisuccinate bovine serum albumin was a gift from dr F. Sweep, University of Nijmegen, The Netherlands. The detection limit is 0.2 µg/dl.

Determination of apoptosis

Number of apoptotic cells in the dentate gyrus was qualitatively scored in Nissl-stained sections of the hippocampus at a magnification of 400x.

Statistics

Completeness of adrenalectomy was determined based on corticosterone plasma levels and on relative saline intake (Richter, 1941); one animal was excluded from the analysis because of incomplete ADX. Plasma corticosterone data were analysed using one-way analysis of variance (ANOVA) or repeated measurements ANOVA, where appropriate. All other data were analysed using two-way ANOVA with treatment and stress as factors (experiment 1), or one-way ANOVA with treatment as factor and stress as covariate (experiment 2). When a significant stress effect or treatment*stress interaction was absent, data of stress and nonstress groups were combined for further analysis on treatment effects. In case stress or interaction effects were significant, stress and nonstress groups were separately analysed applying one-way ANOVA. When appropriate, post-hoc comparisons were performed using Tukey's Honestly Significant Difference test. Statistical difference was accepted at $p < 0.05$. All data are expressed in mean ±SEM.

Results

Experiment 1: Five day treatment with dexamethasone subcutaneously

Two daily subcutaneous injections of dexamethasone for 5 days suppressed basal corticosterone plasma levels both at the onset of the stress response and at the circadian peak ($p<0.05$, table 2). Repeated measurements ANOVA on the stress induced corticosterone response revealed a significant interaction effect of stress and treatment ($F(6,24)=46.7$; $p<0.05$). Although low-dose dexamethasone was not able to completely block the stress-induced rise of corticosterone plasma levels, the levels at 30 minutes after the initiation of stress were still considerably lower in the DEXlow group ($p<0.05$) compared with those in VEH group (figure 1). Further, adrenal weight was significantly reduced in both DEXlow and DEXhigh groups (table 2).

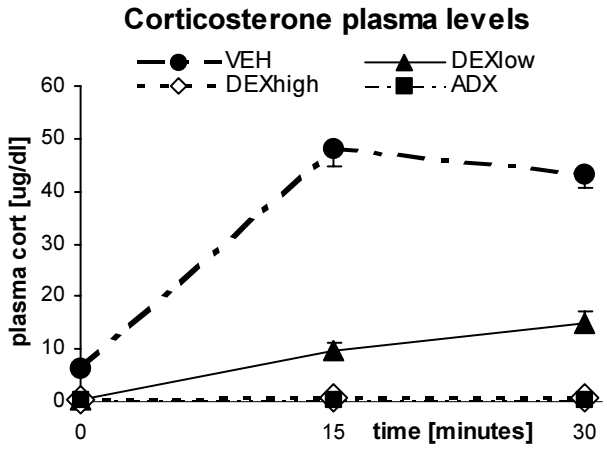

FIGURE 1. Time course of corticosterone secretion in response to a 30 minutes restraint stress after five days of treatment with various doses of dexamethasone or ADX.
Repeated measures ANOVA revealed a significant interaction of time and treatment ($F(6,24)=46,7$; $p<0.0005$). *Post-hoc* analysis revealed that the DEXlow group (twice daily 50 µg/kg) differed significantly from all other groups ($p<0.02$).
Values are expressed as mean±SEM [µg/dl] of 3-5 rats.

TABLE 2. Peripheral glucocorticoid markers affected by five-day dexamethasone treatment or ADX (experiment 1). Nonstress and stress groups are pooled.

Treatment	body weight [% of start weight]	adrenal weight [mg]	thymus weight [mg]
VEH	104% ± 2%	41.0 ± 2.2	522 ± 25.9
DEXlow	93% ± 1% *	25.5 ± 1.7 *	140 ± 7.1 *
DEXhigh	84% ± 2% *,#	19.9 ± 2.0 *	73 ± 5.3 *
ADX	101% ± 2%		608 ± 28.5 *

* $p < 0.05$ compared to SHAM VEH # $p < 0.05$ compared to SHAM DEXlow

Treatment	corticosterone plasma levels [µg/dl] peak	onset stress
VEH	16.07 ± 1.47	6.45 ± 0.79
DEXlow	4.96 ± 0.97 *	0.39 ± 0.03 *
DEXhigh	0.65 ± 0.11 *,#	0.35 ± 0.04 *
ADX	n.d.	0.46 ± 0.09 *

n.d. not determined

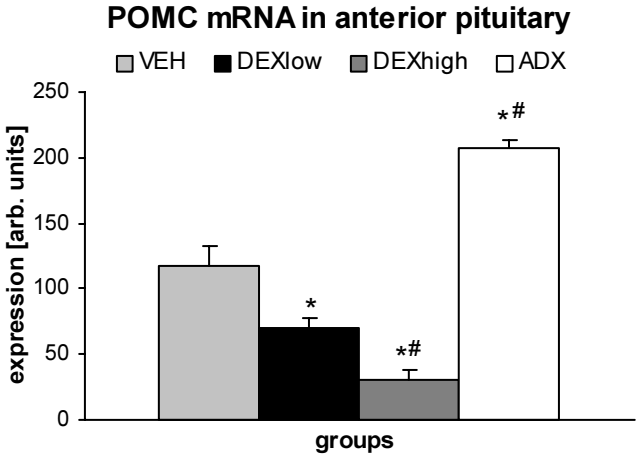

FIGURE 2. The effect of five-day dexamethasone treatment on POMC mRNA expression in the anterior pituitary. As two-way ANOVA revealed no stress effect ($F(1,21)=0.319$; $p>0.05$), stressed and nonstressed rats of the same treatment groups were pooled for further analysis on treatment. Dexamethasone dose-dependently decreased POMC mRNA levels. In contrast, ADX significantly increased POMC mRNA levels. *Post-hoc* analysis showed that the DEXlow group differed significantly from all other groups ($p<0.03$). Bars represent mean ± SEM of 5-8 rats/group in arbitrary units of optical density as measured by *in situ* hybridisation. * significantly different from VEH ($p<0.01$). # significantly different from DEXlow ($p<0.03$).

Two-way ANOVA revealed no stress effect ($F(1,21)=0.319$; $p>0.05$) on POMC mRNA expression in the anterior pituitary consistent with previous studies (Harbuz and Lightman, 1989), but there was a significant treatment effect. In both DEXlow and DEXhigh groups POMC mRNA expression in the anterior pituitary was significantly reduced in a dose-dependent manner ($p<0.05$, figure 2). Intermediate lobe POMC mRNA expression was not affected by treatment nor by stress (data not shown). Stress induced a response of c-fos mRNA expression in the anterior pituitary ($F(1,21)=17.5$; $p<0.05$), but there was no effect of low-dose dexamethasone treatment or ADX (table 3). Only the DEXhigh group showed a reduced stress-induced response of anterior pituitary c-fos mRNA as the stress-induced increase was not significant (Student's t-test).

Other peripheral markers not directly related to the HPA-axis were also strongly affected in the DEXlow group. The thymus wet weight was dramatically reduced (table 2) ($p<0.05$). Final body weight was slightly decreased compared with start of the treatment in contrast to vehicle treated animals which gained weight at a normal rate, which resulted in a significant difference between both groups at the end of the treatment (table 2). In the DEXhigh group body weight was strongly reduced to 84% of starting weight.

The effects of low-dose dexamethasone in the DEXlow group on peripheral markers are all consistent with well-known effects of glucocorticoid action and thus comparable to the effects of high-dose treatment in the DEXhigh group. Likewise, ADX animals showed the typical effects seen after removal of the source of circulating corticosterone, *i.e.* undetectable corticosterone plasma levels, increased POMC mRNA expression and increased thymus weight ($p<0.05$, table 2 and figure 2).

TABLE 3. Effect of stress and five-day dexamethasone treatment on expression of c-fos mRNA in anterior pituitary (exp. 1)

Treatment	c-fos mRNA anterior pituitary
nonstress	
VEH	97.5 ± 27.6
DEXlow	91.4 ± 24.4
DEXhigh	84.3 ± 33.8
ADX	121.0 ± 36.6
stress	
VEH	187.7 ± 9.1 $
DEXlow	196.4 ± 7.6 $
DEXhigh	138.1 ± 32.1
ADX	173.6 ± 8.8

$ $p<0.05$ t-tests respective nonstress group

TABLE 4. Effect of dexamethasone treatment on CRH hnRNA expression in the PVN in absence of stress (experiment 1)

Treatment	CRH hnRNA [arb. units]
nonstress	
VEH	18.5 ± 0.8
DEXlow	14.2 ± 1.4
DEXhigh	12.2 ± 4.0
ADX	19.5 ± 1.5

In contrast to the peripheral effects, the central effects of low-dose treatment are different from those of high-dose treatment. The expected reduction of stress-induced responses of c-fos mRNA and CRH heteronuclear RNA (hnRNA) expression in the parvocellular part of the PVN was indeed evident in the DEXhigh group ($p<0.05$, figure 3). However, in the DEXlow animals the rapid increases of c-fos mRNA and CRH hnRNA after stress were not changed compared to VEH animals or five-day ADX animals (figure 3). Dexamethasone treatment had no effect on the level of c-fos mRNA expression in the PVN in absence of stress, whereas the basal CRH hnRNA expression tended to be affected ($F(3,13)=3.236$, $p=0.057$; table 4).

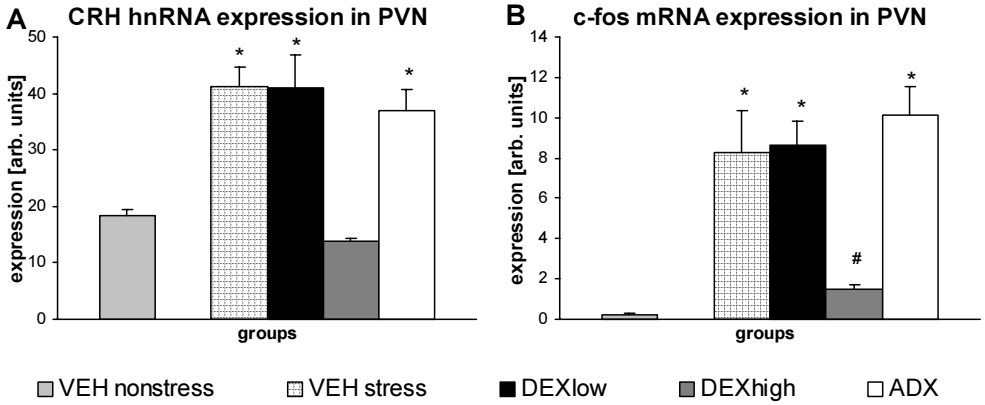

FIGURE 3. The effect of five-day treatment with dexamethasone or ADX on the stress-induced increase of CRH hnRNA (A) and c-fos mRNA (B) levels in the hypothalamic paraventricular nucleus. For reasons of clarity nonstress groups (except VEH) are not shown. Basal CRH hnRNA levels are published in table 3. Two-way ANOVA revealed significant stress and treatment effects ($p<0.02$). In the DEXlow as well as in the ADX group the stress response was not altered, but in the DEXhigh group the response of CRH hnRNA was abolished, whereas only a minor stress response of c-fos mRNA was seen (#, Student's t-test, $p<0.05$). Bars represent mean ± SEM of 3-5 rats/group in arbitrary units of optical density reflecting the amount of ^{33}P-labelled hnRNA respectively mRNA. * $p<0.05$ compared to respective nonstress group and DEXhigh stress group, Tukey's HSD.

Due to high variability within groups CRH mRNA levels are not significantly different (figure 4), but it is clear that in the DEXhigh group the CRH mRNA expression was differently affected than in both ADX and DEXlow groups. Further, another well-known ADX effect, apoptosis of dentate gyrus cells, was not found in the brains of DEXlow animals (nor in DEXhigh animals). In about two-third of the ADX animals pyknotic cells, which are indicative of apoptosis, were abundantly present in the granule cell layer of the dentate gyrus, mainly located in the tip of the inner blade.

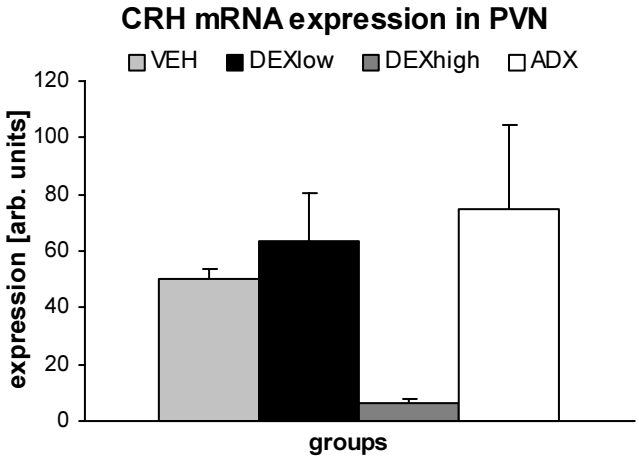

FIGURE 4. Expression of CRH mRNA in hypothalamic paraventricular nucleus of nonstress animals after five-day dexamethasone treatment or ADX. Neither low-dose dexamethasone treatment nor ADX significantly affected CRH mRNA levels. Due to high variability and low number of animals, ANOVA did not reveal significant treatment effects.

Experiment 2: three weeks treatment with dexamethasone in drinking water

As five-day treatment with low doses of dexamethasone was not enough to fully suppress stress-induced corticosterone response and to augment the stress-induced central markers we extended the treatment to three weeks. We switched to administration of dexamethasone through drinking water to minimise possible stress effects induced by daily injections. Dexamethasone treatment did not affect the volume of drinking solution drunk by the animals (table 5). The amounts of dexamethasone ingested by the animals were calculated based on the volume drunk and the concentration of dexamethasone. Animals treated with 0.5 µg, 1.0 µg or

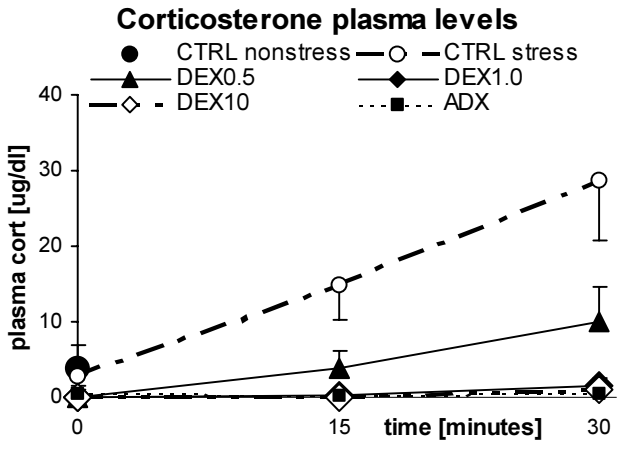

FIGURE 5. Time course of corticosterone secretion in response to a 30 minutes restraint stress after three weeks of treatment with various doses of dexamethasone through the drinking water.
Repeated measures ANOVA revealed a significant interaction of time and treatment ($p<0.005$). Post-hoc analysis revealed that the DEX0.5 group differed significantly from all other groups ($p<0.05$).
Values are expressed as mean±SEM [µg/dl] of 2-7 rats.

TABLE 5. Peripheral glucocorticoid markers after three-week treatment with dexamethasone through drinking water (experiment 2). Nonstress and stress CTRL groups are pooled.

Treatment	body weight [% of start weight]	adrenal weight [mg]	thymus weight [mg]	Volume drunk [ml/day]
CTRL	130% ± 2%	22.2 ± 1.7	558 ± 18	22 ± 0.4
DEX 0.5	106% ± 1% *	13.6 ± 1.3 *	264 ± 58 *	19 ± 0.4
DEX 1.0	94% ± 1% *,#	9.5 ± 0.8 *	145 ± 10 *	19 ± 0.6
DEX 10	52% ± 4% *,#	6.9 ± 0.4 *,#	96 ± 20 *,#	22 ± 1.6
ADX	122% ± 7% #		837 ± 70 *,#	7 ± 0.7 *,#
Saline				31 ± 1.4 *,#

* $p < 0.05$ compared to CTRL stress # $p < 0.05$ compared to DEX 0.5

Treatment	corticosterone plasma levels [µg/dl] peak	onset stress
CTRL	11.6 ± 2.5	3.3 ± 0.72
DEX 0.5	4.6 ± 1.6 *	<0.02 *
DEX 1.0	0.6 ± 0.5 *,#	<0.02 *
DEX 10	<0.02 *,#	<0.02 *
ADX	0.5 ± 0.4 *,#	0.5 ± 0.24 *

10 µg dexamethasone per ml in their drinking water ingested per day 42±1, 91±4 respectively 1447±145 µg dexamethasone per kg average body weight.

Like in the injection study, ADX and treatment with the highest concentration of 10 µg/ml dexamethasone in the drinking water have both the expected effects on peripheral glucocorticoid markers. ADX abolished the corticosterone plasma levels, increased the POMC mRNA levels in the anterior pituitary and increased the thymus weight (table 5, figs. 5 and 6). In the DEX10 animals dexamethasone treatment resulted in a complete suppression of corticosterone plasma levels, a strong reduction of POMC mRNA levels, a severe loss of body weight, and decreased thymus and adrenal weights ($p<0.05$; table 5, figs. 5 and 6).
Peripherally, treatment with low concentrations of dexamethasone in both DEX0.5 and DEX1.0 groups resulted in effects consistent with glucocorticoid actions. Basal levels of plasma corticosterone were completely suppressed and not different from ADX levels and adrenal wet weight was dose dependently decreased, as was the case with thymus wet weight ($p<0.05$, table 5). However, only in the DEX1.0 group the stress-induced increase of corticosterone was completely blocked. In the DEX0.5 group dexamethasone treatment reduced corticosterone plasma levels at 30 minutes after onset of stress to only 35% of control

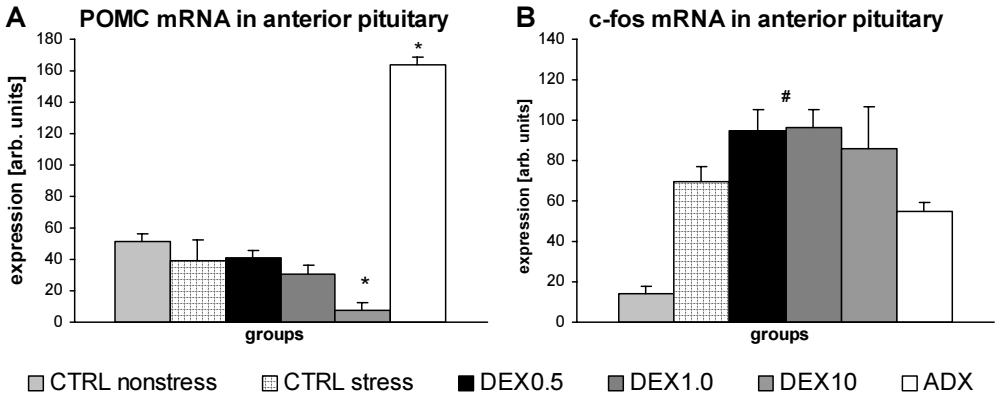

FIGURE 6. The effect of three-week dexamethasone treatment on POMC mRNA expression (A) and stress-induced c-fos mRNA levels (B) in the anterior pituitary. Bars represent mean ± SEM of 2-8 rats/group in arbitrary units of optical density as measured by *in situ* hybridisation.

A) Only in DEX10 animals POMC mRNA levels were decreased, whereas in ADX rats POMC mRNA expression was strongly increased (*, $p<0.04$, Tukey's HSD).

B) Both stress ($p<0.001$) and treatment ($p<0.02$) significantly affected pituitary c-fos mRNA expression. After combining the DEX0.5 and DEX1.0 groups, the stress-induced increase was significantly augmented after treatment with low concentrations of dexamethasone compared to CTRL stress (#, $p<0.03$, Student's t-test).

levels (figure 5). Whereas untreated CTRL animals gained 27-33% body weight during the experimental period, both DEX0.5 and DEX1.0 animals had not gained substantial weight at the end of the experimental period (table 5). The body weight of these animals decreased during the first 5 days of the experiment and stabilised or slightly increased from that day on.
POMC mRNA levels in the anterior pituitary were not altered in both DEX0.5 and DEX1.0 (figure 6A). The c-fos mRNA expression in the anterior pituitary clearly responded to stress in untreated CTRL animals ($p<0.05$, figure 6B), in agreement with a previous report (Senba *et al.*, 1994). Treatment also significantly affected expression levels ($F(4,27)=3.676$, $p<0.05$). ADX levels were lower compared to those of DEX1.0 animals. Dexamethasone treatment seemed to induce a higher c-fos mRNA response than stressed control animals (figure 6B), but this effect was only found to be statistically significant after combining both low-concentration dexamethasone treatment groups.

Similar to the injection study, central markers were differentially affected by treatment with small amounts of dexamethasone compared to treatment with large amounts of dexamethasone. Both DEX0.5 and ADX animals showed increased levels of CRH mRNA in the PVN compared with the CTRL group ($p<0.05$), whereas the DEX10 animals showed a clear reduction in CRH mRNA levels (figure 7). The CRH mRNA expression in the DEX1.0 animals was not changed compared with untreated CTRL animals. Compared to the stressed CTRL group both the c-fos mRNA and the CRH hnRNA response was reduced only in the

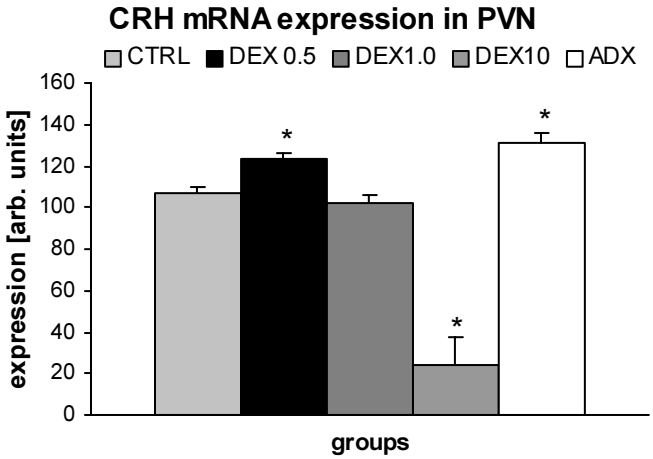

FIGURE 7. Expression of CRH mRNA in hypothalamic paraventricular nucleus after three-week dexamethasone treatment or ADX. In ADX as well as in DEX0.5 animals CRH mRNA levels were significantly enhanced, whereas in DEX10 animals levels were strongly suppressed. Bars represent mean ± SEM of 3-10 rats/group in arbitrary units of optical density reflecting the amount of ^{35}S-labelled mRNA. * $p<0.05$ Tukey's HSD.

DEX10 animals (figure 8). The two lower concentration groups and the ADX group had c-fos mRNA and CRH hnRNA levels not significantly different from the stressed CTRL group. No signs of apoptosis within the granule cell layer of the dentate gyrus could be found in any of the treatment groups.

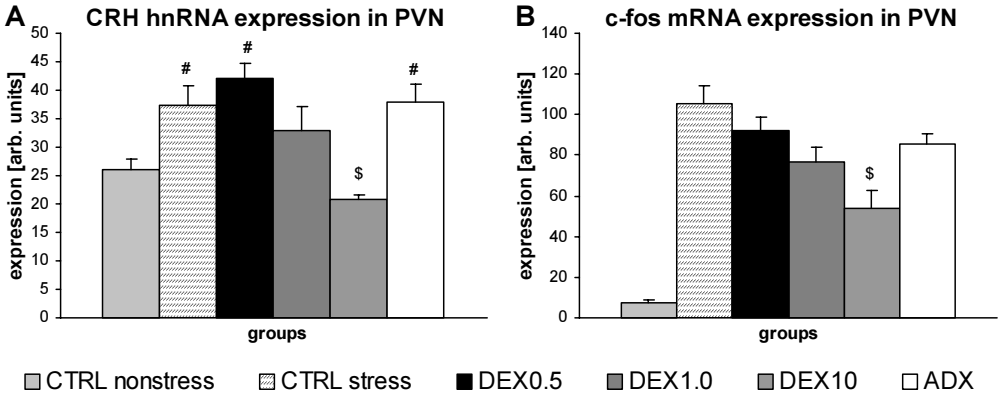

FIGURE 8. Effect of three-week dexamethasone treatment or ADX on the stress-induced increase of CRH hnRNA (A) and c-fos mRNA (B) levels in the hypothalamic paraventricular nucleus. Only the high concentration of dexamethasone reduced the response to stress of both CRH hnRNA and c-fos mRNA ($, $p<0.05$). In ADX, DEX0.5 and DEX1.0 groups expression levels were not significantly altered as compared with CTRL stress levels. Data are expressed as mean ± SEM of 4-8 rats/group. #, $p<0.05$, Student's t-tests.

Discussion

The present study demonstrates the lack of central action of low-dose dexamethasone in a functional way. The findings clearly show the divergence of central and peripheral effects of treatment with small amounts of dexamethasone. Classic markers of peripheral glucocorticoid actions like body weight gain, thymus and adrenal weight and pituitary-adrenal activity were all strongly affected by dexamethasone even after administration of small amounts. Small amounts of dexamethasone administered either by injection for five days or through drinking water for three weeks completely suppressed basal corticosterone plasma levels, whereas circadian peak and stress-induced increases were strongly reduced. In the anterior pituitary POMC mRNA expression is reduced after treatment with small amounts of dexamethasone for five days, although not after treatment for three weeks. On the other hand, strong negative feedback effects of dexamethasone at central glucocorticoid responsive markers were absent after treatment with small amounts. Both stress-induced increases of c-fos mRNA and CRH hnRNA and expression of CRH mRNA in the PVN were comparable to those in untreated control or ADX rats. After three weeks of treatment the CRH mRNA levels were even augmented instead of reduced compared with untreated animals which resembled the effect of ADX. The responsiveness of these central markers to glucocorticoids was confirmed by the strong action of large amounts of dexamethasone. Besides, the affinity of the brain GR for dexamethasone has been shown to be similar to the affinity of pituitary GR (Miller *et al.*, 1992). Thus, the lack of effect on these central markers compared with peripheral markers is not due to lack of responsiveness of these central glucocorticoid targets. Yet, it is probably related to the hampered penetration of dexamethasone into the brain.

In vitro, brain glucocorticoid target areas can easily retain both ^{3}H-dexamethasone and ^{3}H-corticosterone (De Kloet *et al.*, 1975; McEwen *et al.*, 1976). However, whereas ^{3}H-corticosterone can easily enter the brain *in vivo* (De Kloet *et al.*, 1975; Stumpf *et al.*, 1989; Karssen *et al.*, 2001), ^{3}H-dexamethasone can not, although both brain and pituitary express high amounts of GR (De Kloet *et al.*, 1975; Rees *et al.*, 1975; Stumpf *et al.*, 1989). The existence of a blood-brain barrier limiting the uptake of dexamethasone was postulated (De Kloet *et al.*, 1975; Rees *et al.*, 1975; Coutard *et al.*, 1978), but could not be proved until recently. We have demonstrated that these low doses poorly reach the brain glucocorticoid target areas due to the presence of the efflux transporter P-glycoprotein at the blood-brain barrier (Meijer *et al.*, 1998).

As a result of its poor central access to the brain dexamethasone primarily acts on the anterior pituitary to suppress pituitary-adrenal secretion (De Kloet *et al.*, 1974). In contrast, as corticosterone can easily reach the brain, the feedback sites of corticosterone modulating the HPA-axis are likely predominantly localised at multiple sites in the brain (Dallman *et al.*, 1987a; Levin *et al.*, 1988; De Kloet, 1991; Diorio *et al.*, 1993; Dallman *et al.*, 1994). Our data corroborate the concept of a pituitary site of dexamethasone action and are consistent with

reports studying receptor occupancy in pituitary and brain tissue after either short-term or acute dexamethasone treatment (Miller *et al.*, 1990; Miller *et al.*, 1992; Cole *et al.*, 2000). These latter studies have demonstrated that low doses of dexamethasone selectively occupy GR in the pituitary and thymus, whereas GR levels in different parts of the brain were unaffected.

Treatment with large amounts of dexamethasone is effective in suppressing central glucocorticoid markers in addition to its potent peripheral effects. In the current study, the stress-induced responses were clearly reduced and CRH mRNA expression was abolished after administration of large amounts of dexamethasone. This indicates that the barrier formed by P-glycoprotein is not complete. Indeed, after administration in large amounts dexamethasone reaches the brain (Schinkel *et al.*, 1995). Regardless of whether dexamethasone is still hampered to enter the brain at high plasma levels, it will likely reach the brain in sufficient amounts to activate the GR. Large amounts of dexamethasone have been demonstrated to be able to occupy the GR in the hippocampus and hypothalamus (Reul *et al.*, 1987b; Spencer *et al.*, 1990; Miller *et al.*, 1992). All studies reporting effects of dexamethasone on glucocorticoid targets in the brain used high systemic doses or brain implants of dexamethasone (Kovács and Mezey, 1987; Sawchenko, 1987; Imaki *et al.*, 1995; Roozendaal and McGaugh, 1996; Feldman and Weidenfeld, 2002). These and our studies show that systemically administered dexamethasone is able to act on the brain only when administered in large amounts overcoming its P-glycoprotein mediated exclusion from the brain.

In contrast to the well-known inhibitory central effects of large amounts of dexamethasone, we expected to observe augmented central stress responses after treatment with small amounts similar to ADX. Due to depletion of central glucocorticoid receptors from their ligands, both conditions were expected to show lack of glucocorticoid negative feedback in brain and consequently hyperactive CRH neurons. Indeed, CRH mRNA levels were increased after three weeks of either treatment with small amounts of dexamethasone or ADX, although this effect was not seen after five-day treatment. However, the stress responses of c-fos mRNA and CRH hnRNA in the ADX groups as well as in the small amount dexamethasone groups were not augmented compared with those of the control groups in the present study, in contrast to the previously reported amplified central stress response 5 days to one week after ADX (Imaki *et al.*, 1995; Kovacs *et al.*, 2000) or after acute glucocorticoid withdrawal (Herman *et al.*, 1992). However, in concordance with our finding are several other studies reporting no effect of 5 day ADX on induction of c-fos mRNA in hypothalamus after stress (Melia *et al.*, 1994; Helmreich *et al.*, 1996; Brown and Sawchenko, 1997). Lack of an ADX-effect on these parameters in the present study neither confirms nor rejects the complete removal of central glucocorticoid feedback after treatment with small amounts of dexamethasone. On the other hand, ADX and small amount dexamethasone groups never differ significantly from control

groups with regard to the various central glucocorticoid markers, whereas peripheral effects are often opposite to each other. In contrast, groups treated with large amounts of dexamethasone always differ significantly from all groups. Thus, the present study indicates that treatments with amounts less than 1 µg/ml in the drinking water or daily injections of less than 100 µg/kg may result in a brain-selective depletion of glucocorticoids whereas dexamethasone can still act through the peripheral glucocorticoid receptors.

The integrity of peripheral GR mediated actions might be one of the advantageous differences of this paradigm to ADX when studying central effects of corticosterone, even though the peripheral GR system is hyperactivated. Exclusion of small amounts of dexamethasone from the brain may provide a subtle model to study central glucocorticoid action. This model might make it possible to distinguish between direct central effects and indirect peripheral effects subsequently affecting central responses. The need for such a distinction has been exemplified by two recent studies showing that in adrenalectomised rats ingesting sucrose expression of corticotropin-releasing hormone in brain is normalised (Laugero *et al.*, 2001) and centrally infused corticosterone may stimulate HPA-axis activity under some conditions (Laugero *et al.*, 2002). In addition, corticosterone effects can be examined without the potential confounding effects of removal of aldosterone and adrenal catecholamines as after ADX.

This model would be particularly useful in studying GR mediated actions, as this model is hyporesponsive to stress-induced activation of the pituitary-adrenal axis. GR is the main receptor type involved in mediating glucocorticoid negative feedback effects in the brain (De Kloet, 1991). It has been demonstrated that for proper memory functioning the increase in corticosterone and consequently occupation of GR directly after the task is necessary (Oitzl and De Kloet, 1992; Sandi, 1998). Furthermore, in many central processes affected by glucocorticoids the MR/GR balance is critically important in modulating glucocorticoid actions (De Kloet, 1991; De Kloet *et al.*, 1999). It might be worthwhile to use this model in studies concerning the role of GR in learning and memory tasks or in situations of chronic stress.

The impaired penetration of dexamethasone into the brain has important implications for the interpretation of the dexamethasone suppression test (DST). This test is widely used in the clinic often in combination with a CRH challenge, to evaluate the dysregulation of the HPA-axis in depressive patients (Holsboer and Barden, 1996; Holsboer, 2000). Excessive release of CRH and vasopressin (AVP) from PVN neurons into the portal circulation leads to a hyperdrive of the HPA-axis in many of these patients. A low dose of dexamethasone administered the night before does not suppress the basal and exogenous CRH induced ACTH/corticosterone release as is seen in controls. The HPA abnormalities seen in the DST are often described to decreased negative feedback through corticosteroid receptors. The DST may not necessarily provide any proof for this, at least not at suprapituitary levels. As

dexamethasone is administered at a low dose it will mainly act on the pituitary. At this level, the hyperdrive of CRH/AVP may be strong enough to override even properly functioning negative feedback. Dexamethasone suppression in healthy subjects can indeed be overcome by concurrent infusion of CRH and AVP (Von Bardeleben *et al.*, 1985). On the other hand, it can not be excluded that the hyperdrive itself is caused by an aberrant central feedback mechanism, but the DST does not test this.
A better way to probe the efficacy of the central corticosteroid receptor feedback system may be provided by the application of corticosterone. Corticosterone can easily enter the brain (Karssen *et al.*, 2001) and likely acts predominantly on the central feedback target areas (Levin *et al.*, 1988). It would therefore be able to directly suppress the CRH/AVP drive in the PVN. Besides, as corticosterone also activates MR, it would also probe the function of this receptor. Regulation of HPA-axis is partly mediated by MR (De Kloet, 1991; Spencer *et al.*, 1998; Young *et al.*, 1998; Reul *et al.*, 2000b) and may even be the more relevant receptor type involved in dysregulation of HPA-axis seen in stress-related disorders (Reul *et al.*, 2000a; Pariante and Miller, 2001; Makino *et al.*, 2002).

In conclusion, our findings suggest that treatment with small amounts of dexamethasone may deplete glucocorticoids selectively from the brain without removal of glucocorticoid action in the periphery. This condition will specifically affect central glucocorticoid target areas modulating HPA-axis, behavioural adaptation and synaptic plasticity, particularly after stress. Thus, divergence of peripheral and central glucocorticoid effects due to hampered access of small amounts of dexamethasone to the brain may serve as a model for states with disturbed glucocorticoid signalling in the brain.

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Chapter 6

General Discussion

The data presented in the preceding chapters of this thesis have clearly demonstrated the impact of the efflux transporter Pgp expressed at the endothelial cells forming the BBB in modulating access of glucocorticoids to glucocorticoid target areas in the brain. Pgp hampers the uptake into the brain of various synthetic and naturally occurring corticosteroids, like dexamethasone, prednisolone and cortisol. The concentrations of these glucocorticoids in the brain are strongly decreased compared with their plasma concentrations and, when circulating at low plasma levels, these glucocorticoids hardly reach their central targets. Moreover, administration of the potent GR-ligand dexamethasone in small amounts was counter-intuitively found to decrease central glucocorticoid feedback creating a low corticosteroid condition in the brain. The work presented in this thesis has also revealed a remarkable difference between the two main naturally occurring glucocorticoids corticosterone and cortisol, as Pgp appeared to have no effect on corticosterone uptake into the brain. This difference may have implications for central glucocorticoid actions in humans.

P-glycoprotein expression in the brain

In the first chapter data are presented showing the localisation of mdr1 mRNA encoding Pgp in brain. *In situ* hybridisation using DIG-labelled or ^{33}P-labelled riboprobes against mdr1a and mdr1b mRNA demonstrated the presence of mdr1a/mdr1b mRNA around brain capillaries and, surprisingly, in granule cells of the dentate gyrus. Presence of mRNA around brain capillaries suggests that Pgp is expressed at the endothelial cells forming the blood-brain barrier. This finding is consistent with previous studies demonstrating expression of Pgp in endothelial cells at the protein level (Thiebaut *et al.*, 1989; Cordon-Cardo *et al.*, 1990; Sugawara *et al.*, 1990; Lechardeur *et al.*, 1996; Beaulieu *et al.*, 1997). Under normal, healthy conditions Pgp is likely only expressed by endothelial cells, although it can not be excluded that under certain pathological conditions astrocytes may express Pgp as well.
At the protein level, Pgp was found at capillaries throughout the brain. The expression was particularly high in the PVN, which is likely related to its high vascularisation (Sposito and Gross, 1987; Badaut *et al.*, 2000). Within the hippocampus, most Pgp was found at capillaries in the stratum lacunosum moleculare.

Remarkably, mdr1a/mdr1b mRNA was also detected in hippocampal neurons, particularly in the granule cells of the dentate gyrus. Although at the protein level Pgp in the granule cells was below the detection limit, the mRNA signal appears to be specific. Whether Pgp expressed at granule cells plays a role in modulating glucocorticoid actions on the dentate gyrus remains unresolved for now.

Glucocorticoid transport at the blood-brain barrier

The data regarding Pgp-mediated transport of corticosteroids presented in this thesis have corroborated and extended previous literature data regarding the ability of Pgp to transport corticosteroids. Our data show that Pgp transports dexamethasone, prednisolone, cortisol,

cortisone and, to a lesser extent, aldosterone (table 1). It does not transport corticosterone and cortexolone. It is remarkable that, in spite of its broad spectrum of substrates (Schinkel *et al.*, 1994), Pgp distinguishes subtle differences in steroid structure. Comparison of the molecular structures of these steroids reveals that the 17-hydroxyl moiety in combination with an 11-hydroxyl or 11-oxo moiety might determine the ability of MDR1 Pgp to transport steroids (table 1), as was previously postulated with regard to mdr1b Pgp by Bourgeois *et al.* (1993) using another method. Pgp transports steroids having both these hydroxyl-groups (as prednisolone and dexamethasone). Steroids lacking one of these groups (as aldosterone, corticosterone and cortexolone) and steroids without any of these groups are minimally if at all transported. The high affinity MR ligand deoxycorticosterone belongs to the latter group and therefore it should easily be retained in 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.

TABLE 1. Steroid transport capabilities of P-glycoprotein. Summary of data derived from studies described in this thesis and from literature.

corticosteroid	11-OH	17-OH	transport by Pgp
cortisol/hydrocortisone	+	+	+
corticosterone	+	-	- [a]
dexamethasone	+	+	+
prednisolone	+	+	+
cortisone	-	+	+
aldosterone	-/+ [b]	+	+/-
cortexolone/deoxycortisol	-	+	- [c]
deoxycorticosterone	-	-	- [c]
dehydrocorticosterone	-	-	n/a
methylprednisolone	+	+	+
triamcinolone	+	+	+
betamethasone	+	+	+
progesterone	-	-	- / inhibitor [d]
RU486/mifepristone	-	(+)	- / inhibitor [d]

a. Corticosterone might be weakly transported by mdr1b Pgp

b. Aldosterone mainly circulates in the hemiacetal form (see figure 1 Introduction).

c. Both deoxycortisol and deoxycorticosterone may inhibit Pgp function.

d. Both progesterone and RU486 are not transported by Pgp, but inhibit Pgp-mediated transport of other substrates including glucocorticoids

n/a not available

The findings have extended our view on the *in vivo* importance of Pgp in modulating synthetic glucocorticoid action. As synthetic glucocorticoids are hampered to enter the brain, they predominantly act on peripheral glucocorticoid targets when present at low plasma concentrations. At higher plasma levels, direct central action might progressively emerge, although peripheral action may probably still be more pronounced. Central effects at low plasma levels should likely be ascribed to decreased rather than increased central glucocorticoid action, as synthetic glucocorticoids (particularly dexamethasone) are excluded from the brain and, simultaneously, suppress pituitary-adrenal secretion through negative actions at the pituitary level. Furthermore, treatment with synthetic glucocorticoids will lead to a shift in MR/GR balance, as their ability to activate MR is relatively low, whereas they are able to act potently via GR.

Relevance of Pgp-mediated transport of cortisol for endogenous glucocorticoid exposure of the human brain is suggested by the increased corticosterone:cortisol ratio in post mortem brain as compared to human plasma. While in human plasma corticosterone concentrations are only 5% of cortisol levels, in the brain corticosterone levels are 30% of those of cortisol as determined using LC-MS. Although no direct comparison can be made between absolute plasma and brain glucocorticoid levels from the same subjects, these findings do suggest that cortisol levels in brain are 6 times lower than those in blood, which would result in a decrease of total glucocorticoid levels.
The comparison of brain and plasma ratios relies on the unproven assumption that there is no selective transport or clearance of corticosterone by some other factor. Steroid transport may be a more common phenomenon than currently acknowledged, as exemplified by the polar transport of glucocorticoids including corticosterone in the untransfected LLC-PK1 monolayers. Although autoradiography data regarding corticosterone uptake into brain do not provide any indication of transport by other yet unknown steroid transporters, it cannot be excluded that inward transporters may exist at the BBB.

The present data indicate that plasma levels of cortisol, even ‘free’, non-CBG bound cortisol, or dexamethasone may not mirror brain levels. Unfortunately, determination of CSF levels of glucocorticoids may be of limited value, as Pgp is not expressed at the blood side of the blood-CSF-barrier (BCB), but rather at the CSF side (Rao *et al.*, 1999). It may thus pump glucocorticoids into the CSF. Brain and CSF likely constitute different compartments not reflecting linear relationships in glucocorticoid concentrations, in the same way as recently was postulated for drugs that are Pgp substrates (De Lange and Danhof, 2002).
Our autoradiography film data, indeed, showed that in wild type mice radioactive labelling was restricted to the choroid plexus indicating free access of glucocorticoids to this structure and possibly to the ventricles through the BCB. Cortisol, dexamethasone and other Pgp substrates like prednisolone may slowly gain access to the brain through the cerebroventricular system (Rees *et al.*, 1975; Stumpf *et al.*, 1989), or circumventricular organs

and may diffuse into brain areas in the immediate vicinity of the ventricles. However, since the surface of the BBB is approximately 5000 times greater than the surface of the BCB (Pardridge *et al.*, 1981), the uptake in brain tissue as a whole will probably remain considerably reduced even in presence of high plasma levels. This may apply to the PVN even more strongly, as this brain area has a high density of Pgp-expressing capillaries and thus has a high capacity of efflux of Pgp substrates including glucocorticoids.

Cortisol access

Cortisol is one of the first endogenous Pgp substrates identified so far. Active transport of cortisol may be a physiological role of Pgp. In light of the huge number of Pgp substrates presently known, surprisingly few endogenous substrates have been identified. This lack of endogenous substrates and the strongly increased sensitivity of the mdr1a knockout mice to neurotoxic drugs essentially form the basis of the generally accepted view that protection of the brain and/or BBB against xenotoxic compounds is the main role of Pgp at the BBB, like intestinal Pgp protects the whole body against orally supplied xenobiotics. However, multiple additional physiological roles of Pgp have been proposed with regard to transport of endogenous substrates (Johnstone *et al.*, 2000; Garrigues *et al.*, 2002), but it is presently unknown whether any of these proposed additional roles are in any way related to BBB Pgp function. A recently postulated specific function of Pgp at the BBB may be excretion of endogenous substrates out of the brain (King *et al.*, 2001; Lam *et al.*, 2001). The hampered uptake of cortisol suggests that modulating central glucocorticoid feedback or protection of neurons from excess glucocorticoid endangerment may also be among the physiological roles of BBB Pgp in humans. As a consequence, peripheral actions of cortisol might be relatively more potent than central actions.

As glucocorticoids are potent modulators of neuronal activity and function, hampered uptake of cortisol may have strong impact on human brain function. The results presented in chapter 3 do not prove unequivocally that Pgp actually plays a functional role in modulating the actions of cortisol in human brain. Although Pgp is clearly able to transport this glucocorticoid and hampers it from entering the mouse brain, rather high levels of cortisol in post-mortem human brain samples were found. For obvious reasons we were obligated to measure glucocorticoid levels in post-mortem brain samples without accompanying plasma samples from the same subjects, and thus direct comparisons could not be made. However, the increased ratio of corticosterone over cortisol suggested that cortisol was indeed hampered to enter the human brain. Consequently, brain levels of cortisol might be considerably reduced compared to plasma levels in contrast to corticosterone levels, with possible consequences for occupation of corticosteroid receptors.

The ratio of corticosterone over cortisol under physiological conditions may even have been underestimated. Determinations of corticosteroid levels in post-mortem samples may be confounded by the uncertainty of stress pathology in the period prior to death. Extremely

increased plasma glucocorticoid levels might saturate the Pgp transport mechanism enhancing the brain cortisol levels relative to those of corticosterone, although our *in vitro* studies do not provide any indication that Pgp-mediated transport of cortisol is saturated within the physiological range of plasma cortisol levels. Our finding suggesting impaired uptake of cortisol into the brain at physiological levels may be consolidated using other methods like microdialysis in animals in which cortisol is circulating in plasma or in animals receiving exogenous cortisol.

Corticosterone access to rodent brain

The lack of Pgp-mediated exclusion of corticosterone from the brain of wild type mice indicates that Pgp probably does not modulate glucocorticoid action in rodent brain. Several studies have reported corticosterone transport by Pgp, but these studies mostly rely on Pgp encoded by *mdr1b* (Wolf and Horwitz, 1992; Bourgeois *et al.*, 1993; Uhr *et al.*, 2002). This second rodent Pgp may have some capacity to transport corticosterone, but its capacity to transport dexamethasone, prednisolone and cortisol is much larger (Bourgeois *et al.*, 1993).

Since we have used mdr1a single knockout mice to demonstrate the involvement of Pgp in hampering the access of glucocorticoids to the brain, some mdr1b-encoded Pgp might still be present in these mice, which may affect corticosterone uptake into the brain. However, in agreement with the finding that the mdr1b gene is not expressed at the BBB at least under normal *in vivo* conditions (Jette *et al.*, 1995), mdr1b (-/-) mice do not show enhanced brain uptake of various confirmed mdr1b Pgp-substrates (Schinkel *et al.*, 1997). Upregulation of mdr1b expression has been shown to occur in *in vitro* models of BBB (Barrand *et al.*, 1995; Demeule *et al.*, 2001). However, as mdr1b expression has not been shown to be upregulated in brain homogenates of these mice (Schinkel *et al.*, 1994), compensatory upregulation of this Pgp at the BBB of mdr1a (-/-) mice is not likely to have influenced corticosterone brain uptake. Furthermore, it is clear that disruption of the mdr1a gene severely affects the uptake of various other glucocorticoids, despite the efficacy of mdr1b Pgp to transport these glucocorticoids (Bourgeois *et al.*, 1993). Thus, potential presence of mdr1b at the BBB in mdr1a mutant mice is not likely to affect glucocorticoid uptake into the brain of these mice.
Mdr1b mRNA has been found in whole brain homogenates (Croop *et al.*, 1989; Schinkel *et al.*, 1994), which should likely to be ascribed to brain parenchyma cells such as astrocytes and microglia that express this isoform (Lee *et al.*, 2001a) and possibly to granule cells in the dentate gyrus. The lack of mdr1b might have masked the actual effect of Pgp at the BBB on brain uptake of corticosterone in the radioactive uptake study of Uhr *et al* (2002). They showed that the complete absence of both Pgp isoforms in mdr1a/1b double knockouts resulted in a two-fold accumulation of ^{3}H-corticosterone into the brain. Enhanced accumulation of corticosterone in brain of these mice may be attributed to an increased volume of distribution in brain due to enhanced uptake into glial cells. Such an increase is not assumed to affect retention at corticosteroid receptors in neuronal cells.

The presence or absence of mdr1b Pgp may affect brain uptake of corticosterone in indirect ways through effects on steroid metabolism and/or adrenal secretion. Impaired function of hepatic Pgp in both single and double knockout mice might affect glucocorticoid metabolism as altered Pgp expression may affect the steroid metabolising enzyme cytochrome P450 3A4 in the liver (Baron *et al.*, 2001). Although not verified, hepatic metabolism is less likely to be affected in mdr1a knockout mice than in mdr1a/1b double knockouts, as compensatory increased hepatic mdr1b Pgp in mdr1a knockouts (Schinkel *et al.*, 1994) may compensate for loss of mdr1a Pgp. Indeed, in contrast to the mdr1a knockout mice, the mdr1a/1b double knockouts showed increased plasma levels of radioactivity after administration of ^{3}H-corticosterone (Uhr *et al.*, 2002). An altered steroid metabolism might also partly explain the decreased activity of the HPA system seen in mdr1a/1b (-/-) mice (Müller *et al.*, 2003).
In addition, the presence of mdr1b Pgp at the mouse adrenal cortical cells may facilitate corticosterone secretion from the adrenal (Altuvia *et al.*, 1993), which might increase corticosterone plasma levels. Although its role for adrenocortical secretion is not firmly established, the lack of mdr1b could explain the reduced amount of circulating corticosterone in mdr1a/1b knockouts (Uhr *et al.*, 2002). It is not known what a life-long reduction in corticosterone levels implies for the development of the HPA axis.

Another way corticosterone may affect its own uptake into the brain might be through its effects on BBB integrity, which is mediated by GR present in endothelial cells (Gaillard *et al.*, 2001). It has been shown that ADX increases the permeability of the BBB to macromolecules, which was restored by corticosterone replacement (Long and Holaday, 1985). However, a specific effect of corticosterone-induced changes in BBB integrity on glucocorticoid passage has not been reported, and is not likely to occur, as the passage of lipophilic compounds like glucocorticoids is probably not affected by changes in paracellular transport. On the other hand, it is unknown whether corticosterone may affect the expression or functionality of Pgp.

No study so far has revealed corticosterone as a major Pgp substrate in contrast to cortisol. Even the study of Uhr *et al.* (2002) corroborated the much larger effect of the presence of Pgp on the uptake of cortisol into mouse brain compared to its effect on uptake of corticosterone. Transport of corticosterone was not affected by the presence of the single human MDR1 gene in our monolayers, suggesting that Pgp does not hamper entry of this glucocorticoid into human brain. Thus, Pgp is not likely to profoundly affect corticosterone feedback to rodent and human brain.

Aldosterone access

The transport studies in our MDR1 monolayers show that Pgp only weakly transports the high-affinity MR-ligand aldosterone. These results agree with several *in vitro* studies on Pgp-mediated transport of aldosterone. Using comparable monolayers to those in our studies, Ueda *et al* (1992) have demonstrated that aldosterone is moderately transported by the human MDR1 Pgp, while Bourgeois *et al*. (1993) showed that cortexolone 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 ^{3}H-corticosterone and ^{3}H-aldosterone to adrenalectomised rodents both steroids are retained very well in limbic brain structures that abundantly express MR (Birmingham *et al.*, 1984). However, in adrenally intact animals only little aldosterone is extracted from hippocampal cell nuclei relative to corticosterone, probably because the latter steroid circulates in a one hundred to one thousand higher concentration in the blood (Yongue and 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 11ß-steroid dehydrogenase that breaks down corticosterone allowing access of aldosterone to MR (Seckl, 1997). In 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.

Glucocorticoid feedback to the brain

Different roles cortisol and corticosterone?

Whether cortisol acts directly in brain or not, the preferential uptake of corticosterone in human brain suggests that this latter endogenous glucocorticoid may play a more prominent role in human brain function than hitherto recognised (figure 1). In contrast to rodents, both cortisol and corticosterone are circulating in human plasma, although corticosterone is present at tenfold lower levels than cortisol. In human, the presence of Pgp at the BBB might differentiate the time-course of uptake into the brain of cortisol and corticosterone during rises in the plasma corticosteroid levels by slowing down the uptake of cortisol. Due to the differential uptake of cortisol and corticosterone, the human glucocorticoid feedback system might be more complex than the rodent system. The resultant species difference complicates extrapolation of data regarding central glucocorticoid action from rodent to human.

The question arises whether cortisol and corticosterone might affect brain function differently. The differential interaction of Pgp with both hormones is exceptional in the sense that neither pharmacological nor physiological differences between both hormones have been discerned thus far. Both hormones are secreted by the human adrenal cortex upon activation by ACTH and both have a very high affinity for MR combined with a tenfold lower affinity for GR. Therefore, both glucocorticoids are generally considered to act in similar ways on brain function.

However, a more thorough examination of literature data reveals small but consistent differences between both corticosteroids in affinity and transactivation properties of MR. Results from different research groups consistently show that corticosterone has a slightly higher affinity for both rat and human MR (Lan *et al.*, 1981; Krozowski and Funder, 1983; De Kloet *et al.*, 1984a; Arriza *et al.*, 1987), which may underlie the reported higher effectiveness

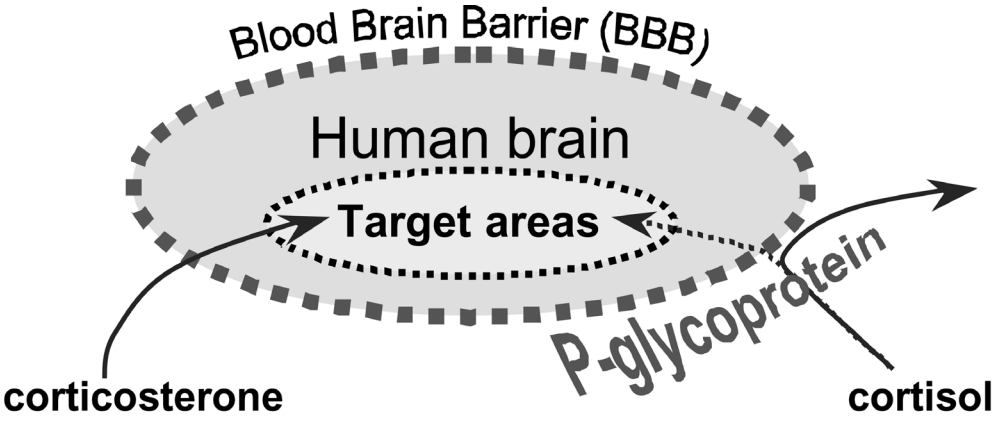

FIGURE 1. Proposed model of glucocorticoid feedback to human brain.
Whereas corticosterone easily enters the brain, the main human adrenal glucocorticoid cortisol is hampered to reach glucocorticoid target areas due to P-glycoprotein mediated efflux at the blood-brain barrier. Thus, corticosterone may play a more important role as mediator of glucocorticoid action in human brain than hitherto recognised.

of corticosterone in promoting human MR transactivation (Lombes *et al.*, 1994; Hellal-Levy *et al.*, 1999). Whether GR properties also differ for both hormones is more difficult to uncover due to lack of literature data. Very few investigations have tested both glucocorticoids in a single study both showing cortisol as the more potent one in transactivation of human GR (Arriza *et al.*, 1988; Hellal-Levy *et al.*, 1999).
Tentatively, corticosterone may be the more active glucocorticoid at the MR in human brain with a potentially different role than cortisol. In this regard, it may be of relevance that the distribution of MR in humans seems to be broader than in rodents, with relatively high levels found in the prefrontal cortex (Lopez *et al.*, 1999). This structure is essential for mood and cognitive processing and may be particularly sensitive to glucocorticoid feedback in humans (Lupien and Lepage, 2001). In addition, a recent study reported that GR levels are relatively low in hippocampus of rhesus monkey in contrast to MR levels (Sanchez *et al.*, 2000). Although this latter finding should be confirmed for human hippocampus, the species-specific distribution of MR suggests that this receptor might have a more pronounced role in mediating glucocorticoid actions in human brain than in rodent brain.

It is presently unknown whether these differences in affinity and transactivational response are actually relevant for the actions of both glucocorticoids on normal human brain functioning. Besides these features there are a lot of other factors which also determine the final response to glucocorticoids and may have presently unknown differential interactions with both hormones. These include co-activators/co-repressors and a variety of transcription factors such as NFκB and AP-1 (Meijer, 2002), but also interaction with membrane-bound receptors (Borski, 2000). Anyway, the difference in relative brain uptake and possibly other features warrants further studies into the potentially different roles of cortisol and corticosterone in

modulating human brain function. The use of cortisol secreting animals would facilitate these studies as species differences in brain uptake of glucocorticoids and receptor distribution complicate extrapolation of rodent data to the human situation.

Dexamethasone feedback actions

To show that impaired uptake of glucocorticoids into brain may actually affect brain function rats treated with different doses of the synthetic glucocorticoid dexamethasone were studied. During the past four decades an increasing body of literature has provided evidence that access of dexamethasone to the brain is impaired, which was demonstrated to be caused by the presence of Pgp at the BBB (Meijer *et al.*, 1998). We have now demonstrated its impaired access in a functional way showing a divergence of direct actions of low-dose dexamethasone on central and peripheral glucocorticoid targets. Dexamethasone circulating at low concentrations does not act at several glucocorticoid responsive genes expressed in the brain. In contrast, these concentrations of dexamethasone potently acted on various peripheral glucocorticoid targets. As both these central and peripheral actions are mediated by a single receptor - the GR - this suggests impaired access to the brain of dexamethasone, although differential cell- or gene-specific sensitivity may provide an alternative explanation (Meijer *et al.*, 2003). This barrier is not complete, as after high-dose treatment dexamethasone turned out to have entered the brain in sufficient amounts to suppress expression of central glucocorticoid responsive genes.

At low plasma concentrations dexamethasone acts primarily at the pituitary level to suppress pituitary-adrenal activity. This is supported by its differential effects on POMC and c-fos mRNA expression in the anterior pituitary. The POMC gene plays a key role in mediating stress and glucocorticoid effects on ACTH/corticosterone secretion. It is well known that CRH and glucocorticoids regulate both basal and stress-induced transcription of the POMC gene in opposite and complex ways (Gagner and Drouin, 1985; Lundblad and Roberts, 1988). Dexamethasone affects POMC expression levels in different ways, through a DNA-binding dependent way and through protein-protein interactions. The dexamethasone-GR complex can influence POMC gene transcription by binding to a negative glucocorticoid responsive element present in the POMC promoter (Drouin *et al.*, 1993). Activated GR may also bind to AP-1 complexes containing c-fos and suppress the CRH-induced transcription of POMC (Autelitano, 1994). Five-day treatment with small amounts of dexamethasone decreases the expression of POMC mRNA, but does not affect the stress induced c-fos mRNA response in the anterior pituitary. As stress-induced pituitary c-fos induces POMC expression, this indicates that dexamethasone acts downstream from pituitary c-fos in its inhibition of POMC transcription.
After three-week treatment with small amounts of dexamethasone the suppression of POMC mRNA has disappeared, but at that time the c-fos mRNA response is augmented. Maximal c-fos induction may prevail over the dexamethasone-mediated inhibition of POMC transcription (Autelitano and Sheppard, 1993) after this long-term treatment, which may explain the lack of

POMC mRNA inhibition, assuming that basal pituitary c-fos mRNA levels were increased similar to the augmented stress-induced c-fos mRNA levels.

Creation of a low central corticoid state

We hypothesised that treatment with small amounts of dexamethasone would create a central ADX-like state without removal of glucocorticoid action in the periphery (see figure 9 introduction). The issue of whether dexamethasone treatment actually creates a low corticosteroid state in the brain is still some matter of debate (Feldman and Weidenfeld, 1995; Lupien and McEwen, 1997; De Kloet *et al.*, 1998; Roozendaal, 2000; Reul *et al.*, 2000b; Belanoff *et al.*, 2001; Feldman and Weidenfeld, 2002). Although our data corroborated the notion that low-dose dexamethasone treatment results in relatively weak central actions, it is less certain that low-dose treatment completely depletes glucocorticoids from the central glucocorticoid targets resulting in a condition resembling that of ADX.

The increased paraventricular CRH mRNA expression levels in both low-dose dexamethasone treated and ADX groups compared to untreated groups supports this latter notion. However, this increase is seen only after extended treatment of three weeks. The stress-induced responses of CRH hnRNA and c-fos mRNA expression in low-dose dexamethasone treated groups do not differ from both ADX and untreated control groups. Furthermore, unlike ADX rats, dexamethasone treated rats do not show apoptosis of granule cells of the dentate gyrus, suggesting that some residual glucocorticoid action may still be present in low-dose treated rats. Protection of granule cells against apoptosis is a MR mediated process and, thus, an increase in the occurrence of apoptosis might be expected if depletion of glucocorticoids from brain after dexamethasone treatment would also abolish the occupancy of hippocampal MR (Sloviter *et al.*, 1989). As dexamethasone does not activate MR *in vivo* (Reul *et al.*, 2000b), the lack of apoptosis is suggestive of presence of at least some corticosterone in brain, although occupation of MR by aldosterone might give an alternative explanation (Woolley *et al.*, 1991).

Clearly, the effects of low-dose dexamethasone treatment on other central corticosteroid responsive markers should be examined to confirm or reject the creation of an ADX-like state. These markers should not only be looked for in the PVN (in which AVP is of particular interest), but also in other glucocorticoid responsive brain areas like hippocampus and amygdala.

Model for central corticosterone action

Even though the complete removal of glucocorticoids from the brain is not yet confirmed, the model clearly shows hyporesponsiveness to activation of pituitary-adrenal secretion. Our findings show that rats treated with low-dose dexamethasone have a strongly reduced circadian rhythmicity of corticosterone plasma levels. Furthermore, they are hyporesponsive to stress-induced activation of pituitary-adrenal secretion. The reduced basal and stress-induced corticosterone secretion in conjunction with the impaired access of dexamethasone to the brain under these conditions may make these rats useful as a model of impaired

glucocorticoid signalling in brain. As apoptosis is sensitive to very low residual levels of corticosterone (Conrad *et al.*, 1997), the lack of apoptosis indicates that this model may provide a way of modulating glucocorticoid signalling in which brain function is less severely disturbed than after complete ADX. In conjunction with the activation of peripheral GR in this model, this may be especially advantageous when studying subtle GR-mediated actions of corticosterone action on brain function.

For instance, involvement of GR in memory processes (Oitzl and De Kloet, 1992) or in neural plasticity, particularly of hippocampal neurons (McEwen, 1999; Duman, 2002) may be studied using this model. Other aspects of brain function that can be studied might be the effects of circadian rhythmicity of glucocorticoid levels on feeding behaviour (Castonguay, 1991; Müller *et al.*, 2000), motor activity and sleep (Bradbury *et al.*, 1998; Born and Fehm, 1998).

Furthermore, this model may be helpful in studying processes in which the MR/GR balance is critically involved, *e.g.* homeostatic control of stress responsiveness, behavioural adaptation and cognition (De Kloet, 1991). Treatment with dexamethasone will, irrespective of the dose administered, always result in a reduced occupation of MR through suppression of endogenous glucocorticoids, as dexamethasone does not bind to MR *in vivo*. This may lead to shifts in the balance between MR- and GR-mediated effects. As both receptor types mediate distinct but co-ordinated actions on neuronal excitability, synaptic plasticity and learning and memory, these shifts in MR/GR balance may disturb brain function and may thus further impair the ability to maintain brain homeostasis.

Balance of dexamethasone feedback and central drive

HPA-axis activity is not only determined by the intensity of glucocorticoid feedback. Dexamethasone suppression of pituitary-adrenal activity might be, at least partly, surpassed by an increased central drive. This may explain the small stress-induced corticosterone response seen in animals treated with the lowest amounts of dexamethasone. It would further explain studies showing that removing the inhibitory hippocampal input either by hippocampectomy or fornix transection causes dexamethasone resistance (Feldman and Conforti, 1980; Sapolsky *et al.*, 1991; Feldman and Weidenfeld, 1995). Furthermore, it is supported by the finding that depletion of hypothalamic norepinephrine and serotonin enhances the dexamethasone negative feedback effect on adrenocortical secretion (Feldman and Weidenfeld, 1991; 1995). The type or intensity of stressors may also influence the extent of dexamethasone suppression (Haracz *et al.*, 1988; Lurie *et al.*, 1989).

Rats treated with 1 µg/ml dexamethasone through their drinking water do not show any stress-induced corticosterone response whereas their central stress response at the level of the PVN was not reduced below those of intact animals, suggesting that in these animals dexamethasone feedback at the pituitary was strong enough to constrain the pituitary-adrenal activity. However, in these animals some dexamethasone may have entered the brain, as suggested by the unaltered CRH mRNA expression of the 1 µg/ml group in contrast to the

increased expression in the 0.5 µg/ml group. Receptor binding studies have indeed shown that doses which are slightly higher than those used in the present study (1-1.5 µg/ml), administered overnight through drinking water, very modestly reduces the available numbers of hippocampal GR in intact rats, while resulting in strongly reduced pituitary GR numbers (Spencer *et al.*, 1990; Miller *et al.*, 1990). Although both studies did not estimate the GR occupation in hypothalamus, these amounts may also slightly reduce available receptor numbers in this area. Thus, also in animals treated with 1 µg/ml dexamethasone through their drinking water the main site of action on the HPA-axis is the pituitary, but for full suppression of the stress-induced secretion of corticosterone some dexamethasone may need to act at the hypothalamic level.

The low central corticoid state created by dexamethasone may even increase the stress responsivity of the paraventricular CRH/AVP-secreting neurons due to removal of glucocorticoid negative feedback to the PVN itself and to brain areas involved in activation of the PVN. Consequently, the central drive on the pituitary in response to stress might be augmented, which may override the dexamethasone-mediated inhibition of ACTH production and secretion. This phenomenon may be viewed as glucocorticoid feedback resistance although the apparent magnitude rather than the potency of feedback has been changed.
Thus, the magnitude of the inhibitory effect of DEX on pituitary-adrenal secretion depends on the balance between the stimulatory action of hypothalamic secretagogues and the inhibitory action of DEX on the anterior pituitary. Differential excitatory influences of extrahypothalamic brain areas on the paraventricular neurons in the PVN may influence the neurochemical composition of the hypothalamic secretion, which subsequently may affect feedback efficacy of dexamethasone. This makes the presented model of central adrenalectomy less useful in conditions that will shift the balance towards increased central drive and decreased feedback efficacy of dexamethasone.

Dexamethasone feedback in depression

The reduced central glucocorticoid feedback after treatment with low-dose dexamethasone suggests that administration of small amounts of dexamethasone might be helpful in treatment of stress-related disorders. Depressed patients often show a state-dependent hyperactive central CRH-system (Raadsheer *et al.*, 1994; Arborelius *et al.*, 1999), which likely underlies their increased cortisol secretion during a 24hr period (Mitchell, 1998; Holsboer, 1999; Gold and Chrousos, 2002). This hypercortisolemia would conceivably be alleviated by a controlled low-dose dexamethasone treatment that leads to a graded lowering of central glucocorticoid levels. Dexamethasone has indeed been shown to have antidepressant properties (Arana *et al.*, 1995; Wolkowitz and Reus, 1999), although rather high doses were used in these studies. A recovery of a disturbed balance of the two corticosteroid receptor types after dexamethasone treatment may also underlie its therapeutic effect.

Pgp-mediated impaired uptake of dexamethasone and the ensuing central low-corticosteroid state may also provide a rationale for the dexamethasone suppression test (DST). This test is widely used in the clinic often in combination with a CRH challenge, to evaluate the dysregulation of the HPA-axis in depressive patients. After administration of a low dose of dexamethasone the night before, depressives consistently show escape from suppression of baseline or CRH-induced cortisol levels (Holsboer, 2000). An imbalance between the central drive and dexamethasone inhibition at the pituitary level may underlie the escape from dexamethasone suppression seen in the DST (Holsboer, 1999). The sustained hyperactive CRH-system of depressed patients may be accompanied with an increased release of AVP (Purba *et al.*, 1996; Holsboer, 2000), which synergises with CRH at the corticotrophic cells to stimulate ACTH secretion. The increased AVP may result in an apparent glucocorticoid feedback resistance at the pituitary level, as AVP-stimulated ACTH-secretion is refractory to glucocorticoid feedback (Aguilera and Rabadan-Diehl, 2000). Consistent with a role of vasopressin in dexamethasone nonsuppression, coadministration of CRH and AVP to dexamethasone-pretreated healthy subjects results in a similar ACTH/cortisol response as in depressed patients (Von Bardeleben *et al.*, 1985), while dexamethasone nonsuppression in hyperanxious rats is due to enhanced vasopressin activity (Keck *et al.*, 2002). In depressed patients, the dexamethasone-induced reduction of central feedback may further aggravate the hyperactive central drive of CRH and particularly AVP leading to an escape from the suppressive effect of dexamethasone on cortisol plasma levels. The augmentation of CRH mRNA levels in the rat PVN after low-dose dexamethasone treatment as found in our study may be supportive to this interpretation.

Glucocorticoid feedback impairment in depression

Impaired dexamethasone feedback in major depression is often ascribed to disturbed central corticosteroid receptor signalling, particularly with regard to the GR (Holsboer and Barden, 1996; Holsboer, 2000; Pariante and Miller, 2001). Supportive data of a key role of GR signalling in depression has been provided by animal studies. Transgenic mice with defective GR function show features that are, although only partially, reminiscent of depression (Holsboer and Barden, 1996; Müller *et al.*, 2002), whereas different types of antidepressants increase GR mRNA and receptor levels (Peiffer *et al.*, 1991; Przegaliñski and Budziszewska, 1993). Disturbed glucocorticoid feedback may be the cause as well as the consequence of the central hyperdrive. It is difficult in a closed-loop system to ascertain whether the HPA-disturbances in depression represent increased central drive or decreased sensitivity to negative feedback or both. Hypercortisolemia due to increased CRH/AVP drive may subsequently lead to corticosteroid receptor downregulation or dysfunction in brain. Alternatively, resistance to negative feedback through GR may cause a disinhibition of hypothalamic CRH neurons leading to hypercortisolemia.

However, evidence of impaired central glucocorticoid feedback in depression is at present not conclusive. As stated above, the dexamethasone depression test does not assess central glucocorticoid feedback. Decreased hypothalamic GR levels in depressed patients have not

been described so far, and, if present, may merely reflect secondary downregulation due to hypercortisolemia as seen in rodent studies (Sapolsky *et al.*, 1986; Pariante and Miller, 2001). In addition, neither GR polymorphism nor deficits in transcription factors or coregulators involved in glucocorticoid signalling, nor defects in glucocorticoid-driven promoters have been demonstrated to be linked to depressive symptomatology thus far. Furthermore, in response to a CRH challenge, depressives show a blunted ACTH response (Holsboer, 1999; Arborelius *et al.*, 1999), which implies increased feedback due to increased cortisol levels. In support of this interpretation, CRH-stimulated ACTH output is normalised after treatment of depressed patients with cortisol synthesis inhibitors like metyrapone (Von Bardeleben *et al.*, 1988).

On the other hand, healthy subjects at a genetic risk for depression more frequently show mild hypercortisolemia and abnormal DEX/CRH test responses than controls, suggesting that altered feedback inhibition may represent a genetic vulnerability factor to depression (Holsboer, 2000). It has to be verified however whether these high-risk probands will actually develop depression.

The notion of impaired GR function in depression is furthermore questionable because of the effectiveness of the GR antagonist RU486 in raising cortisol plasma levels of patients with psychotic major depression (Belanoff *et al.*, 2002), similar to its effects in healthy subjects (Bertagna *et al.*, 1984; Gaillard *et al.*, 1984). At least, it does not support impaired GR function in the PVN.

Moreover, the effectiveness of RU486 in relieving psychotic and depressive symptoms in these patients may suggest that raised levels of cortisol perpetuate the depressive state through GR-mediated positive feedback actions in extrahypothalamic areas like *e.g.* amygdala (Gold and Chrousos, 2002). These facilitatory actions on afferent inputs to the PVN, may be outbalanced in these patients and may disproportionately activate or disinhibit the paraventricular CRH/AVP neurons overriding the normal glucocorticoid negative feedback (Makino *et al.*, 2002). In concert with other imbalances of the stress system such as hyperactivation of CRH and noradrenergic systems, this vicious cycle may sustain depression at least in this set of depressed patients.

It has been shown that activation of GR in the central nucleus of the amygdala increases rather than decreases CRH mRNA levels (Makino *et al.*, 1994; Schulkin *et al.*, 1998). The amygdaloid CRH system may have a stimulatory effect on the PVN (Van de Kar *et al.*, 1991). Interestingly, a recent report showed that in ADX rats chronic intracerebroventricular infusion of corticosterone resulted in increased basal and stress-induced ACTH levels and a tendency for increased paraventricular CRH immunoreactivity (Laugero *et al.*, 2002). Although, at present, no direct evidence mutually linking hypercortisolemia and amygdaloid CRH activation is available, a recent study showed that local implantation of corticosterone into the amygdala prolonged the corticosterone response to a behavioural stressor (Shepard *et al.*, 2003). Within the hippocampus high corticosterone levels acting through GR also appears to disinhibit the HPA-axis (Van Haarst *et al.*, 1997). This suggests that under some conditions

glucocorticoids may indeed be able to activate rather than inhibit the HPA-axis. High-dose RU486 treatment may rapidly reset the balance in facilitatory and suppressive input to the PVN, although the mechanism is poorly understood at present.

Although the emphasis of studies on involvement of corticosteroid receptors in depression has been put on the role of the GR, an increasing amount of evidence indicates that altered MR function may be of relevance as well (Reul *et al.*, 2000b). Post-mortem examination of brains of suicide victims with a history of depression revealed a reduction of MR mRNA levels without alterations of GR mRNA levels (Lopez *et al.*, 1998). Treatment with antidepressants may upregulate MR expression contributing to increased glucocorticoid negative feedback (Brady *et al.*, 1991; Reul *et al.*, 1993; Reul *et al.*, 1994). The MR upregulation may precede both the antidepressant-induced upregulation of GR and the enhanced negative feedback on the HPA-axis (Seckl and Fink, 1992; Reul *et al.*, 1993) and may thus make up the primary cause of restoring normal HPA-axis activity in major depression. The importance of appropriate MR function is further supported by a study showing that systemic administration of antimineralocorticoids impaired the therapeutic effect of the antidepressant amitryptiline (Holsboer, 1999). It was recently postulated that chronic stress might impair the normal CRH-mediated stress-induced upregulation of hippocampal MR leading to a progressively deteriorating hippocampal inhibition of HPA-function resulting in a hyperactive HPA-axis (Reul and Holsboer, 2002).

Taken together, impaired function of GR and especially MR signalling pathways may play a role in the aetiology and pathophysiology of depression. A change in the balance of MR- and GR-mediated actions may underlie the progressive reset of the HPA activity in depressive disorder (De Kloet *et al.*, 1998), whereas antidepressants may restore HPA-axis activity through facilitation of MR function and/or upregulation of MR levels, with subsequent beneficial effects on GR function and/or levels. Modulation of corticosteroid transport at the BBB by Pgp may be a novel strategy to find new treatments of stress-related disorders.

Antidepressants, glucocorticoid feedback and Pgp

An intriguing alternative mechanism of action of antidepressants involving Pgp was recently postulated by Pariante *et al.* (2001) based on *in vitro* findings. They showed that coincubation of dexamethasone or cortisol at low non-saturating concentrations with various antidepressants resulted in enhanced GR function without increased GR levels in L929 fibroblast cells. Coincubation with dexamethasone in presence of a Pgp inhibitor or with corticosterone does not lead to a facilitation of GR function, suggesting involvement of transport mediated by a steroid membrane transporter like Pgp. Recently, it was indeed confirmed that this transporter shows a similar steroid transport profile as Pgp, and RT-PCR demonstrated presence of both mdr1a and mdr1b mRNA (Webster and Carlstedt-Duke, 2002).

This may imply that antidepressants may inhibit the function of Pgp at the BBB, thus increasing uptake of cortisol into the brain and enhancing GR-mediated negative feedback on the HPA-axis, decreasing HPA-axis hyperactivity in depression. Acute facilitation of GR function may precede upregulation of GR levels, implying that GR upregulation may be the consequence of facilitated GR function rather than the cause (Pariante and Miller, 2001). However, as stated before, upregulation of MR preceding GR upregulation may also make up the primary cause of restoring normal HPA-axis activity in major depression.

Effectiveness of other glucocorticoids

The reduced central glucocorticoid feedback after treatment with low-dose dexamethasone raises the question whether treatment with low doses of other glucocorticoids may result in a similar reduction of total brain glucocorticoid levels. Glucocorticoids like prednisolone and hydrocortisone (=cortisol) are Pgp substrates and are able to activate GR as well. Like dexamethasone they might be able to suppress the pituitary-adrenal secretion predominantly at the pituitary level, while they would simultaneously be hampered to enter the brain leading to a low central corticosteroid state. However, there are clear pharmacochemical, pharmacokinetic and pharmacodynamic differences among these three glucocorticoids, which likely interfere with their ability to create a central ADX-like condition. Clear differences in potency to inhibit HPA-axis activity are caused by differences in relative corticosteroid receptor affinities, differences in binding to CBG and differences in metabolism affecting plasma and biological half-life.

The plasma half-life of glucocorticoids is much shorter than the duration of their biological actions, but for both pharmacological parameters glucocorticoids are arranged in the same order. The biological half-life of hydrocortisone is 8-12 hr, whereas the presence of an additional double bond (prednisolone and dexamethasone) or a fluorine atom (dexamethasone) enlarges the plasma half-life and consequently the biological half-life to 12-36 hr (prednisolone) or even 48 hr (dexamethasone) (Jusko and Ludwig, 1992).

Both dexamethasone and prednisolone bind with very high affinity to the GR (<1nM). Hydrocortisone, like corticosterone, binds with very high affinity to the MR (<1nM), whereas it has a 4-10 fold lower affinity to GR. Although dexamethasone has some affinity to the MR *in vitro*, it does not exert any agonistic actions via MR *in vivo* (De Kloet, 1991). Limited access to the MR can not fully explain this discrepancy as Pgp is not likely to protect renal MR and dexamethasone is not a substrate for renal 11ß-HSD type 2 (Reul *et al.*, 2000b). The instability of dexamethasone-MR complexes due to an extremely high dissociation rate of dexamethasone may explain its inability to activate MR *in vivo* (Reul *et al.*, 2000b). Whether this phenomenon also applies to prednisolone is not known, but, anyhow, its mineralocorticoid potency is less than that of cortisol.

Plasma protein binding and particularly binding to CBG interferes with biological activity, as only non-CBG-bound fraction is available for distribution to receptor sites (Pardridge, 1981; Breuner and Orchinik, 2002). Both hydrocortisone and prednisolone, but not dexamethasone,

bind to CBG. The degree of plasma protein binding of prednisolone is dose-dependent due to the low capacity of CBG (Jusko and Ludwig, 1992). Displacement of cortisol by prednisolone due to competition increases the unbound, freely available plasma levels of cortisol (Pugeat *et al.*, 1981). All three steroids may suppress hepatic CBG production, which may increase free cortisol levels, but dexamethasone is much more potent than prednisolone and hydrocortisone (Smith and Hammond, 1992). Of particular importance is the intracellular presence of CBG in corticotroph cells of the anterior pituitary (De Kloet *et al.*, 1984b). Here, CBG may sequester prednisolone and hydrocortisone limiting their accessibility to the pituitary GR.

Taken together, the properties of dexamethasone likely make this hormone the most favourable glucocorticoid in creating a low corticoid condition in the brain. In addition to its ability to be transported by Pgp, its high affinity for the GR combined with its lack of plasma and pituitary CBG binding and its long duration of biological activity favours its potency in acting at the anterior pituitary to inhibit pituitary-adrenal secretion. Due to CBG binding and lower affinity to GR much higher doses (30-70x) of prednisolone and hydrocortisone are needed to suppress the HPA-axis to a similar extent (Baumann *et al.*, 1985; Gispen-de Wied *et al.*, 1993). The use of these high doses makes it likely that some steroid will enter the brain, which in case of hydrocortisone may easily activate MR.

Concluding remarks and perspectives

In conclusion, the findings of the studies described in this thesis have made evident the importance of corticosteroid transport at the BBB in controlling corticosteroid access to the brain. The efflux transporter Pgp may play a crucial role as an intermediate between brain and periphery by controlling transport of corticosteroids at the BBB. Pgp is able to hamper penetration of various corticosteroids into the brain, particularly when these hormones are circulating at low plasma levels. Impaired uptake of synthetic glucocorticoids such as dexamethasone and prednisolone, but also of the naturally occurring glucocorticoid cortisol, likely results in a reduced occupation of central corticosteroid receptors and thus in a diminished response to these glucocorticoids.

Intriguingly, both mouse and human Pgp do not transport corticosterone in contrast to cortisol, which may underlie the increased ratio of corticosterone over cortisol in post-mortem human brain samples compared to plasma. Future investigations will reveal whether corticosterone rather than cortisol may be the major endogenous corticosteroid in mediating corticosteroid actions, particularly via MR, on human brain function, as suggested by the preferential uptake of corticosterone into human brain.

The brain-selective low corticosteroid state created by administration of low-dose dexamethasone to rats might be used as an animal model to specifically study central roles of corticosterone without the potentially confounding effects of reduced peripheral glucocorticoid effects.

At the BBB several other efflux transporters are expressed besides Pgp. For instance, several members of the multidrug resistance-associated proteins (MRP) have been detected at the brain capillaries (Sun *et al.*, 2003). Whether any of these or any yet unknown transporter may also transport corticosteroids remains to be resolved. Recently, it was demonstrated that MRP1 is able to transport corticosterone and deoxycorticosterone (Webster and Carlstedt-Duke, 2002), but the presence of MRP1 at the BBB remains controversial (Taylor, 2002; Sun *et al.*, 2003). Furthermore, steroid membrane transporters might be present at neuronal cells as well, affecting uptake of glucocorticoids directly at the neuronal membrane (Herr *et al.*, 2000; Pariante *et al.*, 2003).

Modulation of Pgp-mediated transport of corticosteroids may influence central glucocorticoid actions, as exemplified by the inhibition of corticosteroid transport by antidepressants (Pariante *et al.*, 2001). Altered uptake of glucocorticoids may reset MR/GR balance and thus HPA-axis activity. Therefore, Pgp may provide an interesting new target to regulate glucocorticoid feedback to the brain in disorders with disturbed central glucocorticoid signalling such as major depression and post-traumatic stress disorder, and possibly also chronic fatigue syndrome and fibromyalgia.

Pgp plays a key role in protection against a wide variety of drugs including anticancer and antiepileptic drugs and HIV protease inhibitors. These drugs may influence transport of endogenous substrates including corticosteroid hormones but also centrally and peripherally acting compounds (King *et al.*, 2001; Lam *et al.*, 2001). Particularly, inhibitors of Pgp transport may interfere with physiological Pgp function. Some steroids (progesterone, RU486) may inhibit Pgp function (Gruol and Bourgeois, 1994). As Pgp impairs the efficacy of treatment of (brain) cancer, much effort has been put in finding reversal agents to bypass Pgp by inhibiting its transport function. Although some of these Pgp modulators are now in clinical trials, the clinical efficacy remains to be established particularly with regard to their potential side effects (Van Zuylen *et al.*, 2000). Inhibition of Pgp may have undesired side effects by increasing the uptake of cortisol into the brain potentially endangering neuronal survival.

Polymorphisms in the MDR1 gene resulting in altered levels and functionality of Pgp have been shown to affect efficiency of this transporter (Hoffmeyer *et al.*, 2000). Regulation of expression and post-translational modification of Pgp presumably also affect Pgp efflux transport function. Various stimuli that evoke cellular stress responses have been shown to affect Pgp expression (Johnstone *et al.*, 2000; Sukhai and Piquette, 2000). However, with regard to BBB Pgp, little is known about these features. Epileptic insults have been shown to induce Pgp expression in the epileptic lesions in both capillaries and glial cells (Sisodiya *et al.*, 1999; Rizzi *et al.*, 2002; Seegers *et al.*, 2002b). Whether stress-related disorders have any effect on Pgp expression is not yet known. Much work should be done to resolve when and

how Pgp operates as a dynamic regulator of the central access of Pgp-substrates including glucocorticoids.

In addition, it should be found out whether corticosteroids can directly affect Pgp functionality. Preliminary data suggest that glucocorticoids might be able to increase Pgp expression at the BBB (Sérée *et al.*, 1998; Aquilante *et al.*, 2000). This would not only affect glucocorticoid access to the brain but also access of other Pgp substrates.

Chapter 7

SUMMARY AND MAIN CONCLUSIONS

The actions of glucocorticoid hormones are essential for healthy functioning of brain and body, particularly during stress. Secretion of these hormones by the adrenals is tightly controlled through the Hypothalamus-Pituitary-Adrenal-axis (HPA-axis). Activity of the HPA-axis follows a circadian rhythm with highest plasma glucocorticoid levels just prior to awakening. Furthermore, secretion of glucocorticoids is stimulated after stress. This stress response plays an important role in the adaptation to a changing environment.
Glucocorticoids affect a wide range of processes in both periphery and brain. They regulate storage and mobilisation of energy, affect immune and inflammatory responses and act on diverse endocrine systems including the HPA-axis. Their effects in the central nervous system are particularly potent. Within the brain they modulate synaptic plasticity and regulate neuronal function, which likely underlie their effects on behavioural adaptation to stress, learning and memory processes and mood.
Glucocorticoids mainly exert their actions through genomic mechanisms altering gene expression. The genomic actions are mediated by two types of receptors, the high affinity mineralocorticoid receptors (MR) and the lower affinity glucocorticoid receptors (GR). In the periphery glucocorticoid actions are mostly mediated by GR, which is abundantly expressed in almost any cell in the body. Within the brain both types of corticosteroid receptors are present although with a different localisation pattern. MR and GR mediate different but partly overlapping aspects of central glucocorticoid action.

To reach their central target areas glucocorticoid hormones have to enter the brain by passing the blood-brain barrier (BBB). The BBB is a dynamic physical and metabolic barrier consisting of specialised endothelial cells that protects the brain from blood-borne compounds, and plays a role in maintaining brain homeostasis. The BBB can strongly interfere with distribution to the brain of endogenous and exogenous compounds. Generally, hydrophilic and large lipophilic compounds are not able to penetrate the brain, as they are not able to pass cell membranes, whereas small lipophilic compounds can easily cross the BBB by passive diffusion through the endothelial cells. However, for a number of highly lipophilic compounds including the synthetic glucocorticoid dexamethasone BBB permeability is unexpectedly low.
The multidrug transporter P-glycoprotein (Pgp) is an important functional component of the BBB and various other tissues with a barrier function. It acts like a ‘gatekeeper’ at the BBB actively keeping a wide variety of lipophilic, potentially neurotoxic substances out of the brain. This transmembrane protein is encoded by the multidrug resistance (MDR) genes, mdr1a in rodents and the highly homologous MDR1 in humans. Studies using mdr1a (-/-) knockout mice which lack functional Pgp at the BBB, have shown that this efflux transporter is responsible for the apparent low permeation of dexamethasone and a wide range of other compounds that should easily penetrate the BBB as expected based on their size and their sufficient high lipid solubility.
Although the importance of the actions of glucocorticoids in brain is commonly accepted, modulation of glucocorticoid access at the BBB level has hardly been a subject of research as

these compounds are considered to readily pass this barrier. Now that it has been demonstrated that transmembrane proteins are able to transport these hormones, this issue becomes an increasingly interesting subject to study. Any process at the BBB that can influence the endothelial crossing of glucocorticoids would directly affect central corticosteroid receptor occupancy and the magnitude of the central response to corticosteroids.

The **aim** of the studies described in this thesis was to examine the interaction of glucocorticoids and the efflux transporter P-glycoprotein expressed at the BBB as a possibly new level at which access to the brain and thus central corticosteroid receptor function may be controlled. Modulation of access of glucocorticoids to the brain may provide a new way to restore aberrant corticosteroid signalling associated with hypercortisolemia, glucocorticoid feedback resistance or MR/GR imbalance.

In **chapter 2**, findings regarding the expression of Pgp in the brain at the level of mRNA are presented. *In situ* hybridisation with a riboprobe recognising both murine mdr1 genes (mdr1a and mdr1b) demonstrates presence of mRNA in the endothelial cells of brain capillaries throughout the rat brain confirming presence of Pgp at the BBB. Surprisingly, expression of mdr1 mRNA was also found in granule cells of the dentate gyrus, a subfield of the hippocampus highly expressing both MR and GR. Its function at this site has yet to be resolved.

In **chapters 3 and 4**, the interactions of various naturally occurring glucocorticoids and synthetic glucocorticoids with Pgp are described particularly with regard to the uptake of these hormones into the brain. The hypothesis that endogenous glucocorticoids would easily reach the central glucocorticoid target areas, whereas Pgp would protect the brain against exogenous synthetic glucocorticoids, was rejected.
Autoradiographic studies in adrenalectomised mice with a disrupted mdr1a gene confirmed that the synthetic glucocorticoid prednisolone like dexamethasone is hampered to enter the mouse brain and to reach glucocorticoid receptors. Presence of Pgp did not affect the uptake of the endogenous rodent glucocorticoid corticosterone into mouse brain. In sharp contrast to corticosterone, the main human glucocorticoid hormone cortisol was hampered to enter the brain of mice expressing Pgp at the BBB, but not of mice lacking Pgp.
Although the absence of cortisol in the normal physiology of the mouse might explain this differential interaction with mouse Pgp, the remarkable difference between the cortisol and corticosterone transport capability of Pgp was also demonstrated for human Pgp using an *in vitro* model. Pig kidney epithelial cells form monolayers when seeded on filters and when stably transfected with human Pgp MDR1 cDNA these monolayers express high levels of Pgp at their apical side. Polar translocation of Pgp substrates to the apical side of these monolayers is reminiscent of Pgp function at the human BBB. Human Pgp-mediated polar transport of cortisol, prednisolone and dexamethasone, but also of the naturally occurring corticosteroids

cortisone, 11-deoxycortisol and, although to a lesser extent, aldosterone was demonstrated with these monolayers. Inhibition of transport by a selective Pgp blocker confirmed involvement of Pgp. In contrast, Pgp did not transport corticosterone in this system.
To further corroborate the differential uptake of corticosterone and cortisol in human brain as suggested by the mouse and monolayer findings, cortisol and corticosterone levels were measured in extracts of human post-mortem brain samples and plasma using liquid chromatography-mass spectrometry (LC-MS) to determine the ratio of corticosterone over cortisol (**chapter 3**). In contrast to rodents, both cortisol and corticosterone are circulating in human plasma, although corticosterone is present at ten- to twentyfold lower levels than cortisol. While in human plasma corticosterone concentrations are only 5% of cortisol levels, in the brain corticosterone levels are 30% of those of cortisol as determined using LC-MS.

> *Thus, in contrast to corticosterone, which readily enters rodent and human brain, cortisol, the main endogenous glucocorticoid in human, appears to be partially excluded from rodent and human brain.*

The differential uptake of corticosterone and cortisol may have important implications for glucocorticoid feedback to human brain. The LC-MS data suggest that cortisol levels in human brain are 6 times lower than those in human blood. The total glucocorticoid levels in human brain may thus be lower than assumed based upon plasma levels.
Furthermore, the preferential uptake of corticosterone in human brain suggests that this endogenous glucocorticoid may play a more prominent role in human brain function than hitherto recognised. Due to the differential uptake of cortisol and corticosterone, the human glucocorticoid feedback system might be more complex than the rodent system. Whether this is actually the case remains to be resolved, but literature data indicate that corticosterone may be the more effective glucocorticoid at the human MR, suggesting that corticosterone might have a potentially different role than cortisol in brain.

In **chapter 5** the data showing the functional consequences of Pgp-mediated exclusion of dexamethasone from the brain on central gene expression are presented. It was hypothesised that glucocorticoid feedback to the brain would be reduced as a consequence of depletion of glucocorticoids from the brain induced by treatment with low-dose dexamethasone. Dexamethasone acting at the level of the pituitary potently suppresses corticosterone secretion. As dexamethasone poorly enters the brain due to the presence of Pgp at the BBB, administration of low amounts of dexamethasone depletes the brain from endogenous glucocorticoids, for which dexamethasone does not appropriately substitute. Peripherally, dexamethasone replaces corticosterone at the GR. The resulting condition is a brain-selective hypocorticoid state.
To confirm the working hypothesis, dexamethasone was chronically administered to rats for 6 days or three weeks. As expected, dexamethasone circulating at low concentrations did not feed back at several glucocorticoid responsive genes expressed in the hypothalamic

paraventricular nucleus (PVN). Stress-induced responses of c-fos mRNA and CRH hnRNA were not reduced, whereas CRH mRNA expression was even increased after three weeks of treatment. After high-dose treatment dexamethasone turned out to be able to enter the brain in sufficient amounts to activate GR. Expression of glucocorticoid responsive genes was strongly reduced indicating that the barrier formed by Pgp is not able to completely exclude dexamethasone from entering the brain. In contrast, various peripheral glucocorticoid targets were strongly affected by dexamethasone independent of the amount administered.

> *After low-dose treatment, dexamethasone-induced effects on brain function should probably be ascribed to decreased rather than increased central glucocorticoid action.*

Other glucocorticoids that are substrates of Pgp are less likely to create this central adrenalectomy-like condition, as the high affinity to GR, lack of plasma binding and long-lasting activity favours the potency of dexamethasone to completely inhibit pituitary-adrenal secretion at low plasma levels. To suppress pituitary-adrenal secretion to the same extent high doses of cortisol (about 70-fold those of dexamethasone) are needed, which will likely reach the brain as well activating both MR and GR.

In **conclusion**, the findings presented in this thesis have made clear the importance of glucocorticoid transport at the BBB in controlling glucocorticoid access to the brain. The data show that Pgp is able to hamper penetration of various corticosteroids into the brain, particularly when these hormones are circulating at low plasma levels. Efflux transporters like Pgp may play a crucial role as an intermediate between brain and periphery by controlling transport of corticosteroids at the BBB. As exemplified by the dexamethasone study, impaired uptake of synthetic glucocorticoids, but also of the naturally occurring glucocorticoid cortisol, likely results in a reduced occupation of central corticosteroid receptors and thus in a diminished response to these glucocorticoids.
Intriguingly, both mouse and human Pgp do not transport corticosterone in contrast to cortisol. Future investigations will reveal whether corticosterone rather than cortisol may be the major endogenous corticosteroid in mediating corticosteroid actions, particularly via MR, on human brain function, as suggested by the preferential uptake of endogenous corticosterone into human brain.
The brain-selective low-corticosteroid state created by administration of low-dose dexamethasone to rats might be used as an animal model to specifically study central roles of corticosterone without the potentially confounding effects of reduced peripheral glucocorticoid effects.

Chapter 8

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Chapter 9

SAMENVATTING

Stress en glucocorticoïden

Stress-gerelateerde ziekten zoals depressie gaan vaak samen met overmatige secretie van glucocorticoïd hormonen zoals cortisol. Veel symptomen van deze ziekten zijn inderdaad te wijten aan deze 'stresshormonen', maar dat betekent niet dat glucocorticoïden altijd schadelijk zijn. Zij spelen juist een onmisbare rol in de normale fysiologie van mens en dier en met name in de respons op stress.

Het begrip 'stress' zoals gebruikt in dit proefschrift houdt meer in dan alleen negatieve aspecten. Elke verandering in het lichaam of in de omgeving die de bestaande evenwichtssituatie (homeostasis) bedreigt leidt tot een stressreactie. Stress activeert het brein en dit leidt via stimulatie van de Hypothalamus-Hypofyse-Bijnier-as (HHB-as) tot een verhoging van de concentraties van glucocorticoïden in het bloed (zie figuur 2, hoofdstuk 1). In muizen en ratten is dit corticosteron, in de mens is dit zowel cortisol als corticosteron. Naast effecten in vele perifere organen, hebben deze corticosteroïd hormonen ook weer effecten in de hersenen. In de hersenen beïnvloeden ze genexpressie, moduleren ze het functioneren van neuronen en brengen ze veranderingen in gedrag en geheugen teweeg. Zonder deze hormonen kunnen mens en dier zich niet staande houden in een omgeving die continu verandert.

De werking van glucocorticoïden wordt strikt gereguleerd. Niet alleen door de vele hersengebieden die de activiteit van de HHB-as reguleren, maar ook op andere niveaus. Via negatieve terugkoppeling op de hersenen en de hypofyse onderdrukken glucocorticoïden hun eigen secretie. Een niveau van regulatie dat tot nu toe weinig aandacht heeft gekregen, is de regulering van de toegang van glucocorticoïd hormonen tot de hersenen. Om de receptoren in de hersenen, via welke het signaal wordt doorgegeven, te bereiken, moeten glucocorticoïden de bloed-hersen-barrière (BHB) passeren. Deze barrière schermt de hersenen af van mogelijk schadelijke invloeden van buitenaf. Gezien hun krachtige effecten op de hersenen, kan regulatie van de toegang van glucocorticoïden tot het centraal zenuwstelsel wel eens van grote invloed zijn op de werking van de hersenen.

Lipofiele stoffen zoals corticosteroïd hormonen zouden in principe de BHB moeiteloos moeten kunnen passeren. Echter, aan de bloedkant van deze barrière bevindt zich P-glycoproteïne, een transporteiwit dat allerlei heel verschillende lipofiele stoffen (waaronder veel medicijnen zoals cytostatica en anti-epileptica) tegenhoudt. Het belemmert ook de opname van het synthetisch glucocorticoïd dexamethason in het brein.

Doel onderzoek

Het **doel** van de studies beschreven in dit proefschrift was uit te zoeken wat de rol van de efflux transporter P-glycoproteïne (Pgp) is in het reguleren van de toegang van endogene en synthetische glucocorticoïden tot de hersenen. De verwachting was dat met name synthetische

glucocorticoïden uit de hersenen geweerd zouden worden, terwijl natuurlijke hormonen de BHB gemakkelijker zouden passeren.

Expressie P-glycoproteïne

In **hoofdstuk 2** worden experimenten beschreven waarbij gekeken is naar de expressie van het Pgp in de hersenen. De aanwezigheid van mRNA van het gen dat codeert voor het Pgp (mdr1a) werd aangetoond in de endotheelcellen die de BHB vormen. Ook op eiwitniveau werd de aanwezigheid van Pgp in deze cellen bevestigd. Verbazingwekkend was dat mRNA ook aangetroffen werd in neuronale cellen van de gyrus dentatus, een subgebied van de hippocampus. Deze hersenstructuur speelt een belangrijke rol bij leren en geheugenprocessen en is ook erg gevoelig voor glucocorticoïden. Het is nog niet duidelijk wat de rol van het Pgp in dit hersengebied is.

Opname glucocorticoïden

In experimenten beschreven in de **hoofdstukken 3 en 4** is gekeken naar de opname van verschillende glucocorticoïden in de hersenen. Hierbij zijn gewone muizen en knock-out muizen die, doordat het mdr1a gen is uitgeschakeld, de efflux transporter Pgp missen, gebruikt. Deze dieren zijn geïnjecteerd met radioactief gelabelde glucocorticoïd hormonen. Na een uur zijn de dieren gedood en de hersenen en andere organen, waarin de radioactiviteit is opgenomen, verwijderd. Met autoradiografie waarbij gevoelige films werden blootgesteld aan de radioactieve straling in de coupes van de hersenen werden verschillen in de opname van radioactiviteit in de hersenen van beide typen muizen bekeken. Radioactiviteit werd ook gemeten in homogenaten van de kleine hersenen, andere organen en bloedplasma.
Deze experimenten lieten zien dat het synthetische hormoon prednisolon, net als dexamethason, moeizaam het brein binnendringt door de aanwezigheid van het Pgp op de BHB. In de hersenen van gewone muizen was minder radioactiviteit aanwezig dan in hersenen van muizen die het Pgp missen. In andere organen en plasma werd geen verschil gevonden.

Verschil in hersenopname van cortisol en corticosteron

Verrassend was het grote verschil tussen de opname van het muiseigen hormoon corticosteron en het natuurlijke menselijke hormoon cortisol. Opname van cortisol wordt bemoeilijkt door het Pgp, maar corticosteron komt gemakkelijk het brein in.
Beide hormonen lijken erg op elkaar qua molecuulstructuur en affiniteit voor de twee verschillende typen corticosteroïd receptoren en grote verschillen in welk opzicht ook zijn niet eerder gerapporteerd. Kleine verschillen zijn er echter wel, maar daar is nooit aandacht aan besteed. Corticosteron komt ook voor in de mens, zij het in veel lagere bloedconcentraties dan cortisol. Gezien hun zeer vergelijkbare eigenschappen wordt altijd aangenomen dat corticosteron nauwelijks een eigen rol - naast cortisol - speelt in de mens. Het door ons in muizen gevonden verschil wijst echter op een mogelijk verschil in toegang tot de menselijke hersenen van beide hormonen. Om uit te zoeken of het verschil in interactie met het Pgp de opname van cortisol in het menselijke brein bemoeilijkt terwijl het geen effect heeft op de corticosteronopname, zijn twee lijnen van onderzoek gevolgd.

Eerst is vastgesteld of het humane Pgp hetzelfde verschil in transport laat zien. Varkensniercellen die getransfecteerd waren met MDR1 cDNA coderend voor het humane Pgp werden gebruikt om het transport van glucocorticoïden door het humane Pgp te bestuderen. Monolayers van deze cellen transporteren Pgp-substraten van het onderste compartiment naar het bovenste (zie figuur 10, hoofdstuk1) en zijn een geschikt model voor de humane BHB. Deze monolayers en die van niet-getransfecteerde cellen werden gebruikt om transport van radioactief gelabelde corticosteroïd hormonen te bestuderen. Uit deze *in vitro* proeven bleek dat ook het humane eiwit niet in staat is corticosteron te transporteren maar wel cortisol en ook dexamethason en prednisolon. Er is dus geen verschil in substraatspecificiteit tussen het humane en het muizen-Pgp.
Dit opmerkelijke verschil suggereert dat corticosteron makkelijker in de humane hersenen kan worden opgenomen dan cortisol. Deze suggestie wordt verder onderbouwd door resultaten van de corticosteroïd-bepalingen in humane postmortem hersenmonsters en plasmamonsters. Met behulp van vloeistof-chromatografie/massa-spectrometrie werd vastgesteld dat de corticosteron/cortisol ratio in de hersenen zesmaal hoger is dan in het plasma. Er is dus, vergeleken met cortisol, relatief meer corticosteron aanwezig in het brein dan in het plasma. Dit wijst erop dat corticosteron inderdaad makkelijker het humane brein binnendringt dan cortisol.
Deze preferentiële opname van corticosteron in de hersenen vergeleken met cortisol suggereert dat corticosteron een belangrijker rol kan spelen in de menselijke hersenen dan totnogtoe aangenomen. In hoeverre dit ook echt consequenties heeft, zal verder onderzoek moeten uitwijzen.

Dexamethason effect op de hersenen

Een mogelijk gevolg van de aanwezigheid van Pgp op de BHB zou kunnen zijn dat cortisol, maar ook synthetische glucocorticoïden als dexamethason en prednisolon, minder sterke effecten hebben in het brein dan in de rest van het lichaam. Dit werd verder onderzocht in experimenten waarin ratten behandeld werden met lage doses dexamethason (**hoofdstuk 5**).
Door de werking van het Pgp zullen lage concentraties dexamethason nauwelijks in het brein worden opgenomen en dus nauwelijks een direct effect hebben op het functioneren van de hersenen via de daar aanwezige receptoren. Als krachtig glucocorticoïd zal dexamethason onder deze omstandigheden echter wel sterke effecten hebben op perifere organen en bijvoorbeeld leiden tot een verkleining van de thymus en een afname van het lichaamgewicht. In ratten die gedurende drie weken met lage doses dexamethason behandeld werden, waren glucocorticoïd effecten in de hersenen inderdaad zwak vergeleken met effecten in de periferie. Na behandeling met hoge doses dexamethason werden wel duidelijke directe effecten in de hersenen gevonden, wat erop wijst dat bij deze hoge doses dexamethasone in voldoende mate het brein binnendringt om daar de receptoren te bezetten. Deze resultaten bevestigen dat effecten van dexamethason, mits de dosis niet te hoog is, minder sterk zijn in het brein dan daarbuiten.

Een zeer interessant bijkomend gevolg van deze behandeling met lage doses dexamethason is dat de lage concentraties effectief genoeg om de endogene corticosteron-secretie te onderdrukken door de remmende invloed van dexamethason op de HHB-as op het niveau van de hypofyse, die buiten de BHB ligt (zie figuur 9, hoofdstuk 1). Dit leidt dit tot een hersen-selectieve depletie van glucocorticoïden, waarbij in de hersenen zowel corticosteron als dexamethason concentraties laag zijn. Deze verlaging van glucocorticoïd concentraties leidt tot een vermindering van de effecten van glucocorticoïden in de hersenen. In de periferie buiten de BHB daarentegen neemt dexamethason de glucocorticoïd werking van corticosteron over. Inderdaad waren, in de ratten die langdurig met lage doses dexamethason behandeld waren, sommige effecten op genexpressie in de hersenen vergelijkbaar met effecten van het verwijderen van corticosteron door de bijnier weg te nemen (adrenalectomie). Het gevolg van de behandeling met lage doses van het sterke glucocorticoïd dexamethason leidt dus, door de activiteit van het Pgp op de BHB, tot een paradoxale vermindering van de werking van glucocorticoïden in de hersenen.

Conclusies

De belangrijkste **conclusie** die uit de studies zoals beschreven in dit proefschrift getrokken kan worden is de efflux transporters P-glycoproteïne op de bloed-hersen-barrière een cruciale rol kan spelen als intermediair tussen de hersenen en de rest van het lichaam door de opname van glucocorticoïden in de hersenen te belemmeren. Niet alleen synthetische glucocorticoïden maar ook het natuurlijke hormoon cortisol komen moeizaam het brein binnen doordat Pgp deze hormonen uit het brein weert. Het verschil in opname van cortisol en corticosteron suggereert dat het glucocorticoïd systeem van mensen complexer is dan totnogtoe aangenomen. Corticosteron, het hormoon waar eigenlijk in humaan onderzoek nooit aandacht aan besteed is, kan in de mens naast cortisol wel eens een belangrijke rol spelen in het doorgeven van glucocorticoïd signalen naar de hersenen.

Van grote praktische betekenis is verder dat blootstelling van de hersenen aan overmaat cortisol mogelijk beperkt kan worden door behandeling met lage doseringen dexamethason. Dit kan leiden tot een nieuwe aanpak van stress-gerelateerde ziekten die gekenmerkt worden door verhoogde cortisol secretie, zoals depressie.

Addendum

EFFLUX TRANSPORTER P-GLYCOPROTEIN, CORTISOL AND UPTAKE OF THE GLUCOCORTICOID RECEPTOR ANTAGONIST C-1073 (MIFEPRISTONE) IN BRAIN;

IMPLICATIONS FOR TREATMENT OF PSYCHOTIC DEPRESSION

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Abstract

Psychotic depression is characterised by a high rate of hypercortisolemia. The glucocorticoid receptor (GR) antagonist C-1073 (mifepristone or RU486) has recently been demonstrated to rapidly relieve patients of both psychotic and depressive symptoms. We hypothesise that C-1073 blocks excess cortisol action at the GR level in brain regions involved in cognitive and emotional processing. As only very high doses are effective, the question arises whether effective blockade of central GR by C-1073 may be impeded by hampered penetration of C-1073 into the brain. Alternatively, since C-1073 is known to inhibit the blood-brain-barrier transporter P-glycoprotein (Pgp) that transports cortisol, C-1073 may facilitate uptake of cortisol into the brain counteracting its own antagonistic action.

To determine whether C-1073 itself is hampered to enter the brain, Liquid Chromatography-Mass Spectrometry-Mass Spectrometry steroid profiles were made of brain and plasma extracts of rats treated with high doses for several days. Our results show that concentrations of the various compounds were similar in brain and plasma both at one hour and at three hour after the last administration. Clearance of circulating C-1073 and metabolites was very rapid. Levels were very high at one hour but considerably lower at three hours after the last administration.

To investigate whether C-1073 and/or its metabolites are able to inhibit cortisol transport mediated by Pgp, we have used monolayers of epithelial cells stably expressing human Pgp. Our data show that 10 μM C-1073 alone blocked Pgp-mediated cortisol transport. A mix of C-1073 and its three main metabolites at therapeutically relevant concentrations also inhibited transport of cortisol.

In conclusion, penetration of C-1073 and metabolites into the rat brain may not be impaired. Central action of C-1073 (and metabolites) may be impeded by rapid clearance and inhibition of Pgp-mediated cortisol-efflux. The latter might result in an increased retention of cortisol in brain on top of the C-1073-induced disinhibition of cortisol secretion, requiring higher C-1073 concentrations in brain to overcome the agonistic cortisol action. Additionally, it may result in increased activation of the central mineralocorticoid receptor. The resultant enhanced shift of the MR/GR balance may reset cognitive and neuroendocrine systems.

Introduction

Disturbances of cognitive, emotional and neuroendocrine functions in affective disorders are frequently associated with hypercortisolemia. Since it is hypothesised that excess cortisol action in specific brain areas might be causally linked to these dysfunctions (Holsboer, 2001; Belanoff *et al.*, 2001a), the use of glucocorticoid receptor (GR) antagonists may provide an effective new strategy in treatment of these psychopathologies. Strong evidence supporting this hypothesis has recently been provided with regard to psychotic major depression (PMD) (Belanoff *et al.*, 2002). PMD is a distinct depressive syndrome different from nonpsychotic depression in various aspects including presence of delusions or hallucinations and neuroendocrine dysfunction. Patients show high rates of hypercortisolemia (Belanoff *et al.*, 2001b) and of nonsuppression on the dexamethasone suppression test (Nelson and Davis, 1997). The GR-antagonist C-1073 (RU486 or mifepristone) was demonstrated to rapidly relieve patients from both psychotic and depressive symptoms (Belanoff *et al.*, 2002). After a brief 7-day treatment with high doses of 600-1200 mg/day patients showed dramatic improvement in their scores on Brief Psychiatric Rating Scale and Hamilton Rating Scale for Depression.

We hypothesise that C-1073 blocks excess cortisol action at the GR level in brain regions involved in cognitive and emotional processing, *e.g.* frontal cortex and limbic brain regions. As only very high doses are effective, the question arises whether effective blockade of central GR by C-1073 may be impeded by hampered penetration of C-1073 into the brain.
Alternatively, C-1073 may facilitate uptake of cortisol into the brain at the blood-brain-barrier (BBB) by inhibition of the efflux transporter P-glycoprotein (Pgp) (Gruol *et al.*, 1994). Since we have shown that Pgp transports cortisol out of the brain (Karssen *et al.*, 2001), C-1073 may be able to inhibit Pgp-mediated cortisol efflux at the BBB, resulting in enhanced cortisol uptake in brain. The subsequently increased cortisol brain levels would require higher brain levels of C-1073 to effectively block central GR.
This counteracting effect will be on top of the C-1073-induced disinhibition of cortisol secretion due to blockade of cortisol negative feedback on the HPA-axis at the pituitary and hypothalamic levels. Under normal conditions, the latter effect is only seen when GR is activated in presence of increased glucocorticoid levels *i.e* during the circadian peak in both humans and animals (Gaillard *et al.*, 1984; Raux-Demay *et al.*, 1990; Van Haarst *et al.*, 1996). In hypercortisolemic depressed patients, this effect may be seen during the whole day (Kling *et al.*, 1989; Belanoff *et al.*, 2002; Schatzberg unpublished).

For measurement of brain uptake of GR-antagonists LC/MS/MS steroid profiles were made of brain and plasma extracts of rats treated with various doses of C-1073. *In vivo*, C-1073 is rapidly converted into three main metabolites, a mono-demethylated (RU42633), a di-demethylated (RU42848) and a hydroxylated (RU42698) (Deraedt *et al.*, 1985). Since

these metabolites retain activity as GR-antagonists (Deraedt *et al.*, 1985; Heikinheimo *et al.*, 1987a), they were also determined in this study.
To investigate whether C-1073 and/or its metabolites are able to influence cortisol transport mediated by Pgp, we have used monolayers of pig kidney epithelial cells (LLC-PK1) stably expressing human MDR1 Pgp (Karssen *et al.*, 2001).

Materials and methods

Animal experiments

Young adult male Wistar rats (Charles River, Germany) were housed under a 12-12 hour light-dark cycle with lights on at 7:00hr in a temperature (21°C) and humidity controlled room. Food and drinking water were available *ad libitum*. Before and during experiments rats were handled daily. 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 (The Netherlands).

Animals were treated with various doses of C-1073 for five days. Body weight was monitored throughout this period. At the last day after the last injection the animals were killed by decapitation. Brain and plasma were collected and frozen until further use. Adrenals and thymus were also dissected and weighed.
C-1073 was subcutaneously administered at a dose of 16 mg/kg once daily or at a dose of 50 mg/kg twice daily. In a third experiment C-1073 suspended in an aqueous solution containing 0.25% carboxymethylcellulose/0.2% Tween-20 was administered by gavage once a day. To test whether C-1073 disinhibits the HPA-axis the animals where decapitated at the end of the day during the circadian rise of corticosterone levels. Groups treated with vehicle were also included in all experiments.

Corticosteroid determination in brain and plasma

Using Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LC/MS/MS) steroid profiles were made of samples of the cortex and plasma. Samples were prepared for assay by dichloromethane/ethanol extraction essentially as previously described (Karssen *et al.*, 2001). The brain samples (weighing about 350 mg) were homogenised in 2 ml 0.1 M perchloric acid with a Potter-Elvehjem tissue homogeniser (10 times up and down, 1000 rpm). To check for differences in recovery 100 ng of dexamethasone was added to each sample. The homogenates were transferred with a 4 ml wash of dichloromethane (DCM) to screw-capped glass tubes. After adding an extra 4 ml DCM the tubes were shaken on a horizontal reciprocating shaker for 30 minutes and subsequently centrifuged at 1000x g at 4°C for 10 minutes. The DCM layer was transferred to a clean coned tube and rinsed with 1 ml water, centrifuged at 700x g for 10 minutes. Then, the DCM-layer was transferred to a long tube and evaporated to dryness under nitrogen. To maximise the amount transferred, the extracts were redissolved in 750 µl ethanol and after transferring to an eppendorf, evaporated again. The final extracts were resuspended

in 100 µl 25% methanol and centrifuged at 13000 rpm for 5 minutes. To avoid potential dissimilarities between different extraction methods, 250 µl plasma samples were extracted in the same way.

The LC/MS/MS assays were performed on a Triple Stage Quadrupole mass spectrometer (ThermoFinnigan TSQ-Quantum, San Jose, USA) with an atmospheric pressure chemical ionisation interface. A modification of the method of Van der Hoeven *et al.* (1997) was used. The analysis was performed in positive ionisation mode using selective reaction monitoring of C-1073, its three main metabolites, corticosterone and dexamethasone. The $[M+H]^+$ precursor ions were fragmented using argon as collision gas. The *m/z* ratios of the most abundant product ions were alternately scanned. The ion-source temperature and the nebulisation heater were kept at 200°C and 400°C, respectively. The voltages on the corona needle and on the electron-multiplier were set at 10 µA. Each experiment, a new standard series was made in 25% methanol with concentrations ranging from 1-500 ng/ml of all steroids. Dexamethasone (1µg/ml) was used as an internal standard. A Surveyor LC System (ThermoFinnigan) was used to inject 20 µl of the standard or extraction samples. A gradient of methanol-water (containing 1 g/l acetic acid) changing from 50/50% to 90/10% at a flow rate of 500 µl/min separated the steroids on an ADS C_{18} column. All samples were measured in duplo. The detection limit of this assay was 1-5 ng/ml for each steroid.
Steroid concentrations were calculated from a standard plot of area under the curve versus concentration. The standard curves usually displayed an r^2 of more than 0.95. Presented data are corrected for recovery of dexamethasone, which was in the order of 25-50%.

Transepithelial transport and inhibition studies

In order to examine the inhibitory action of RU486 on Pgp-mediated cortisol transport 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 encoding P-glycoprotein (LLC-PK1:MDR1) (Schinkel *et al.*, 1995) as previously described (Karssen *et al.*, 2001).
During culturing and experiments complete medium, which consisted of DMEM supplied with HEPES (25 mM) and glucose (4.5 g/l) and supplemented with penicillin (100.000 U/l), streptomycin (100 mg/l), L-glutamine (2 mM) and 10% (v/v) foetal calf serum, was used. The cells were seeded on microporous polycarbonate membrane filters (0.4 µM pore size, 12 mm diameter; Costar, USA) at a density of $120*10^3$ cells/cm^2. 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. One hour later 8 µl of a 100x stock of GR antagonists or vehicle (ethanol) was added at the apical side. RU486 was added alone at a final concentration of 10 or 100 µM. In a separate experiment a mix of RU486 and metabolites at therapeutically relevant concentrations was added. At the start of the experiment (t=0), 8 µl of a 100x stock of ^{3}H-cortisol (Amersham Pharmacia Biotech, UK; specific activity 63 Ci/mmol) in ethanol (final concentration 15 nM) was added in triplicate at the apical or basal side respectively. To measure the transepithelial transport from the apical to the basal side or from basal to the

apical side, the appearance of radioactivity in the compartment opposite that to which activity was added, was examined. Over the four hours of study 75 µl samples were taken once every hour from this compartment. These samples were counted in a Tricarb ß-counter together with eight µl samples of the 100x stock. From the other compartment 75 µl aliquots were removed each time to ascertain an undisturbed action of Pgp. Basal to apical and apical to basal transport is presented as percentage of total activity 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 and De Boer, 2000).

Statistical analysis

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

The steroid profiles of rat brain and plasma made with LC/MS/MS show that three hours after the last treatment with 16 mg/kg the C-1073 levels were low in both brain and plasma (figure 1). Brain levels were 19.7±13 ng/gr tissue, whereas levels in plasma were 12.6±5.5 ng/ml. There was, however, quite a large variability in C-1073 levels between animals. In addition, plasma levels did not correlate with brain levels. The concentrations of the three metabolites were below the detection limit, except for RU42633 levels, which were very low but detectable (data not shown).

Corticosterone levels in brain at the same time point late in the afternoon were about 8-10% of plasma levels in both treatment groups (figure 1). Due to the low number of animals no significant differences in corticosterone levels in blood plasma or in brain were found between

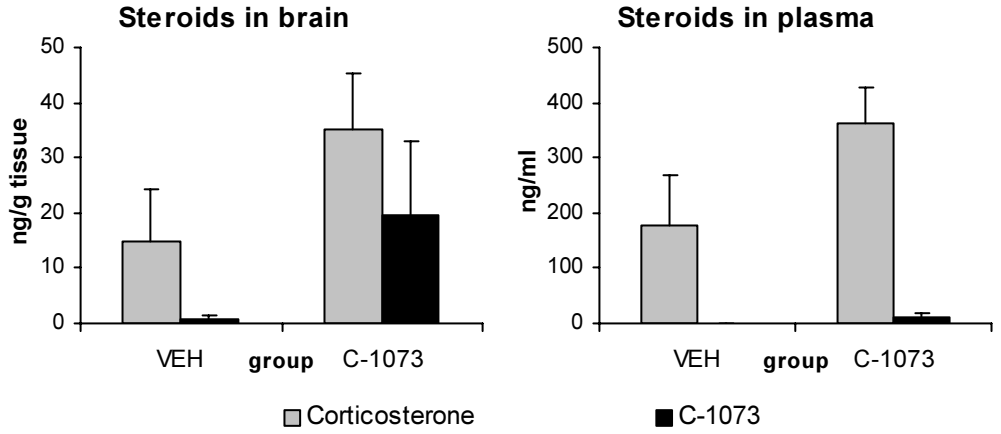

FIGURE 1. Steroid levels at 3 hours after last administration of 16 mg/ml C-1073; levels of C-1073 were in the same range in both brain and plasma (10-20 ng/ml). Brain levels of corticosterone were 10-12 times lower than plasma levels in both groups. N=2-4, shown is mean+SEM. Note the difference in scale.

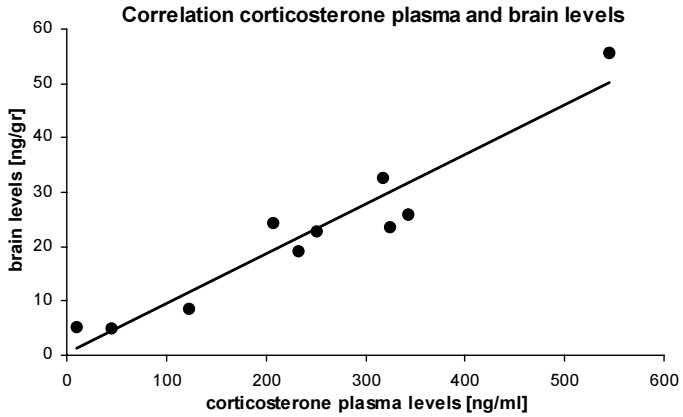

FIGURE 2. Representative example of the strong correlation between corticosterone brain and plasma levels. At 3 hours after the last administration of C-1073 (8 or16 mg/kg) or vehicle brain and plasma levels correlated significantly (r^2=0.92; p<0.005).

vehicle and C-1073 treated animals. There was a strong significant correlation between corticosterone plasma and brain levels (figure 2).

Extremely high brain and plasma levels of C-1073 were found in animals treated with a high dose of 50 mg/kg at one hour after the last administration in the morning (figure 3).

After oral treatment….?? (Experiment in progress)

C-1073 treatment did neither affect adrenal and thymus weight nor body weight after any treatment schedule.

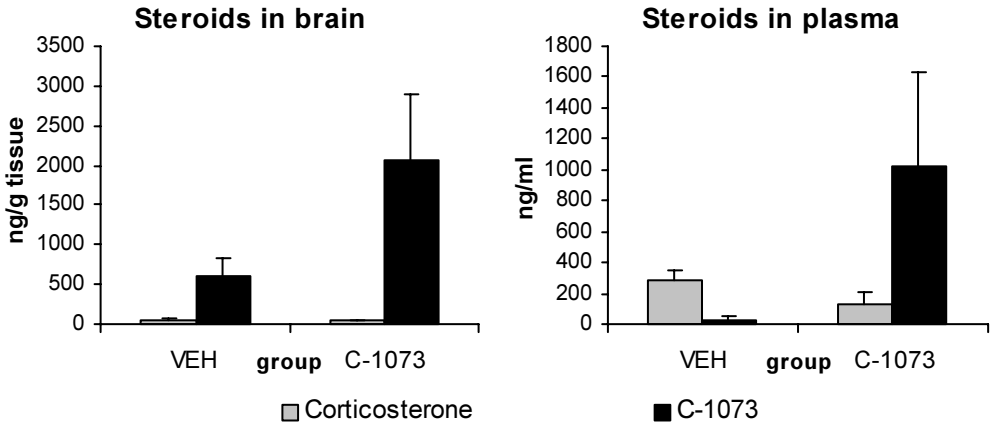

FIGURE 3. At one hour after the last administration of 50 mg/kg C-1073, C-1073 were extremely high in both brain and plasma. For unknown methodological reasons false positive results were obtained in vehicle treated animals. Although less strong, again a significant correlation was found between corticosterone brain and plasma levels (not shown).

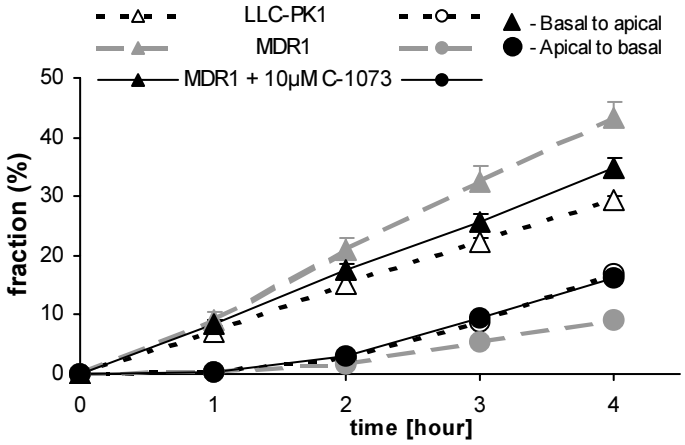

FIGURE 4. In presence of 10 µM C-1073, transport of ^{3}H-cortisol in MDR1-transfected monolayers is inhibited and not different from transport of cortisol in nontransfected monolayers. Data are presented as mean±SEM of three wells.

Inhibition of cortisol transport

Our monolayer data demonstrate that 10 µM C-1073 is able to block Pgp-mediated cortisol transport (figure 4). In MDR1-monolayers cortisol was transported in a highly polarised fashion. In presence of C-1073 this transport was inhibited. ANOVA followed by post-hoc analysis shows that at t=4 in presence of C-1073 MDR1 transfected monolayers were not different from untransfected monolayers with regard to transport of ^{3}H-cortisol, while both were statistically different from the untreated MDR1 monolayers. 100 µM C-1073 was not able to further enhance the inhibitory action on cortisol transport (data not shown).

At a concentration of 2.5 µM – a therapeutically relevant level – C-1073 inhibited transport to a minor extent (data not shown). As the three main metabolites of C-1073 are structurally similar to C-1073, they may inhibit Pgp as well. Therefore we tested the ability to inhibit cortisol transport of a mix of C-1073 and metabolites at therapeutically relevant concentrations. A mix of C-1073, RU42848, RU42698 (2.5 µM each) and 6 µM RU42633 affected cortisol transport in MDR1 monolayers to a similar extent as 10 µM C-1073 alone (figure 5). C-1073 did not affect C-1073 transport in untransfected monolayers (data not shown).

Discussion

We have examined the interactions of the GR antagonist C-1073 at the blood-brain barrier, that may affect its action in the brain. Some of our findings may explain why high oral doses are needed to see an effect of C-1073 on brain function. Limited access of C-1073 to the brain is not likely to occur, as plasma and brain levels in our rats were similar. However, our data suggest that C-1073 and its active metabolites may rapidly disappear from the brain. Our data further demonstrate that C-1073 and its metabolites are able to inhibit Pgp-mediated transport

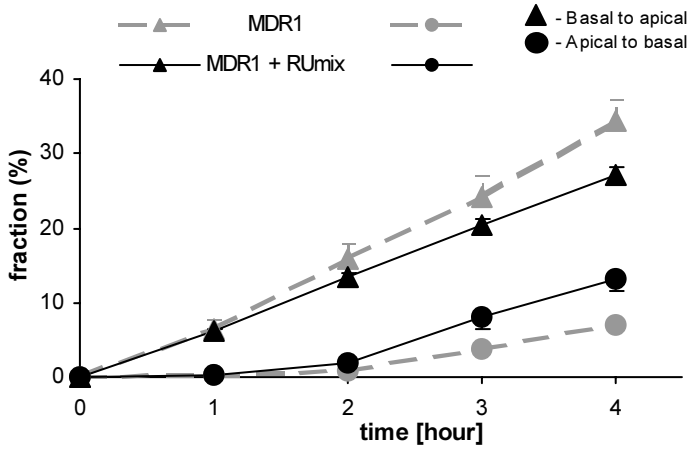

FIGURE 5. A mix of C-1073 and its three main metabolites at therapeutically relevant concentrations (see text) inhibits transport of ^{3}H-cortisol in MDR1 transfected monolayers.

of cortisol. This inhibition would presumably result in increased cortisol brain levels, which would require higher C-1073 concentrations in brain to compensate for increased cortisol action. Besides rapid clearance and/or facilitation of cortisol uptake, the increased activity of the HPA-axis resulting from blockade of glucocorticoid feedback is also likely to be responsible for the high systemic doses needed.

When administered systemically, very high doses of C-1073 are needed to be effective in the brain as an antiglucocorticoid in both humans (Gaillard *et al.*, 1984; Belanoff *et al.*, 2002) and animals (De Kloet *et al.*, 1988; Ratka *et al.*, 1989). Indeed, to effectively block competitive binding of endogenous glucocorticoid to central GR, high levels in the brain are needed. However, it is intriguing that when administered directly in the brain, a 100,000 fold lower dose is enough to effectively block corticosterone action in rats (De Kloet *et al.*, 1988; Ratka *et al.*, 1989; Van Haarst *et al.*, 1996). Our findings indicate that impaired penetration of the BBB may not explain the poor effectiveness of systemic doses as compared to central doses. The similar levels in brain and plasma as shown by us are, however, in contrast to previous studies. Shortly after a single oral administration of 5 mg/kg ^{3}H-RU486 total brain levels of radioactivity were about 37% of plasma levels, although it was stated that some brain areas including cortex were selectively labelled (Deraedt *et al.*, 1985). In another study, Zucker rats were orally treated with 10 mg/kg twice a day for 4 days (Heikinheimo *et al.*, 1994). Six hours later, brain concentrations were 28% of those in plasma. These discrepancies are presently unexplained for, but might be related to dose, route of administration, timing and/or rat strain. It is, however, clear that C-1073 is able to cross the blood-brain barrier at least to a considerable extent.

Our data may further provide alternative explanations for the high doses needed. Rapid clearance and/or preferential uptake into other tissues may account for the large difference

between the two time points measured in the present study. Whereas at one hour after the last injection levels were extremely high, at three hours after the last administration levels were very low. Although the different doses administered may explain some of this, a rapid clearance from blood and brain due to uptake into other extravascular compartments is a more likely explanation. High rate of extravascular diffusion and fast elimination has previously been demonstrated after administration of a low dose of 5 mg/kg to rats (Deraedt *et al.*, 1985).

The high variability of C-1073 between animals and the discrepancy between brain and plasma found in the present study are difficult to explain, but do not appear to be related to the method of detection using extraction and LC/MS/MS measurements. Corticosterone plasma levels correlate well with corticosterone brain levels, supporting the applicability of the employed method for measurement of steroid levels.

It might be difficult to extrapolate the present findings to humans. C-1073 follows a profoundly different pharmacokinetic profile in rat and human (Deraedt *et al.*, 1985). In humans, C-1073 has a complex pharmacokinetic profile due to binding to the low capacity/high affinity serum binding protein, α_1-acid glycoprotein, which binds about 95% of circulating C-1073 (Heikinheimo *et al.*, 1987b; Heikinheimo *et al.*, 1989; Philibert *et al.*, 1991). After the redistribution phase following oral intake of doses of C-1073 higher than 100 mg the plasma levels of C-1073 and metabolites do not change for over 24 hours. With higher doses administered, only the duration of this period of stable levels increases while the plasma levels remain unchanged at 2.5 μM. The saturation of α_1-acid glycoprotein probably explains the long plasma half-life in human (Deraedt *et al.*, 1985). The rat α_1-acid glycoprotein does not bind C-1073, which explains the low levels in plasma and also may result in faster plasma clearance (Philibert *et al.*, 1991). However, adipose tissue may function as a depot that through continuously supplying C-1073 to the circulation may extend the period of activity of C-1073 (Heikinheimo *et al.*, 1994).
It is not known how plasma binding may affect brain uptake of C-1073. One study shows that concomitant treatment of rats with C-1073 and the human α_1-acid glycoprotein results in a decreased first-pass uptake in liver and uterus (Steingold *et al.*, 1990), but the effect of prolonged concomitant treatment was not studied. If only free levels would be available for uptake into the human brain as might be suggested by the reported low levels in cerebrospinal fluid -about 4% of plasma concentration- (Sartor and Figg, 1996), our findings in rat might model the human situation quite well. Further studies should shed light on this issue.

Interestingly, our study provides a new alternative mechanism of action not related to the GR antagonism of C-1073, but to its potency to block transport of substrates of the efflux transporter Pgp. Like other antidepressants (Pariante *et al.*, 2001), C-1073 promotes accumulation and intracellular action of Pgp substrates and it inhibits transport of Pgp substrates (Lecureur *et al.*, 1994; Gruol and Bourgeois, 1994; Gruol *et al.*, 1994; Gruol *et al.*,

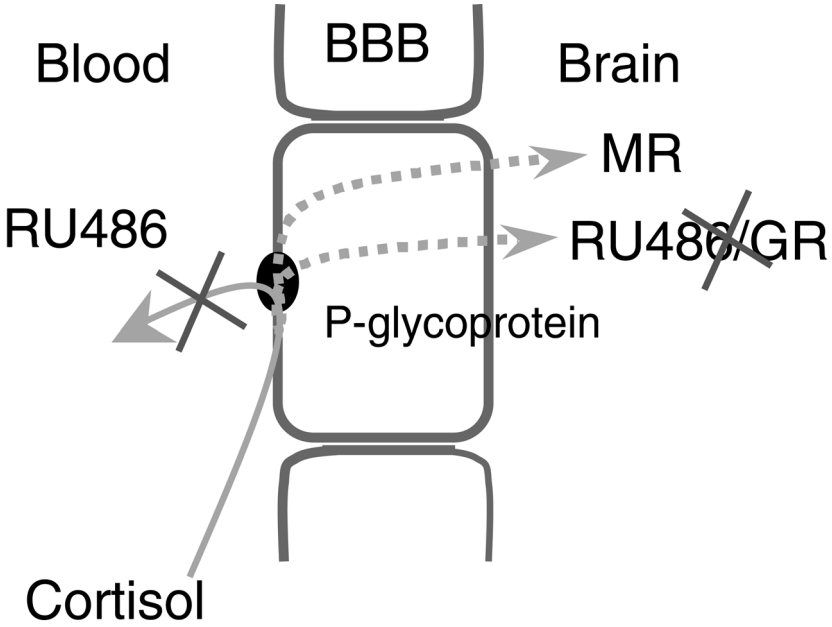

FIGURE 6. Hypothesised C-1073-induced facilitation of cortisol brain uptake through inhibition of the efflux transporter P-glycoprotein. Under normal conditions cortisol is hampered to enter the brain due to active outwards directed transport at the blood-brain barrier mediated by P-glycoprotein. In presence of C-1073 (RU486) this efflux is blocked facilitating uptake of cortisol into the brain. The ensuing increased cortisol concentration will not lead to increased activation of GR, since this receptor is blocked by the high concentrations of C-1073. However, it may lead to increased activation of the MR which would affect cognitive performance and neuroendocrine regulation.

2002). Accumulation studies show that C-1073 is probably not transported itself by Pgp (Gruol and Bourgeois, 1994).

The present study shows that C-1073 is able to block transport of cortisol. Due to the presence of Pgp at the BBB, uptake of cortisol into the brain is hampered (Karssen *et al.*, 2001) and thus C-1073 would presumably be able to increase cortisol brain levels (figure 6). At a concentration of 10 µM C-1073 already completely suppressed Pgp-mediated cortisol transport. As levels of C-1073 generally do not rise above 2.5 µM, we also tested this concentration. Although at this concentration C-1073 alone only minimally reduced cortisol transport, a mix of C-1073 and its metabolites inhibited cortisol transport to a much higher degree. Since we have chosen concentrations based upon literature data on plasma levels in humans after C-1073 treatment (Lähteenmäki *et al.*, 1987), this suggests that Pgp-inhibition of cortisol efflux by C-1073 and its metabolites might indeed result in an increased uptake of cortisol in brain *in vivo*. This would require higher C-1073 concentrations in brain to overcome the agonistic cortisol action.

Our data may explain the high doses needed in treatment of psychotic depression, but they do not explain the rapid onset of action of C-1073. We hypothesise that blockade of GR in specific brain areas involved in cognitive and emotional processing is the principal mechanism of action. Patients with psychotic depression show specific impairments in prefrontal and hippocampal function not found in patients with nonpsychotic depression (Schatzberg *et al.*, 2000). These abnormalities might be caused by the excessive glucocorticoid activity characteristic of these patients, possibly via enhanced prefrontal dopaminergic activity (Schatzberg *et al.*, 1985; Schatzberg *et al.*, 2000). This glucocorticoid-induced enhancement might be blocked by C-1073 treatment, which may break the vicious glucocorticoid/dopamine cycle.

Blocking the GR also results in increased cortisol levels in brain (both by blocking the feedback loop and by facilitation of cortisol brain uptake at the blood-brain barrier). Since C-1073 has negligible affinity for the mineralocorticoid receptor (MR) (Philibert, 1984), the higher cortisol levels may result in increased activation of this receptor, which exerts an inhibitory action on the HPA-axis (Dallman *et al.*, 1987; De Kloet *et al.*, 1998). Furthermore, MR and GR do mediate distinctly different and sometimes even opposing actions of glucocorticoid hormones on neuronal function and behaviour (De Kloet, 1991; De Kloet *et al.*, 1998). Treatment with C-1073 will shift the MR/GR balance, which is important for proper cognitive and neuroendocrine functioning (Oitzl *et al.*, 1997; De Kloet, 2002). The resultant alteration of central glucocorticoid signalling might normalise the set points of hormonal and cognitive function in the patients. Further studies examining the effects of C-1073 on central GR and MR occupation are presently under way.

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CURRICULUM VITAE

Adriaan Martijn Karssen werd op 5 mei 1972 geboren te Delft. Hij behaalde in 1990 zijn VWO-diploma aan het Christelijk Lyceum Dr. W.A. Visser 't Hooft te Leiden. Aansluitend begon hij met zijn studie Biologie aan de Rijksuniversiteit Leiden. In 1996 werd het doctoraalexamen met als specialisatierichting Medische Biologie succesvol afgelegd.

Tijdens deze studie deed hij zijn hoofdvakstage bij de sectie Dynamische Morfologie van het Instituut voor Evolutionaire en Ecologische Wetenschappen van de Rijksuniversiteit Leiden onder begeleiding van drs. A.J. Tellegen en prof. dr. J.L. Dubbeldam. Hier deed hij een neuroanatomische studie van de tectofugale zenuwbanen in de wilde eend, via welke visuele informatie de activiteit van craniocervicale spieren kan beïnvloeden. Tevens werd de cursus 'Voortplanting, ontwikkeling en veroudering' aan de Vrije Universiteit te Amsterdam als bijvak gevolgd.

Een tweede onderzoeksstage werd uitgevoerd bij de divisie Medische Farmacologie van het Leiden/Amsterdam Center for Drug Research (LACDR) onder begeleiding van drs. O.C. Meijer en prof. dr. E.R. de Kloet. Tijdens deze stage onderzocht hij de rol van corticosteron als mogelijke mediator van de effecten van de serotonine 1A ($5HT_{1A}$-) receptor agonist flesinoxan op de expressie van $5HT_{1A}$-receptor mRNA in de hippocampus.

In de zomer van 1997 startte hij als gastmedewerker aan de Universiteit Leiden bij Medische Farmacologie, waar hij een nieuwe bindingsassay voor corticosteroïd receptor bepalingen verder ontwikkelde en uittestte.

Vanaf januari 1998 tot april 2002 werkte hij als assistent in opleiding aan zijn promotieonderzoek bij Medische Farmacologie met als promotor prof. dr. E.R. de Kloet. Dit project werd uitgevoerd in samenwerking met de divisie Farmacologie van het LACDR. Het resultaat van dit onderzoek staat beschreven in dit proefschrift.

Vanaf april 2002 is hij op hetzelfde lab werkzaam op een project dat gefinancierd wordt door het kleine Californische bedrijf Corcept Therapeutics. Hij verricht onderzoek naar de opname in hersenen van de glucocorticoïd receptor antagonist C-1073, een 'oud' medicijn met een nieuwe toepassing als snelwerkend antidepressivum. Een voorpublicatie van dit werk kan in het addendum bij dit proefschrift gevonden worden.

Vanaf voorjaar 2004 zal hij als postdoc werkzaam zijn aan Stanford University (Californië, VS) bij dr. D. M. Lyons en prof. dr. A. H. Schatzberg.

PUBLICATIONS

Book chapters

AM Karssen, OC Meijer and ER De Kloet (2003) Corticosteroids and the blood-brain barrier. In: Steckler T, Kalin NH, Reul JMHM (eds). Handbook on stress, immunology and behaviour. Elsevier Science, Amsterdam, The Netherlands, Ch. 3.2.5: (in press)

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NAWOORD

Met het afronden van dit proefschrift is dan eindelijk voor mij de eindstreep als promovendus in zicht, de doctorstitel. Zover ben ik natuurlijk niet slechts in mijn eentje gekomen. Veel mensen hebben, ieder op eigen wijze, bijgedragen aan de totstandkoming van dit proefschrift.
Het werk zoals beschreven in dit proefschrift is uitgevoerd onder de begeleiding van Ron de Kloet. Het project was een samenwerking met Liesbeth de Lange en Bert de Boer van de divisie Farmacologie van het LACDR. Vanaf het tweede jaar, na zijn terugkeer uit de Verenigde Staten, is ook Onno Meijer er nauw bij betrokken geweest.
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Further, I would like to thank Joe Belanoff, who as director and founder of Corcept Therapeutics provided the grant that gave me the opportunity to work on an interesting project and to finish this thesis in the meantime.
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