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
cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticosterone

cortisolone/deoxycortisol
deoxycorticoster
Introduction and objectives

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. Cortisolone (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.
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-flight-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 prefrontal 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,
Introduction and objectives

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). 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.,
Introduction and objectives

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.

<table>
<thead>
<tr>
<th>corticosteroid</th>
<th>receptor affinity</th>
<th>plasma binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortisol/hydrocortisone</td>
<td>MR/GR</td>
<td>CBG/albumin</td>
</tr>
<tr>
<td>corticosterone</td>
<td>MR/GR</td>
<td>CBG/albumin</td>
</tr>
<tr>
<td>dexamethasone</td>
<td>GR</td>
<td></td>
</tr>
<tr>
<td>prednisolone</td>
<td>GR/(MR)</td>
<td>CBG/albumin</td>
</tr>
<tr>
<td>cortisone</td>
<td>GR</td>
<td>albumine</td>
</tr>
<tr>
<td>dehydrocorticosterone</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>cortexolone/deoxy cortisol</td>
<td>(GR)</td>
<td>CBG/albumin</td>
</tr>
<tr>
<td>deoxy corticosterone</td>
<td>MR</td>
<td>n/a</td>
</tr>
<tr>
<td>aldosterone</td>
<td>MR</td>
<td>albumine</td>
</tr>
<tr>
<td>RU486/mifepristone</td>
<td>GR antagonist</td>
<td>AAG</td>
</tr>
</tbody>
</table>

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. Cortisololone 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
Introduction and objectives

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
Chapter 1

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 3H-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 ³H-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 ³H-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](Schinkel1999.png)

**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 clefs and little fenestration or pinocytosis, and they have a relatively high number of mitochondria. 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.
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.
Chapter 1

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 $\alpha$-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).
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.

<table>
<thead>
<tr>
<th>Genbank entry</th>
<th>Homology (^a)</th>
<th>Gene/protein</th>
<th>Protein entry</th>
<th>Homology (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_133401 (^c)</td>
<td>100</td>
<td>Rat mdr1a</td>
<td>NP_596892 (^c)</td>
<td>100</td>
</tr>
<tr>
<td>AF286167</td>
<td></td>
<td></td>
<td>AAK83023</td>
<td>99</td>
</tr>
<tr>
<td>M81855</td>
<td>85</td>
<td>Rat mdr1b</td>
<td>P43245</td>
<td>84 / &gt;90</td>
</tr>
<tr>
<td>NM_011076</td>
<td>90</td>
<td>Mouse mdr1a</td>
<td>NP_035206</td>
<td>95 / 98</td>
</tr>
<tr>
<td>NM_011075</td>
<td>83</td>
<td>Mouse mdr1b</td>
<td>NP_035205</td>
<td>83 / &gt;90</td>
</tr>
<tr>
<td>XM_02059</td>
<td>83</td>
<td>Human MDR1</td>
<td>XP_02059</td>
<td>87 / &gt;93</td>
</tr>
<tr>
<td>NM_000927</td>
<td></td>
<td></td>
<td>NP_000918</td>
<td></td>
</tr>
</tbody>
</table>

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
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 fibrillar 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; Séréé 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 β-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 thymoma 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 choroid 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).

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.