Effects of stress and corticosterone on the hippocampus: linking gene transcription to physiology
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Chapter 1

General introduction
Chapter 1
1.1. Stress and corticosteroids

"Everybody knows what stress is and nobody knows what it is"
Hans Selye (Selye 1973)

Stress and the HPA axis
The term stress was first defined by Hans Selye as “a syndrome produced by diverse noxious agents”. He described three stages of this syndrome: an initial brief alarm reaction, followed by a prolonged period of resistance, and a final stage of exhaustion (Selye 1936). At present, stress is generally defined as a condition in which the physiological or psychological balance, or homeostasis, of an organism is threatened. Stressors are events that (potentially) threat homeostasis and thus induce physiological and behavioral responses directed to reinstate stability (de Kloet et al. 2005; de Kloet et al. 1999; McEwen and Wingfield 2003). The magnitude of the organism’s stress response is influenced not only by the disturbed homeostasis, but also by the adversity of the stressor and the sense of controllability (de Kloet et al. 1998; Kim and Diamond 2002).

Exposure to a stressor leads to various endocrine responses on different time-scales (for an overview see (Sapolsky et al. 2000)). A universal component and major hallmark of the stress response is activation of the hypothalamus-pituitary-adrenal (HPA) axis (Box 1). The HPA axis is driven by circadian and ultradian periodic pattern generators (Pecoraro et al. 2006). Stress causes activation of the HPA axis, resulting in an increase in corticosterone secretion from the adrenal glands, which overrides the normal circadian rhythm. This increase in circulating corticosterone levels has a broad range of physiological effects, including immune system suppression, increased cardiovascular tone, increased circulating glucose levels, and inhibition of reproductive behavior (Sapolsky et al. 2000).

Apart from inducing these peripheral effects, corticosterone can also pass the blood-brain barrier. Among other things, the hormone negatively affects the expression of CRH and VP in the hypothalamus, as well as the release of ACTH from the pituitary gland (Dallman and Jones 1973; Feldman et al. 1992; Kovacs et al. 2000; Pinnock and Herbert 2001). The suppressive effect of (synthetic) corticosteroids on HPA axis activity differs between different rodent strains, indicating that genetic differences are important in the regulation of the stress response (Gomez et al. 1998; Harizi et al. 2007; Thoeringer et al. 2007).

Corticosteroid receptors
Corticosteroid receptors are not only expressed in the periphery, but are also present in the brain (McEwen et al. 1968). Over twenty years ago, it was recognized that two corticosteroid receptor types are expressed, which differ in both their localization and their affinity for corticosterone (Reul and de Kloet 1985). Both the mineralocorticoid
Box 1 - The HPA axis

The hypothalamus-pituitary-adrenal axis (HPA axis; Figure 1) is activated by various environmental factors. In response to stress, parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotrophin releasing hormone (CRH) and vasopressin (VP). These hormones stimulate the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH then reaches the adrenal glands, where the production and release of corticosterone (in rodents; cortisol in humans) is upregulated (Lightman et al. 2002; Volpi et al. 2004). Corticosterone exerts diverse effects throughout the body, including the brain. Via a negative feedback mechanism, corticosterone inhibits the release of CRH by the hypothalamus as well as the release of ACTH by the pituitary gland, thereby ultimately regulating its own release (Canny et al. 1989; Pinnock and Herbert 2001; Plotsky et al. 1993; Sawchenko 1987). Corticosterone can also bind to corticosteroid receptors in higher brain areas, such as the hippocampus, which in turn have indirect control over the HPA-axis (Feldman and Weidenfeld 2001; Jacobson and Sapolsky 1991).

Throughout the day, corticosterone is released in pulses with variable amplitude: the highest amplitudes of corticosterone pulses are found at the start of the active phase (in the evening for rodents, in the morning for man), while pulses are much smaller at the start of the inactive phase (Atkinson et al. 2006; Windle et al. 1998a; Young et al. 2004b). Exposure to stress in the rising phase of this ultradian cycle will give a large increase in corticosterone secretion, whereas stress in the falling phase will lead to a much smaller release of corticosterone (Windle et al. 1998a; Windle et al. 1998b). After chronic stress, the frequency of corticosterone pulses was found to be increased, causing enhanced mean corticosterone levels in these animals (Windle et al. 2001).

Figure 1: Schematic sagittal view of the rat brain and adrenal gland in which various components of the HPA axis are indicated.
receptor (MR) (Arriza et al. 1987) and the glucocorticoid receptor (GR) (Hollenberg et al. 1985) were cloned soon after that. The MR has a high affinity for corticosterone (Kd = 0.5 nM) and is therefore occupied to a large degree under basal HPA conditions. This receptor is mainly expressed in limbic brain areas, such as the hippocampus (Ahima and Harlan 1991). The GR has a tenfold lower affinity for corticosterone (Kd = 5.0 nM) and is thus only substantially occupied when circulating corticosteroid levels are high, e.g. after stress. This receptor is widely expressed throughout the brain (Fuxe et al. 1985; Reul and de Kloet 1985).

Both the MR and the GR belong to the nuclear receptor superfamily and act as transcription factors (Lu et al. 2006). The two receptors show a large degree of sequence homology (Stolte et al. 2006) and consist of several functional domains (Giguere et al. 1986). The N-terminal region contains the ligand-independent activation domain 1 (AF1), and is involved in transactivation and transrepression of downstream genes (Freedman 1999; Fuse et al. 2000). The DNA binding domain (DBD) is responsible for binding of the receptor to the hormone responsive elements (GREs) of target genes, and is involved in transactivation but not transrepression (Luisi et al. 1991; Umesono and Evans 1989). The ligand binding domain (LBD) contains the ligand-dependent activation domain 2 (AF2), which interacts with agonists and various coregulators (Freedman 1999; Hollenberg and Evans 1988). In rats, both the MR and GR gene consist of 8 exons (Lu et al. 2006). The N-terminal and the DBD each consist of two exons. The remaining four exons together form the LBD.

Although encoded by a single gene, the MR has various possible mRNA variants as a result of alternative splicing. In rat, this results in MRα, MRβ, and MRγ mRNA isoforms, which are differentially expressed within the hippocampus (Kwak et al. 1993; Vazquez et al. 1998). In human tissue, only MRα and MRβ are expressed, in a tissue-specific manner (Zennaro et al. 1997). At least two functional translation initiation start sites exist, leading to MR-A and MR-B proteins (Pascual-Le Tallec et al. 2004). Various posttranslational modifications further add to receptor variability. The MR protein can be phosphorylated (Alnemri et al. 1991), although the functional consequences of this modification are unclear. Also, the receptor can be sumoylated and ubiquitinated (Pascual-Le Tallec et al. 2003; Tirard et al. 2007). Several of these modifications of the MR were found to affect transcriptional activity of the protein (Pascual-Le Tallec et al. 2004; Pascual-Le Tallec et al. 2003; Tirard et al. 2007; Zennaro et al. 2001).

Like the MR, the GR exists in several different isoforms. At least two isoforms can be expressed as a result of alternative RNA splicing. Of these, the GRα is the most common isoform, which is highly expressed in the brain (Pujols et al. 2002). The GRβ isoform does not bind corticosterone, but can form heterodimers with GRα, thereby repressing GRα-mediated transactivation (Bamberger et al. 1995; De Castro et al. 1996; Oakley et al. 1999). Via this mechanism, GRβ can contribute to corticosterone resistance (Leung et al. 1997; Sousa et al. 2000; Webster et al. 2001). However, since GRβ is expressed to a very low level in the brain (de Rijk et al. 2003; Pujols et al. 2002), this is
not likely to have a large impact on brain functioning. Apart from alternative splicing, additional GR variants are generated from translational mechanisms (Lu and Cidlowski 2005; Yudt and Cidlowski 2001) and various post-translational modifications such as phosphorylation, ubiquitination, sumoylation, and acetylation (Bodwell et al. 1991; Ito et al. 2006; Tian et al. 2002; Wallace and Cidlowski 2001). Importantly, several of the resulting GR variants have different transcriptional efficacies (Ito et al. 2006; Kino et al. 2007; Le Drean et al. 2002; Lu and Cidlowski 2005; Wallace and Cidlowski 2001; Webster et al. 1997).

**Mechanism of action**

For corticosteroids to affect gene transcription, a first prerequisite is to enter the (brain) cell. The multidrug membrane transporter P-glycoprotein captures corticosteroids from the cell membrane and pumps them out, thus preventing them from entering the cell (Goodsell 1999). In rodents, several isoforms of P-glycoprotein are expressed. The mdr1a (multidrug resistance gene product) is predominantly expressed in the blood brain barrier (Regina et al. 1998) and does not expel corticosterone from the brain (Karssen et al. 2001). Conversely, mdr1b is responsible for the efflux of corticosterone from brain cells (Uhr et al. 2002; Wolf and Horwitz 1992). This form of P-glycoprotein is predominantly expressed in the hippocampus (Karssen et al. 2004; Kwan et al. 2003).

**Figure 2:** Schematic overview of how corticosteroids affect transcription. Mdr = multidrug resistance gene product; hsp = heat shock protein; TF = transcription factor; SRC = steroid receptor coactivator (here used as an example for all coactivators); NCoR = nuclear receptor corepressor (here used as an example for all corepressors). See text for details.
When corticosterone enters the cell, it can be converted into inactive 11-dehydrocorticosterone by the enzyme 11β-hydroxysteroid dehydrogenase-2 (11β-HSD-2) (see for review (Seckl 1997)). The related enzyme 11β-HSD-1 catalyzes the reverse reaction. 11β-HSD-1 mRNA is abundantly present in the hippocampus (Jamieson et al. 1999; Moisan et al. 1990; Wan et al. 2002), whereas 11β-HSD-2 mRNA is expressed to a rather low level in the adult hippocampus (Robson et al. 1998; Zhou et al. 1995). Hippocampal 11β-HSD-1 expression is attenuated by chronic stress (Jamieson et al. 1997), suggesting a homeostatic mechanism to reduce negative effects of chronic high corticosteroid levels on the brain (Seckl and Walker 2001).

In the absence of corticosterone, the MR and GR predominantly reside in the cytoplasm (Figure 2), in a complex with heat shock proteins (hsp) (Hedman et al. 2006; Smith and Toft 1993). In particular hsp70 and hsp90 are essential chaperones for opening of the hydrophobic cleft in the ligand-binding region, and thus for receptor functioning (Morishima et al. 2000; Picard et al. 1990). Upon binding of corticosterone, the receptors dissociate from the hsp complex and translocate into the nucleus (Nishi et al. 2001) where they can regulate gene transcription in basically two different ways.

First, in a process termed transrepression, monomeric MR or GR can bind via protein-protein interactions to transcription factors that have been activated via other signaling cascades (Gottlicher et al. 1998). Most frequently, this results in mutual transcriptional antagonism between the transcription factor and the corticosteroid receptor. The most studied transrepression interactions are those of the GR with transcription factors NF-κB (McKay and Cidlowski 2005; Ray and Prefontaine 1994), AP-1 (Heck et al. 1994), and CREB (Guardiola-Diaz et al. 1996), but many other interactions exist.

Second, in response to activation by glucocorticoids, corticosteroid receptors can form homo- or heterodimers (Nishi et al. 2004) and bind to glucocorticoid responsive elements (GREs) on the DNA (Zilliacus et al. 1995) in a process termed transactivation. Via this mechanism, the activated receptors increase or decrease gene transcription directly or indirectly via the recruitment of coregulatory proteins. These coregulators either promote (coactivators) or attenuate (corepressors) transcriptional activity. The ratio between corepressors and coactivators has been shown to affect the dose-response curve of corticosterone signaling via the GR (Szapary et al. 1999; Wang et al. 2004).

The best-studied family of coactivators is the steroid receptor coactivator (SRC) family, consisting of SRC-1 (a and e), SRC-2, and SRC-3. All SRC family members are expressed in the rodent hippocampus (Meijer et al. 2006). Coactivation of the MR and GR by SRCs depends highly on the specific context. For example, SRC-1e was found to efficiently coactivate transcription from promoters containing 3 GRE’s, while SRC-1a was more efficient when a promoter with 1 GRE was expressed (Meijer et al. 2005). Also, in different cell types different SRC subtypes are recruited by GR activation (Grenier et al. 2004). SRCs bind with different affinities to various nuclear receptors (Ding et al. 1998; Meijer et al. 2005). Finally, partial agonism of mifepristone at the GR is higher when
SRC-1a rather than SRC-1e is overexpressed (Meijer et al. 2005). Thus, SRCs act in a promoter-, cell type-, receptor-, and ligand-specific manner. Apart from the SRC family, many other coactivators exist, such as CBP/p300 (Chakravarti et al. 1996), Zac-1 (Huang and Stallcup 2000), Hic5 (Yang et al. 2000), FLASH (Obradovic et al. 2004), and PGC-1 (Knutti et al. 2000).

The best characterized corepressors are the mutually related proteins nuclear receptor corepressor (N-CoR) (Jepsen et al. 2000) and silencing mediator of retinoid and thyroid receptors (SMRT) (Chen and Evans 1995). Both proteins are highly expressed throughout the hippocampus (van der Laan et al. 2005). N-CoR only binds the GR when it is in complex with an agonist (Wang and Simons 2005), whereas SMRT interacts with the GR in complex with agonists as well as antagonists (Wang et al. 2004). Other known corepressors include DAXX (Obradovic et al. 2004) and PIAS-1 (Pascual-Le Tallec et al. 2003).

Recently, rapid non-genomic effects of corticosterone on hippocampal CA1 neurons were described. These rapid effects likely involve a membrane MR (Dallman 2005; Karst et al. 2005). This thesis, however, will focus on the slower, gene-mediated effects of corticosteroids via the nuclear MR and GR.

**Chronic stress and depression**

Short-term exposure to stress is thought to promote adaptation to the environment (see also section 1.3), maintaining stability as the environment changes. This process is also termed ‘allostasis’ (McEwen and Wingfield 2003). On the other hand, repeated or chronic stress may cause ‘allostatic overload’, in which the cumulative costs for allostasis to the body increase dramatically (McEwen and Wingfield 2003). This can result in mal-adaptation and various physical and psychiatric diseases such as depression (de Kloet et al. 2005; de Kloet et al. 1999; McEwen 2001; Stokes 1995).

Alterations in HPA axis activity are well established in patients suffering from depression. Anatomically, HPA axis hyperactivity in depressed patients is reflected in increased pituitary gland volume (Axelson et al. 1992; Krishnan et al. 1991) and enlarged adrenal glands (Amsterdam et al. 1987; Dorovini-Zis and Zis 1987; Nemeroff et al. 1992; Rubin et al. 1995). Hormone levels are also affected in depression. Hypercortisolism has been found in 40 to 60% of depressed patients, especially in those suffering from severe or psychotic depression (Belanoff et al. 2001b; Parker et al. 2003). Increased cortisol levels were found in saliva, urine, and plasma; in addition, a flattened circadian cortisol rhythm was described (Belanoff et al. 2001b; Galard et al. 1991; Gold et al. 1986; Keller et al. 2006; Rubin et al. 1987; Rybakowski and Twardowska 1999). In bipolar depression, a correlation between plasma corticosterone levels and severity of depression has been found (Rybakowski and Twardowska 1999). Apart from increased corticosteroid levels, increased levels of CRH and ACTH have also been found in depression (Catalan et al. 1998; Nemeroff et al. 1984; O’Toole et al. 1997).
There is thus a clear association between HPA axis dysregulation and the incidence of depression. Importantly, there are several indications that this relationship is causal. First, in depressed patients, HPA axis normalization precedes the clinical effect of the antidepressant and was found to be a prerequisite for clinical improvement (Amsterdam et al. 1988; Heuser et al. 1994; Heuser et al. 1996; Holsboer et al. 1982; Ravindran et al. 1997; Rubin et al. 1995; Rubin et al. 1987). Patients who were clinically remitted but still showed HPA axis dysregulation were found to be likely to relapse into a depressive episode within a few weeks (Holsboer et al. 1982). Second, patients suffering from Cushing’s disease, which is characterized by hypersecretion of corticosteroids, show depressive comorbidity in 70% of the cases. The severity of the depression in these patients is directly related to the circulating levels of cortisol (Murphy and Wolkowitz 1993). Third, stressful life events were found to have a substantial causal relationship to the onset of depression (Kendler et al. 1999).

Several endocrine challenge tests are used as a diagnostic tool in depression research, of which the combined dexamethasone/CRH (dex/CRH) test has the highest sensitivity: on the basis of this test, over 80% of the tested subjects can be correctly identified as patients or non-patients (Heuser et al. 1994). In the dex/CRH test, subjects are pretreated with the synthetic glucocorticoid dexamethasone and receive an injection with CRH the following day. Cortisol levels are monitored in response to the CRH injection. Dose-response curves in this test were found to be shifted towards higher dexamethasone doses in depressed patients, i.e. more dexamethasone was needed to suppress the CRH-mediated increase in cortisol secretion (Modell et al. 1997). These results suggest a disturbed negative feedback mechanism in depressed patients. Another indication for this disturbed feedback comes from mice with a reduced GR function, that show a disinhibited HPA axis and stress-induced depressive-like behavior (Ridder et al. 2005). These and other findings have led to the GR-hypothesis of depression, i.e. the hypothesis that a reduction in number or function of GRs leads to the pattern of HPA-axis dysfunction that is reported in depressed patients (Holsboer 2000; Neigh and Nemeroff 2006). Interestingly, in patients suffering from bipolar or psychotic depression, symptoms were alleviated within a week by treatment with mifepristone, a GR antagonist (Belenoff et al. 2001a; Belanoff et al. 2002; Flores et al. 2006; Simpson et al. 2005; Young et al. 2004a). In rats, the effect of treatment with the selective serotonin reuptake inhibitor fluoxetine was fastened and augmented by additional treatment with a GR antagonist (Johnson et al. 2007). Together, this makes the GR a promising target for future treatment of certain types of depression.

1.2. The hippocampus

The MR and the GR are both abundantly expressed in the hippocampus (Reul and de Kloet 1985). For this reason, much of the research on how stress affects brain function
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has focused on this brain area. In the next section (1.3), I will describe some effects of stress and corticosteroids on the hippocampus. Here, I first highlight the anatomical and functional characteristics of this brain structure.

**Anatomy of the hippocampus**

The hippocampus, a major target for corticosterone, is a large, elongated structure situated between the thalamus and the cerebral cortex in the rat brain. The hippocampal formation is generally divided into four distinct areas: the dentate gyrus (DG), the hippocampus proper or cornu ammonis (CA), the subicular complex, and the parahippocampal cortex (Amaral and Witter 1989; Lopes da Silva et al. 1990). In this thesis, the term ‘hippocampus’ refers to the hippocampus proper and the DG combined. In a cross-section of the hippocampus, two interlocked C-shaped cell layers can be easily distinguished: the granule cell layer of the DG, and the pyramidal cells of the CA region (see Figure 3). The CA cell layer is subdivided into areas CA1 and CA3, with the small transitional CA2 region between them (Lorente de No 1934).

![Diagram of hippocampus](image)

**Figure 3**: Localization of hippocampus in a coronal section of the rat brain (A) and a schematic representation of the hippocampus containing an overview of the main projections between the hippocampal subfields (B). Sb = subiculum; Ent = entorhinal cortex. See text for details concerning hippocampal connections.

In the DG, three different layers are recognized: the molecular or dendritic layer, where the perforant path fibers terminate; the granule cell layer containing the cell bodies of the principal cells; and the polymorphous or hilar region which is populated by a variety of neuronal cell types. The CA region also consists of several layers: the stratum lacunosum-moleculare, containing bundles of fibers and dendritic terminals; the stratum radiatum, where sparse cell bodies as well as the Schaffer collaterals are found;
the stratum pyramidale, containing the pyramidal cell bodies; the stratum oriens, where the basal dendrites of the CA1 pyramidal cells are found; and the alveus, which consists of axons of pyramidal cells and incoming fibers (Amaral and Witter 1989; Lopes da Silva et al. 1990).

Within the hippocampus, communication largely follows a unidirectional trisynaptic pathway (Amaral and Witter 1989; Lopes da Silva et al. 1990). From layer II of the entorhinal cortex, the perforant pathway projects mainly to the granule cells in the DG, but also to CA3 pyramidal cells. From layer III of the entorhinal cortex, projections lead to the CA1 area and the subiculum. Cells in the DG project with their mossy fibers to the entire curvature of the CA3 area, which in turn projects with Schaffer collaterals to the apical dendrites of CA1 pyramidal cells. From the CA1 area, projections lead to the subiculum, and from there back to the entorhinal cortex, thus forming a loop. Apart from this pathway, neurons from CA1, CA3 as well as DG also give rise to axons that terminate in the contralateral hippocampus (Van Groen and Wyss 1988).

**Function of the hippocampus**

The hippocampus is a functional part of the limbic system, which is associated with novelty and fear related responses (Purves 2004). An important case study addressing the specific function of the hippocampus and surrounding areas is that of patient H.M. This patient had his amygdala, hippocampal gyrus, and anterior two-thirds of the hippocampus removed, in an attempt to decrease his epileptic symptoms. The operation led to severe anterograde memory impairment, without affecting other cognitive functions or intelligence (Scoville and Milner 1957). A later case study described patient R.B., who exhibited amnesia after suffering a bilateral lesion of the CA1 area due to ischemia (Zola-Morgan et al. 1986). This case study indicates that damage to only the hippocampus, without affecting surrounding areas, results in a clinically significant and long-lasting impairment in declarative memory. Later animal and human studies confirmed the notion that the hippocampus is specifically important for declarative, but not implicit memory (Eichenbaum 2000; Squire and Zola 1996; Zola-Morgan and Squire 1993).

A clear example of declarative memory is spatial memory, which is often tested in rodents with the Morris water maze experiment. In this test, animals are required to find a hidden platform in a circular pool, using visual cues. Spatial memory in this type of experiment was found to strongly depend on hippocampal functioning (Morris et al. 1982; Winson 1978). Also in humans, the hippocampus is important for spatial memory, as was shown in several PET studies (Maguire et al. 1998; Maguire et al. 1997). Another indication that the hippocampus is important for spatial memory comes from the discovery of so-called place cells (Leutgeb et al. 2005). These are hippocampal principal cells that fire when an animal is in a specific location in its environment. Place cells were found in animals as well as in humans (Ekstrom et al. 2003; O'Keefe and Dostrovsky 1971; Wilson and McNaughton 1993).
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Hippocampal long-term potentiation (LTP) is characterized by a long-term change in synaptic efficacy, and is believed to underlie learning and memory processes (Kim and Diamond 2002). LTP in the CA1 area was found to be necessary for consolidation of spatial memory (Tsien et al. 1996). Conversely, impairments in LTP in the mossy fiber-CA3 and perforant path-DG pathways are not correlated with a spatial memory deficit (Chen and Tonegawa 1997). The DG however does play a crucial role in pattern separation, meaning that small changes in spatial input result in large changes in DG output to the CA3 area (Leutgeb et al. 2007; McHugh et al. 2007). Furthermore, the DG is essential for detecting novelty of spatial information (Lee et al. 2004). The CA3 area plays a crucial role in rapid learning and pattern completion, meaning that slightly different spatial inputs result in the same output from CA3 to CA1 (Leutgeb et al. 2007; Nakazawa et al. 2002; Nakazawa et al. 2003).

1.3. Stress and the hippocampus

Corticosteroid receptors are differentially expressed in the various hippocampal subfields. The MR is highly expressed in all hippocampal cell layers (van Eekelen and de Kloet 1992). This holds true for the α, β, as well as the γ splice variant, although some differences in expression between these subunits were found (Vazquez et al. 1998). The GR, on the other hand, is expressed to a high level in the CA1-2 area and the DG in the adult rat, but very weakly in the CA3 area (Fuxe et al. 1985; Rosenfeld et al. 1988; van Eekelen and de Kloet 1992). This results in a large degree of colocalization of both receptors in the CA1 and DG, but not the CA3 area (Han et al. 2005; van Steensel et al. 1996).

Due to the high expression of MRs as well as GRs, CA1 and DG neurons are very sensitive to stress and corticosteroids. Already some decades ago, corticosteroid receptors in the hippocampus were shown to be functionally relevant in a study where peripherally administered corticosterone decreased the firing rate of hippocampal neurons (Pfaff et al. 1971). Since then, many targets for corticosteroids have been identified that play a role in neuronal excitability. Among these targets are voltage-dependent ion channels, in particular voltage-dependent calcium channels (VDCCs); neurotransmitter mediated responses via ligand gated or G-protein coupled receptors; and ion transporters (de Kloet et al. 2005; Joels 2001).

In general, basal properties of the cell are not affected by corticosterone. Changes in cell physiology only become apparent when the membrane potential of the cell is shifted away from the resting level (Joels and De Kloet 1989; Kerr et al. 1989). MR activation is thought to play an important role in maintaining excitability: when predominantly the MR is activated, voltage-dependent calcium influx is small, restricting cell firing frequency accommodation and afterhyperpolarization (AHP) amplitude (Joels 2001; Joels and de Kloet 1990). At the same time, inhibitory inputs from serotonergic
fibers are attenuated (Joels 2001; Joels and de Kloet 1992). Conversely, GR activation leads to enhanced calcium influx, which will cause enhanced AHP and therefore attenuated transfer of excitatory information (Joels 2001; Karst et al. 1994; Kerr et al. 1992). The increase of modulatory 5-HT_{1A} receptor mediated responses adds to the attenuation of excitation (Joels 2001; Joels and de Kloet 1990; 1992). It should be noted that these effects of MR and GR activation on cellular physiology have been mainly studied in the hippocampal CA1 area. In other brain regions, such as the DG, different effects of MR and GR activation have been reported (Joels 2006).

In this paragraph, I will describe some effects of stress and corticosteroids on hippocampal cells that are relevant for this thesis, focusing on VDCCs and G-protein coupled 5-HT_{1A} receptors.

**Voltage-dependent calcium channels**

Currents flowing through voltage-dependent calcium channels (VDCCs) are one of the main targets for corticosterone. Many different VDCC subunits are expressed in the hippocampus (Box 2). Calcium influx through VDCCs has many functions, including regulation of gene expression, mRNA stability, excitation, synaptic plasticity, and even neuronal survival (Catterall et al. 2003; Walker and De Waard 1998).

Voltage-dependent calcium currents in the hippocampal CA1 area are modulated by corticosterone with a U-shaped dose dependency (Joels 2006; Joels et al. 1994). Under basal conditions, when predominantly the MR is activated, calcium influx through VDCCs is limited (Karst et al. 1994). Excitability of the cells is thus maintained and cellular viability is promoted (Joels 2001; Joels et al. 1994). However, after activation of the GR in addition to the MR, as well as when circulating corticosterone levels are extremely low, voltage-dependent calcium currents are dramatically increased (Karst et al. 1994; Karst et al. 1997b; Kerr et al. 1992). This increased calcium influx can help to normalize activity after stress-induced arousal, but may also lead to enhanced vulnerability to cell death upon additional stimuli (Joels 2001; Joels et al. 1994). In the rodent CA1 area, enhanced calcium influx particularly through sustained high-voltage activated VDCCs was found upon activation of the GR (Joels et al. 2003; Karst et al. 1994; Kerr et al. 1992). It was recently shown that the L-type but not N-type current is affected (Chameau et al. 2007).

The effect of high levels of corticosterone on calcium currents depends on a GR-mediated genomic mechanism, as was shown in a study using transgenic GR^{dim/dim} mice in which homodimerization and subsequent DNA binding of GRs is prevented (Karst et al. 2000). However, whether direct transcriptional regulation of calcium channel subunits composing the L-type VDCC is involved in this process, is not quite clear. In a recent PCR study, mRNA expression of the α1C and α1D subunits, which can both compose the pore of the L-type channel, was not affected by corticosterone (Chameau et al. 2007). In the
Box 2 – Voltage-dependent calcium channel subunits

Stress or corticosterone incubation can influence calcium currents flowing through voltage-dependent calcium channels (VDCCs) in hippocampal cells. VDCCs consist of a pore-forming and voltage-dependent α1 subunit and several auxiliary subunits (Figure 4) (Catterall et al. 2003; Isom et al. 1994; Tsien et al. 1991; Walker and De Waard 1998). The largely extracellular α2-δ subunit is composed of two proteins linked together via a disulphide bridge and associates with the α1 subunit to increase current density, accelerate current activation and inactivation, and shift the current-voltage relation in a hyperpolarizing direction (Klugbauer et al. 2003). The cytoplasmic β subunit is important for surface expression of the VDCC and affects kinetics of activation and voltage dependence as well as stabilization of gating modes (Birnbaumer et al. 1998). A transmembrane γ subunit is sometimes present and decreases VDCC activity by causing a hyperpolarizing shift in the inactivation curve (Black 2003). Apart from the different possible compositions of calcium channels, channel properties can also be affected by posttranslational modifications. For instance, both α1 and β subunits can be phosphorylated, which increases the calcium influx through the channel (Nunoki et al. 1989).

An overview of the different nomenclatures as well as voltage activation properties of calcium channel α1 subunits is listed in Table 1.

![Figure 4: Schematic representation of the subunit structure of a voltage-dependent calcium channel in the membrane.](image)

<table>
<thead>
<tr>
<th>Calcium channel</th>
<th>α1 Subunit</th>
<th>Type</th>
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<th>Properties</th>
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<td>HVA</td>
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<td>Ca,1.2</td>
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<td>HVA</td>
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<td>R</td>
<td>yes</td>
<td>L/HVA</td>
</tr>
<tr>
<td>Ca,3.1</td>
<td>α1G</td>
<td>T</td>
<td>yes</td>
<td>LVA</td>
</tr>
<tr>
<td>Ca,3.2</td>
<td>α1H</td>
<td>T</td>
<td>yes</td>
<td>LVA</td>
</tr>
<tr>
<td>Ca,3.3</td>
<td>α1I</td>
<td>T</td>
<td>yes</td>
<td>LVA</td>
</tr>
</tbody>
</table>

Table 1: Overview of the various types of VDCCs and their corresponding α1 subunits; expression in rat hippocampus and voltage properties. LVA = low voltage activated; HVA = high voltage activated.
same study, though, increased expression of the β4 subunit was found, which might partly account for the enhanced current by increasing trafficking of the channel to the cell membrane (Arikkath and Campbell 2003; Birnbaum et al. 1998).

Not many studies have addressed the effect of corticosteroids on calcium currents in the dentate gyrus. Adrenalectomy (ADX) was found to time-dependently alter voltage-dependent calcium currents in the DG: currents were enhanced 1-2 days after ADX, but decreased when recorded after 3-7 days (Kärst and Joels 2001). In accordance, in the first few days after ADX, increased mRNA expression of the α1C subunit was found (Nair et al. 2004). At the start of this thesis, the effect of GR activation on calcium influx in the DG was not known.

5-HT_{1A} receptor-mediated responses
The hippocampus receives many projections from serotonergic fibers and is rich in 5-HT receptors (Box 3) in all hippocampal subfields. 5-HT is involved in several functions in the central nervous system: serotonergic neurons modulate electrical activity and responsivity to external stimuli. Because of the widespread distribution of the serotonergic system, a large variety of functions is affected by 5-HT, including motor output, body weight regulation, sleep, learning, food intake and sexual activity. Furthermore, 5-HT is involved in anxiety and affective disorders (Stahl 2000).

Apart from the 5-HT_{1A} receptor, which is discussed in detail below, several other hippocampal 5-HT receptors (i.e. 5-HT_{2}, 5-HT_{4}, and 5-HT_{7}) can be affected by corticosterone (Bijak et al. 2001; Birnstiel and Beck 1995; Mendelson and McEwen 1991; Watanabe et al. 1993; Yau et al. 2001; Zahorodna et al. 2006). Stimulation of these receptor types leads to depolarization, which is slower in onset and longer lasting than the 5-HT_{1A} receptor-mediated hyperpolarization (Barnes and Sharp 1999; Hoyer et al. 2002). In this thesis, however, only the corticosterone-mediated effects on the 5-HT_{1A} receptor system are discussed.

Acute effects of corticosteroids on 5-HT_{1A} receptor-mediated responses
Like calcium currents, corticosterone affects 5-HT_{1A} receptor-mediated (Andrade and Nicoll 1987) responses in the hippocampal CA1 area with a U-shaped dose dependency (Hesen and Joels 1996; Joels 2006; Joels et al. 1994). When corticosteroid levels are low, the response of CA1 pyramidal neurons to 5-HT is small (Beck et al. 1996; Joels and de Kloet 1992; Joels et al. 1991). In the absence of corticosterone, however, an enhanced response to 5-HT is found (Hesen and Joels 1996; Hesen et al. 1996). Also after acute stress, a single injection with corticosterone, or in vitro corticosterone incubation, 5-HT_{1A} receptor-mediated responses are increased (Joels and de Kloet 1992; Joels et al. 1997). This effect can be reversed by treatment with the GR antagonist mifepristone (Hesen and Joels 1996; Joels and de Kloet 1992; Joels et al. 1997) and was found to depend on GR homodimerization (Karst et al. 2000). This indicates that
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Box 3 – The 5-HT\textsubscript{1A} receptor

At present, 16 different receptors for serotonin (5-hydroxytryptamine; 5-HT) are known to be expressed in the brain. These receptors can be divided into 7 families, i.e. 5-HT\textsubscript{1} (5-HT\textsubscript{1A}, 5-HT\textsubscript{1B}, 5-HT\textsubscript{1D}, 5-HT\textsubscript{1E}, and 5-HT\textsubscript{1F}), 5-HT\textsubscript{2} (5-HT\textsubscript{2A}, 5-HT\textsubscript{2B}, and 5-HT\textsubscript{2C}), 5-HT\textsubscript{3} (5-HT\textsubscript{3A}, 5-HT\textsubscript{3B}, 5-HT\textsubscript{3C}), 5-HT\textsubscript{4}, 5-HT\textsubscript{5} (5-HT\textsubscript{5A} and 5-HT\textsubscript{5B}), 5-HT\textsubscript{6}, and 5-HT\textsubscript{7} (lower case indicates receptors that have not been demonstrated to definitely function in native systems) (Barnes and Sharp 1999; Hoyer et al. 2002; Kroeze et al. 2002). With the exception of the 5-HT\textsubscript{3} receptor, which is a ligand-gated ion channel (Maricq et al. 1991), all 5-HT receptors are G-protein coupled receptors (Raymond et al. 2001) (Figure 5). Many of these 5-HT receptors are expressed in the hippocampus (Andrade 1998; Barnes and Sharp 1999).

One of the best characterized 5-HT receptors is the 5-HT\textsubscript{1A} receptor, which is linked to a G-protein coupled inwardly rectifying K\textsuperscript{+} channel (GIRK or K\textsubscript{ir}; see Figure 5) (Andrade et al. 1986; Andrade and Nicoll 1987; Luscher et al. 1997) and is highly expressed in the hippocampus, where it is located postsynaptically (Zgombick et al. 1989). The 5-HT\textsubscript{1A} receptor plays a role in many processes, including motor output, body weight regulation, sleep, learning, food intake and sexual activity (Stahl 2000). Furthermore, the receptor has been implicated in depression and antidepressant treatment (Cryan and Leonard 2000; Graeff et al. 1996; Lesch and Gutknecht 2004).

Many potential modulators of 5-HT\textsubscript{1A} receptor-mediated responses exist. For example, activity of the G-protein can be affected by members of the protein family regulators of G-protein signaling (RGS) (Willars 2006). These RGS proteins increase endogenous GTPase activity (Berman et al. 1996; Watson et al. 1996), and some members (i.e. at least RGS1, RGS3, and RGS4) dramatically accelerate 5-HT\textsubscript{1A} receptor-mediated K\textsuperscript{+} current waveforms (Douplnik et al. 1997). In another study, RGS4, RGS10 and RGSZ1 were found to be effective inhibitors of G-protein signaling in response to activation of 5-HT\textsubscript{1A} receptors (Ghavami et al. 2004).

Additionally, various proteins can affect surface expression of the GIRK channel. A first example is serum- and glucocorticoid-regulated kinase (SGK), which is expressed as one of three different isoforms (SGK1-3) (Kobayashi et al. 1999). SGK1 regulates cell surface expression of many ion channels (Lang et al. 2005) including GIRK (Gamper et al. 2002; Lang et al. 2003; Yoo et al. 2003). Another example is neural cell adhesion molecule (NCAM), which is involved in regulation of GIRK surface expression (Delling et al. 2002). In NCAM-knockout mice, sensitization to activation of the 5-HT\textsubscript{1A} receptor was found (Stork et al. 1999), indicating that NCAM reduces surface expression of the GIRK channel that is responsible for the 5-HT\textsubscript{1A} receptor-mediated response in wild-type animals.
transcriptional regulation involving the GR is a prerequisite for the altered physiological responses to occur.

Data on the effect of corticosterone on 5-HT_{1A} receptor mRNA expression is somewhat less conclusive. After ADX, increased 5-HT_{1A} receptor gene expression was found in all hippocampal subfields (Chalmers et al. 1993; Mendelson and McEwen 1992; Zhong and Ciaranello 1995). In the CA1 area, administration of high doses of corticosterone was found to decrease 5-HT_{1A} receptor expression in some studies (Czyrak et al. 2002; Lopez et al. 1999), while others reported no effect (Meijer and de Kloet 1994; Meijer and de Kloet 1995). Taken together, no changes in mRNA expression of the 5-HT_{1A} receptor have been found in the CA1 area after corticosterone application or stress that could account for the altered functional 5-HT_{1A} receptor-mediated response.

In the DG, occupation of the MR was found to normalize 5-HT_{1A} receptor gene expression after ADX, while additional occupation of the GR leads to decreased expression (Lopez et al. 1999; Meijer and de Kloet 1994). However, functional responses to 10 µM 5-HT were increased by activation of the GR in addition to the MR in adrenalectomized animals, while responses to 30 µM 5-HT were not affected by occupation of the GR (Karten et al. 2001). Thus, the altered mRNA expression was not reflected in similar changes in 5-HT_{1A} receptor-mediated responses.

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**Effects of chronic high corticosterone on 5-HT_{1A} receptor-mediated responses**

Aside from the acute effects of corticosterone, long-term exposure to corticosteroids also affects the serotonergic system. 5-HT_{1A} receptor-mediated responses were found to be attenuated by exposure to high levels of exogenous corticosterone for 2 or 3 weeks (Karten et al. 1999; Mueller and Beck 2000). A 21-day chronic unpredictable stress paradigm also decreased the functional 5-HT_{1A} response (van Riel et al. 2003). Concerning transcriptional changes, some studies have shown that chronic restraint as well as chronic social stress results in a downregulation of 5-HT_{1A} receptor mRNA expression and binding in the hippocampus (Lopez et al. 1998; Watanabe et al. 1993). However, other studies have shown no difference in 5-HT_{1A} receptor gene expression in response to chronic stress or chronic high levels of exogenous corticosterone (Karten et al. 1999; Lopez et al. 1999; van Riel et al. 2003).
1.4. Transcriptional changes as a basis for altered physiology

The effects of high doses of corticosteroids on voltage-dependent calcium influx and 5-HT$_{1A}$ receptor-mediated responses depend on homodimerization and subsequent DNA binding of the GR (Karst et al. 2000). The GR-mediated changes in physiology develop in a delayed manner (de Kloet et al. 1998; Joels 2001; Joels and de Kloet 1992) and changes in 5-HT$_{1A}$ receptor-mediated responses, AHP amplitude, and voltage-dependent calcium current amplitude were shown to depend on de novo protein synthesis (Karst and Joels 1991; Kerr et al. 1992). Together, these findings indicate that a GR-mediated genomic mechanism - most likely by transactivation - is essential for the effects of corticosterone on cellular excitability (Figure 6).

Many genes in the hippocampus are regulated by corticosteroids. In a large-scale gene profiling study, over 200 genes have been identified that are regulated by MR or GR activation (Datson et al. 2001). In a different study using micorarrays, GR-induced transcriptional changes in the hippocampus were shown to be time-dependent (Morsink et al. 2006). Specifically, 1 hour after 20 min incubation with 100 nM corticosterone exclusively down-regulated genes were observed, while 3 hours after GR activation both up- and down-regulated genes were found. After 5 hours, gene expression was almost back at baseline. This suggests that the first wave of genomic effects of corticosterone is realized predominantly via transrepression, followed by a wave of transactivation.

However, although these large-scale studies revealed many corticosteroid-regulated genes, they are not very informative about gene transcripts that underlie changes in neuronal excitability, since their abundance is often too low to be reliably detected. The sensitivity of these large-scale studies was also compromised by the fact

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**Figure 6:** Schematic representation of the link between gene expression, protein levels, and changes in cellular physiology via ion transporters, G-protein coupled receptors, and ion channels.
that the whole hippocampus or hippocampal slices were used, possibly diluting cell- or subregion-specific effects of corticosterone. This is important because the transcriptomes of different hippocampal subregions were shown to be quite different from each other (Datson et al. 2004; Lein et al. 2004; Zhao et al. 2001). Thus, the availability of coactivators and corepressors as well as mediators of downstream pathways may differ between the regions, which could lead to regionally specific effects of corticosterone.

Physiological effects of corticosterone on cells in the CA1 area have been repeatedly described to have a U shaped dose dependency (Joels 2006). This consistent pattern across various physiological parameters may point to the fact that corticosteroids target a gene that is involved in many different responses. However, transcriptional regulation of candidate genes that might underlie the physiological effects does not follow the same pattern. Thus, to date no direct link has been found between changes in cellular excitability and corticosteroid-responsive genes, leaving the molecular mechanism behind the GR-mediated effects on hippocampal physiology to be clarified. Possible explanations for this lack of direct link are, apart from technical considerations: (i) the changes in receptor or channel mRNA levels are transient and precede the effects on neuroexcitability (Joels et al. 2003; Morsink et al. 2006), or (ii) other genes that modify translational processes, trafficking, or functioning of the receptor or channel are regulated by corticosterone.

The main question of this thesis is whether and how transcriptional regulation induced by stress and corticosteroids can be linked to functional changes in principal cells of the rodent hippocampus.

1.5. Outline of this thesis

Assumption
Glucocorticoid receptors are transcriptional regulators of several genes in the hippocampus. As a result of transcriptional regulation, physiological properties of hippocampal cells are altered.

Questions
In this thesis, we tried to get more insight into the relationship between altered physiology and transcriptional changes induced by stress or corticosterone in the hippocampus. Several approaches can be used to study this relationship.

A first approach tries to explain physiological changes known to occur in response to corticosterone by studying transcriptional regulation of several candidate genes that might underlie these physiological changes. It was found earlier that 5-HT_{1A} receptor-mediated K^+ currents in hippocampal CA1 cells were increased in response to acute stress or corticosterone application (Hesen and Joels 1996; Joels and de Kloet 1992;
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Joels et al. 1997). Transcriptional changes were found to be necessary for this effect (Karst et al. 2000). In contrast, chronic high corticosterone levels resulted in a decrease in 5-HT<sub>1A</sub> receptor-mediated responses (Karten et al. 1999; van Riel et al. 2003). Transcriptional changes that might underlie these phenomena were studied with a candidate gene approach.

- In **chapter 2**, we examined the effect of corticosterone on hippocampal mRNA expression of RGS4 and SGK1. These two candidate genes might be related to the increase in 5-HT<sub>1A</sub> receptor-mediated responses after acute corticosterone application.

- In **chapter 3**, possible transcriptional changes underlying the decrease in 5-HT<sub>1A</sub> receptor-mediated responses after chronic high corticosterone levels were studied. Hippocampal mRNA expression of RGS4, SGK1 as well as NCAM was studied in rats subjected to high corticosterone levels for 21 days.

A second possible approach is to look at the effects of known transcriptional changes by examining whether these changes in transcription are translated to changes in physiology. After chronic stress, alterations in mRNA expression of calcium channel subunits were found in the hippocampal dentate gyrus. These transcriptional changes were only apparent when slices obtained from chronically stressed animals were acutely incubated with corticosterone (Qin et al. 2004).

- In **chapter 4**, we examined whether the earlier observed changes in mRNA expression of calcium channel subunits after chronic stress were translated into changes in whole cell calcium currents in dentate granule cells. We also studied the effects of treatment with the GR antagonist mifepristone on calcium currents in control and chronically stressed rats.

A third approach is to ask the question whether mRNA, protein expression, and physiological function of calcium channels change in parallel by GR activation. The chronic stress study indicated regional differences in the effects of chronic stress as well as acute corticosterone application in the CA1 area compared to the dentate gyrus (Karst and Joels 2007; van Gemert and Joels 2006). Differences in corticosteroid effects on mRNA and protein expression might underlie these regional differences in corticosteroid effects on physiology.

- In **chapter 5**, the effect of acute corticosterone application on mRNA and protein expression of calcium channel subunits as well as on calcium currents was studied in two hippocampal subfields: CA1 area and dentate gyrus.

The experimental findings of this thesis are summarized and discussed in **chapter 6**.