Gene expression in identified rat hippocampal neurons: Modulation by corticosteroids and stress
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Chapter 1

General Introduction

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Chapter 1

The term stress was originally described by Hans Selye (Selye, 1936) as "a syndrome produced by diverse noxious agents". At present, it could be generally defined as any factor under any condition that seriously perturbs either physiological or psychological homeostasis of an organism. In response to stress, amongst other things the hypothalamic-pituitary-adrenal axis gets activated resulting in raised release of the stress hormone corticosterone in rodents (cortisol in human). Corticosterone enters the brain, targets many regions including the hippocampus, where it modulates hippocampal functions by activating receptors – i.e. the high affinity mineralocorticoid receptor and low affinity glucocorticoid receptor, which are nuclear transcription factors. Activation of the mineralocorticoid receptor is required to maintain physiological homeostasis while activation of the glucocorticoid receptor is necessary to restore disturbed physiological balance. Actions of the two types of receptors are believed to function via altered transcription of corticosteroid responsive genes. The main aim of this thesis was to attempt to reveal how expression changes of corticosteroid responsive genes in synaptically active neurons may contribute to altered neuronal activity, in particular in the hippocampal dentate gyrus and CA1 area. We addressed this issue in small samples of biologically relevant brain tissue and under conditions of differential activation of corticosteroid receptors. By correlating expression changes with altered electrophysiological properties in identified hippocampal principal neurons, we hoped to link physiology and its underlying molecular mechanism. The results may help obtain more insight in the genes involved in stress related neurological disorders, and thus to narrow down candidate genes for further functional analysis.

1.1 Stress and stress hormones

When animals are exposed to a stressful condition, very often the balanced physiological homeostasis is disturbed, which triggers a sequence of complex neuro-endocrinological reactions in order to cope with challenged stimulations, the so-called stress response. Various stressors induce corresponding stress responses, thus, nature and duration of the stressor determine the degree and specificity of the stress response (Kim and Diamond, 2002).

The stress response acts as a double edged sword: on the one hand, temporarily switching on the stress response prevents further disturbance and intends to restore the disturbed homeostasis; on the other hand, if the stress response cannot be switched off it may enhance in the long run susceptibility and vulnerability of the cells to pathological states. Nevertheless, regardless of the diverse stressors inducing various types of stress responses, one universal component of the stress response is a well characterized activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1). Thus, higher brain structures like the hippocampal formation integrate and process external stressor signals, then send integrated internal signals to lower brain structures. Stimulated by input from higher brain structures e.g. the hippocampus, corticotrophin-releasing hormone (CRH) together with its co-secretagogue vasopressin (VP) are released from the paraventricular nucleus (PVN) located close to the median eminence of the hypothalamus, reaching the adenohypophysis or anterior pituitary via the portal vessels. In the anterior pituitary, another peptide adrenocorticotropic hormone (ACTH), is synthesized and secreted. CRH has been found to play an important role in mediating stress responses by binding to two types of receptors. It has been shown that the CRH receptor-1 mediates a fast immediate mode that activates the
sympathetic/flight-or-fight system (Chrousos, 1998); by contrast CRH receptor-2 mediates a slow-sustained adaptive mode that activates the para-sympathetic system (Chrousos and Gold, 1992; Hsu and Hsueh, 2001). VP is considered as a modulator of ACTH secretion (Kovacs, 1998) that potentiates the effect of CRH (Gillies et al., 1982), possibly through the PKC signalling pathway (Thibonni er et al., 1998). Expression of CRH, VP and ACTH have been shown to be regulated by stress (Kovacs et al., 2000; Pinnock and Herbert, 2001).

When ACTH is secreted from the pituitary it reaches the adrenal gland where in rodents it stimulates synthesis and release of an important stress hormone corticosterone (cortisol in humans). Circulating via the blood stream, corticosterone is delivered widely over the body, including the central nervous system (Dallman et al., 1994). Within the stress HPA axis, corticosterone is found to be the major feedback factor controlling HPA activity by adjusting production and release of CRH and ACTH, at the level of hypothalamus and pituitary respectively (Akana et al., 1992). Interestingly, the synthetic steroid dexamethasone (DEX) was found to bind mainly in the anterior pituitary (De Kloet et al., 1975). Later on, the presence of a membrane protein, the multiplied drug resistance P-glycoprotein, was found to be mainly responsible for expelling DEX from the brain (Meijer et al., 1998). The HPA axis activity is not only determined by stress but also has a natural rhythm controlled by the biological clock. The release of corticosterone peaks in the evening and lowers in the morning for rodents like the rat, but goes in the opposite direction for humans, peaking in the morning (Bradbury et al., 1994; Kalsbeek et al., 1996). However, the altered release of corticosterone in response to stress is so strong that it overrides the release pattern of corticosterone encoded by the normal biological clock.

Collectively, HPA axis activation is one of the major hallmarks of the stress response and among other things characterized by enhanced release of the stress hormone corticosterone, which is considered to be a key indicator of stress HPA axis activity.

**Figure 1** Schematic description of the HPA axis. In response to stress, the HPA axis gets activated to cope with the stress. Firstly, corticotrophin-releasing hormone (CRH) as well as vasopressin (VP) are produced in parvocellular neurons of the paraventricular nucleus (PVN) in the hypothalamus, which receives transsynaptically input from the limbic hippocampal formation. Secondly, released from the PVN, CRH reaches the anterior pituitary, where in turn ACTH is secreted into the circulation. Upon stimulation of the adrenal glands by ACTH, the stress hormone corticosterone (in rodents/cortisol in human) is synthesized and released (Dallman et al., 1994; Lightman et al., 2002). Under normal physiological conditions, release of corticosterone responds to a circadian rhythm, peaking in the morning for human or evening for rats (Bradbury et al., 1994; Kalsbeek et al., 1996). Via the blood stream, corticosterone is delivered all over the body, including the central nervous system. The hippocampus is a primary target for corticosterone in the brain. The two types of corticosteroid receptors are abundantly expressed in the hippocampus. Under basal corticosterone levels, there is predominant occupation of the high affinity MR, whereas GR is additionally activated in response to stress.
1.2 Corticosteroid receptors

When released from the adrenal gland and circulating via the blood stream, corticosterone targets most organs of the body. Most importantly for the present thesis, corticosterone passes the brain-blood-barrier, i.e. enters the brain. Corticosterone functions by binding to its receptors. The finding in the late sixties of the presence of corticosteroid receptors in limbic structures, which are known to be involved in learning, memory and cognition (Douglas, 1967; Benson, 1984; O'Keefe, 1990; Squire et al., 1992; Alvarez et al., 1995), for the first time pointed to a recognition site at extra-hypothalamic brain regions (McEwen et al., 1968; Gerlac h and McEwen, 1972). The functional relevance of these sites was underlined a few years later by the observation that peripherally administrated corticosterone suppressed the firing rate of hippocampal neurons (Pfaff et al., 1971). In the eighties it was found that there are in fact two distinct types of corticosteroid receptors, the glucocorticoid (GR) and mineralocorticoid receptor (MR) (Reul and de Kloet, 1985). The discrimination of GR and MR laid down a basis for exploring the effects of differential activation of GR and MR on hippocampal neuronal functions. The concept of two types of corticosteroid receptors was later confirmed by the cloning of GR and its splice variants (Hollenberg et al., 1985) as well as the cloning of MR (Arriza et al., 1987).

The two types of receptor bind corticosterone differentially. MR has a high affinity for corticosterone (Kd=0.5nM), i.e. 10 times higher than GR binding affinity (Kd=5.0nM) (Reul and de Kloet, 1985). Expression of corticosteroid receptors is well documented. MR is mainly expressed in the limbic structures. All subregions of the hippocampus as well as amygdala and septum highly express MR (Ahima and Harlan, 1991). Compared to the restricted expression of MR, expression of GR was found across the whole brain, including hippocampus. Within the hippocampus, the CA1 and dentate gyrus subregions but not CA3 highly express GR (Fuxe et al., 1985). Co-expression of GR and MR not only exists in hippocampus (Van Eekelen et al., 1988), but also in the amygdala and medial prefrontal cortex (Helm et al., 2002).

The GR and MR belong to a super family of nuclear receptors that act as transcriptional factors. GR and MR share great sequence homology. Chemically, the sequence of GR as well as MR contains highly conserved DNA binding and ligand binding domains, and an N and C terminal domain.

The GR is encoded by one gene. Its common form is the GRα isoform, most dominantly expressed in the hippocampus. As standard nuclear transcription factor, the general signalling pathway is well documented (see section 1.3; Parker and Schimmer, 1994; Tenbaum and Baniahmad, 1997; Barrett and Spelsberg, 1998; Hart, 2002; McKenna and O'Malley, 2002). The GRβ isoform is generated due to mRNA splicing at the 3' end (Hollenberg et al., 1985; Encio and Detera-Wadleigh, 1991; Bamberger et al., 1995). Just like the common GRα form, GRβ is capable of initiating transcription (Bamberger et al., 1995) but it is much less expressed compared to GRα in the brain (Oakley et al., 1996). It has been shown that GRβ forms either homodimers or heterodimers with MR to block GRα mediated transactivation (Bamberger et al., 1995; de Castro et al., 1996) and contributes to corticosterone resistance (Leung et al., 1997; Sousa et al., 2000; Strickland et al., 2001; Webster et al., 2001). Although functionally the activity of GRβ seems opposite to that of GRα, it is very unlikely that GRβ would have substantial impact on hippocampal function because of its extremely low expression in brain (Oakley et al., 1996).
Figure 2  General structure of the corticosteroid receptors transcript. Both receptors (MR and GR) belong to the nuclear receptor super family. The genetic sequences of GR or MR comprise distinct protein regions: the N terminal region containing a regulatory activation domain 1 (AF1) that is reported to bind to other nuclear factors such as AP1 (Wallberg et al., 2000; Hsiao et al., 2003); a DNA binding domain (DBD) that is required for transactivation but not transrepression (Luís et al., 1991); a ligand binding region (LBD) that contains activation domain 2 (AF2) which interacts with agonists and coregulators such as SRCs (Leo and Chen, 2000); and the less defined far C terminal. For rats the full length of the GR as well as MR gene contains 8 exons. The N-terminal contains two exons. The DBD also has two exons, which display two highly conserved Zinc finger motifs (Evans, 1988; Naar et al., 1991; Umesono et al., 1991). The remaining four exons exist in the LBD.

With respect to MR, splicing at the untranslated region of the 5' end of MR results in three variants: MRα, β, and γ, which are all expressed in the hippocampus. However, all three splicing forms translate into one identical protein (Arriza et al., 1987; Kwak et al., 1993; Zennaro et al., 1995). Classically, MR is known to form homodimers to regulate gene transcription. However, additional features of MR have been reported. It is clear that MR can form heterodimers with the GR (Trapp et al., 1994). The heterodimers of MR and GR were found to suppress expression of the 5HT-1A gene (Ou et al., 2001). It has been found that the N terminal domain of MR and GR display opposite properties that might determine opposite transcriptional activities (Derfoul et al., 2000).

1.3 Corticosteroid receptors as transcription factor

Corticosteroid receptors are key players in transcriptional signalling pathways. However, the efficacy and specificity of actions of corticosteroid receptors are modulated by various factors, both spatially and temporally, at extracellular, intracellular and nuclear levels.

Firstly at the extracellular level, the free amount of corticosterone determines the availability of corticosterone for its receptors. The availability of corticosterone is controlled by the following three factors: i) plasma corticosterone binding proteins-globulins (CBG): Corticosterone bound with CGB, is regarded as the non-free form and thus has no biological function (Mendel, 1989; Smith and Hammond, 1992; De Kloet, 1997; Breuner and Orchinik, 2002); ii) the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD): Type I enzyme 11β-HSD converts inactive corticosterone to the active state, while type II enzyme 11β-HSD oxidizes active corticosterone to the inactive form (Seckl, 1997). Since the 11β-HSD type II isoform was found not to be expressed in hippocampus, excessive corticosterone would readily saturate its high affinity receptor MR and fully activate the low affinity GR in the hippocampus (Edwards et al., 1988); iii) multidrug resistance P-glycoprotein (mdrPgp): glucocorticoids are actively transported out over the cellular membrane by mdrPgp. The
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pumping action is most apparent for synthetic drugs such as the glucocorticoid agonist DEX (Cordon-Cardo et al., 1989; Bourgeois et al., 1993; Karssen et al., 2002), a fact clearly demonstrated by mdrPg p  knock out studies (Schinkel et al., 1995). Free corticosterone penetrates through the cell membrane to the intracellular compartment to bind corticosteroid receptors.

Secondly, at the intracellular level release of corticosteroid receptors from a protein complex additionally determines the efficacy of action. In the inactivated state, most corticosteroid receptors form a protein complex together with heat shock proteins (Hsps) and immunophilin (Smith and Toft, 1993; Hutchison et al., 1994; Nathan and Lindquist, 1995). In the presence of corticosterone, corticosteroid receptors dissociate from the protein complex and bind to corticosterone, as activated receptors. The activated receptors translocate to the nucleus to promote transcriptional regulation.

Lastly and most importantly, at the nuclear level corticosteroid receptors regulate responsive genes at the transcriptional level. In general, corticosteroid receptors directly target an element named glucocorticoid response element (GRE) of responsive genes. It is generally thought that homodimerization and DNA binding are necessary to regulate gene transcription, a common feature of members of the nuclear factor super family. However, this common rule is not exclusively applicable to the GR. Evidence showed that GR homodimerization is not essential for GRE binding to the genes of phenylethanolamine N-methyltransferase (PNMT) (Adams et al., 2003) and Na/K ATPase beat 1 (Derfoul et al., 2000). In addition, GRs with a deletion of exon 2, which disrupts DNA binding, still retain the potential to regulate transcription (Mittelstadt and Ashwell, 2003). This has led to the notion that GR homodimers can repress transcriptional activities through interaction with other transcription factors. There are quite a number of proteins interacting with GR in the nucleus. So far this was reported for AP-1 (Diamond et al., 1990; Kovacs et al., 2000), NF-kB (Gottlicher et al., 1998; Sheppard et al., 1998), STAT3 (Zhang et al., 1997a; Zhang et al., 1997b), Est2 (Mullick et al., 2001), STAT5 (Stoecklin et al., 1999), HIF-1 (Kodama et al., 2003), peroxisome proliferators activated receptor λ coactivator 1 (PGC-1) (Knutti et al., 2000; Rosenfeld and Glass, 2001) and the protein 14-3-3σ (Kino et al., 2003). Among these, the nuclear transcription factors NF-kB and GR have been identified to repress their transcriptional activities mutually (de Kloet, 2003).

Apart from the above mentioned proteins interacting with corticosteroid receptors, another group of molecules known as steroid receptor coactivators (SRC), has been found to modulate transcriptional activities of corticosteroid receptors (McKenna et al., 1999), in particular transactivation. The SRC family consists of SRC-1, 2 and 3 (McKenna et al., 1999). SRC-1 was found to be broadly expressed in hippocampal principal neurons (Ogawa et al., 2001), indicating a strong coexpression with corticosteroid receptors. The splice variant SRC-1a interacts with GR (Ma et al., 1999; Meijer, 2002). SRC-2 is found to be much less expressed in the brain and specifically interacts with MR (Fuse et al., 2000). Complementary to enhanced transcription by coactivators from the SRC family, another class of molecules defined as corepressors was shown to reduce transcriptional activities of corticosteroid receptors. The corepressors that are expressed in the brain at least comprise NCoR and SMAT (Jeppsen et al., 2000; McKenna and O'Malley, 2002). Preferably, corepressors decrease antagonist-receptor induced gene transcription (Zhang et al., 1998). The mechanism of action of corepressors of corticosteroid receptors awaits more detailed study. Altogether, coactivators and corepressors of corticosteroid receptors control gene
transcription efficacy, possibly in a promoter dependent manner. However, the exact molecular mechanisms need to be further addressed (Figure 3).

**Figure 3** Modulation of the gene transcription machinery by corticosteroid receptors. In the presence of corticosterone, corticosteroid receptors dissociate from a protein complex containing heat shock proteins (hsp) and bind to corticosterone yielding the activated form. The activated corticosteroid receptors then translocate to the nucleus to exert modulation of gene transcription by two modes. The first mode is the transactivation (A). GR homodimers target responsive genes by binding to a so-called glucocorticoid responsive element (GRE), which is a 20-base palindromic sequence (TGGTACAAATGTTCT) (Beato et al., 1987). The GRE is the key mediator for transactivation and it is further divided into three classes based on properties of interacting with the GR, categorized as simple GREs, composite GREs and tether GREs. Full transactivation requires participation of SRCs. The second mode is the so-called transrepression (B). Here, corticosteroid receptors are not necessary to bind to the GREs but interact with other nuclear factors, forming heterodimers to repress transcription of responsive genes. It is generally accepted that monomer but not homodimers of GR are involved in the transrepression pathway. For instance, the nuclear transcription factor NFkB interacts with GR monomers repressing their transcription mutually.

Compared to GR interacting proteins, the candidates for MR are relatively less defined. However, a recent study using the yeast two-hybrid approach revealed a group of proteins interacting with MR. Thus, the death associated proteins DAXX and FLICE associated huge (FLASH) interact with MR and GR, but the fas associated factor -1 (FAF-1) only interacts with MR (Obradovic et al., 2004).

### 1.4 Actions of corticosteroids

At the transcriptional level, by virtue of the transactivation or transrepression machinery, corticosteroid receptors regulate expression of responsive genes, which is directly reflected at the cellular level with respect to physiological functions. Several distinct
classes of proteins were found to be functionally modulated by corticosterone (Figure 4): i) voltage gated ion channels, in particular voltage dependent calcium channels (VDCC); ii) neurotransmitter activated ion channels either activated via ligand or G protein coupled receptors; iii) ATP dependent pumps and transporters (not in figure). The actions of corticosteroid receptors on these classes of proteins have been extensively addressed elsewhere (Joels, 1997, 2001).

![Diagram](image)

**Figure 4** Schematic representation of cellular actions of corticosterone in hippocampal neurons. At the physiological level, corticosterone modulates several distinct classes of molecules including ion channels, ligand gated (ionotropic) receptors and the G protein-coupled receptors (Joels, 1997). Among the ion channels, the L type VDCCs are the most sensitive to corticosterone, whereas ligand gated ionotropic channels, i.e. AMPA, NMDA and GABA receptors are found to be less affected. The functional alterations induced by corticosterone are believed to be genomic effects involving expression changes of corticosterone responsive genes.

Earlier studies suggested that at least calcium currents mediated by L type VDCCs are modulated by corticosterone (Kerr et al., 1992; Karst et al., 1994). The effect displayed a U-shaped dose dependency (Joels et al., 1994). Thus, predominant activation of MR with basal corticosterone levels allows limited Ca influx (Karst et al., 1994). This will maintain the excitability of the cells and promote cellular viability (Joels et al., 1994). By contrast, activation of GRs in addition to MRs as well as absence of corticosterone results in enhanced Ca influx into CA1 neurons, which promotes the susceptibility to undergo degeneration upon an additional stimulus (Kerr et al., 1992; Karst et al., 1994). In the rat hippocampal CA1 neurons enhanced calcium influx through sustained (possibly L – type ) VDCCs after GR activation was demonstrated both in vivo and in vitro (Karst et al., 1994; Joels et al., 2003). Furthermore, the calcium current increase in CA1 neurons was identified to be a GR mediated genomic effect, as concluded from a study on transgenic GR\(_{\text{dim/dim}}\) mice in which the formation of GR homodimers is prevented (Karst et al., 2000). However, whether expression changes of L type VDCCs directly contribute to the altered calcium current is not clear. Furthermore, it needs to be examined whether L type VDCCs are direct genomic targets of GR. Experiments addressing these questions are described in Chapter 2 of this
thesis. Compared to ion channels, ligand gated (amino acid) receptors are assumed to be less affected by corticosterone and the effects are fast and reversible, suggesting possibly a non-genomic process (Joels and de Kloet, 1993). Similarly to what has been described for VDCCs, corticosterone also affects the G-protein coupled 5HT-1A receptor in a U-shaped dose dependent manner (Hesen et al., 1996).

Studies using genetic manipulation of corticosteroid receptors further elaborated their functional relevance. Thus, downregulation of GR by the antisense technique altered HPA axis activity (Pepin et al., 1992). In animals with a deletion of exon III of the GR in brain by the Cre/LoxP system, a reminiscent symptom appeared (Tronche et al., 1999). A variety of other transgenic mice lines were created to study GR mediated signalling. Point mutation in or knocking out of the GR was applied, as shown in the mice lines GR<sup>dim/dim</sup> (Reichardt et al., 1998), GR<sup>null</sup> (Finotto et al., 1999) and GR<sup>hypo</sup> (Cole et al., 1995). The various data from these transgenic mice showed that protein-protein interactions rather than GR homodimerization is essential for survival. However, the corticosterone effects on Ca influx and 5HT-1A receptor transmission described above were found to depend on GR homodimerization (Karst et al., 2000).

Given the differential affinity of MR and GR for corticosterone and the often opposing functional effects exerted by the two receptors types, fluctuation of corticosterone concentration has profound impact on hippocampal functions (McEwen, 1999). This impact is even more evident for two extreme situations commonly applied to study corticosteroid receptor functions, i.e. 1) the depletion of endogenous corticosterone by removal of the adrenal gland (ADX), so that no activation of corticosteroid receptors occurs; and 2) models for stress paradigms that lead to long-term exposure of an animal to elevated corticosterone levels. During the period of stress, the low affinity GR in addition to high affinity MR gets activated.

<i>i) Depletion of corticosterone by ADX – no activation of MR and GR</i>

With basal corticosterone levels (below 5μg/dl for rat), mostly the high affinity receptor MR will be occupied (Reul et al., 1987). With depletion of endogenous corticosterone by ADX, both GR and MR become unoccupied. Therefore, this model is indicated to study the relevance of MR mediated events in the brain.

The effects of ADX are apparent and specific. Cell loss of dentate granule neurons in particular occurs, whereby approximately 25% of dentate granule neurons undergo apoptotic death, while the neighbouring cells sharing the same microenvironment do not (Sloviter et al., 1989; Sapolsky et al., 1991). The apoptotic death is MR dependent, as replacement by a low dose of corticosterone fully prevents cell death (Sloviter et al., 1989; Woolley et al., 1991; Hu et al., 1997). Besides cell loss, atrophy of dendritic trees of dentate granule cells appears in response to ADX. However, this seems to have little effect on ionic currents measured in the soma of granule cells (Wossink et al., 2001).

Parallel to induction of apoptosis, cell proliferation also occurs in the dentate gyrus after ADX, seen in a similar pattern as apoptosis. The induction of new born cells was shown to be repressed by corticosterone application in ADX rats, but MR activation does not seem to be sufficient to reverse this process (Fischer et al., 2002).

ADX has profound effects on ion currents and neurotransmission in hippocampal principal neurons. At the single dentate granule neuron level, enhanced calcium current was noticed by whole cell patch clamp recording (Karst and Joels, 2001). In addition, ADX led
to increased expression of 5HT-1A receptor mRNA in dentate granule neurons (Chalmers et al., 1993; Meijer and de Kloet, 1994, 1995; Le Corre et al., 1997) and CA1 neurons (Chalmers et al., 1993; Zhong and Ciaranello, 1995) although the expression change of 5HT-1A receptors did not correlate with functional responses (Karten et al., 2001).

Outside the hippocampus, an increased miniature inhibitory post-synaptic current frequency was observed in parvocellular neurons in the paraventricular nucleus of the hypothalamus, which could point to a local compensatory mechanism after loss of hormonal feedback (Verkuylen and Joels, 2003). For more details of functional changes after ADX I refer to comprehensive reviews (Joels et al., 1997; Joels, 2001).

In spite of relatively well characterized effects of corticosterone on hippocampal functions in particular at the physiological level, the molecular mechanisms underlying the functional alterations after ADX still need to be further addressed. Concerning the fact that apoptosis occurs three days after ADX in part of the dentate granule neurons while the rest of the granule neurons which share the same microcellular environment do resist apoptosis (Sloviter et al., 1989), it is still unclear what molecular mechanisms selectively determine the cell fate. In this thesis this question will be pursued in Chapter 3. In general, the potential determinants of apoptosis in response to ADX in whole dentate gyrus region can be grouped in two categories: the intrinsic factors of gene expression as well as extrinsic factors such as growth factors (receptors) (Hansson et al., 2000) or synaptic strength (Karten et al., 2001). At the start of this thesis, the expression changes of apoptotic molecules, growth factors (receptors) and other putative candidates had not been examined yet at the single neuron level.

**ii) Elevation of corticosterone by chronic stress – prolonged activation of GR in addition to MR**

When animals are exposed to stressful situations, the HPA axis activity will be enhanced resulting in increased release of corticosterone. Elevated corticosterone levels lead to substantial activation (up to 90%) of GR in addition to MR (Reul and de Kloet, 1985; Spencer et al., 1990). Acute as well as chronic stress paradigms have been found to modulate hippocampal functions markedly (Joels et al., 1994; Joels, 1997; Joels et al., 1997; Joels, 2001). The short-term effects of GR (+MR) activation were briefly discussed earlier, in section 1.4. Below I will focus on the effects of chronically elevated corticosterone levels.

Chronic stress results in dendritic atrophy of CA3 neurons (Magarinos et al., 1996; Galea et al., 1997) that eventually may induce cell loss (Sapolsky et al., 1985). The structure of the dentate gyrus is also affected by chronic stress. One of the features of chronic stress is synaptic vesicle reorganization in mossy fiber terminals of the dentate gyrus (Magarinos et al., 1997). Furthermore, neurogenesis in the dentate gyrus is suppressed by chronic stress (Gould et al., 1997). Recent studies in which an unpredictable chronic stress paradigm was used supplied more details about the effect of chronic stress. Thus, both cell proliferation and apoptosis were found to be suppressed by chronic stress but this suppression was reversible when animals were allowed to recover from stress (Pham et al., 2003; Heine et al., 2004). It has been suggested that the structural changes of hippocampus resulting from chronic restraint stress could be prevented by lithium treatment (Wood et al., 2004).

Functionally, chronic stress also markedly affected the hippocampus. For instance, chronic unpredictable stress enhanced AMPA but not NMDA receptor mediated responses of dentate granule cells, which would make neurons more excitable and enhances vulnerability
to additional stimuli (Karst and Joels, 2003). Similarly, enhanced glutamate mediated neurotransmission was observed in the CA3 region with a chronic immobilization paradigm (Kole et al., 2002). Comparable to the elevated corticosterone level during the chronic stress paradigm, long-term injecting of corticosterone for 56 days was seen to alter mitochondria volume in the CA3 area (Coburn-Litvak et al., 2004). In the CA1 area chronic stress resulted in suppression of 5-HT (1A) receptor-mediated responses, possibly due to posttranslational modification of the receptor (van Riel et al., 2003). Moreover, chronic stress has been found to suppress LTP in the dentate gyrus (Kim and Diamond, 2002; Alfárez et al., 2003) and CA3 region (Pavlides et al., 2002), which might impair learning and memory (Bodnoff et al., 1995) (Figure 5). At the PVN level, a reduced mIPSCs frequency with no changes in amplitude appeared after chronic stress (Verkuyl and Joels, 2004).

A number of neurotransmission genes were found to be modulated by long term exposure to corticosterone. With a binding study, the NMDA receptor subunits NR2A and NR2B were found to be upregulated by long term exposure to corticosterone (Weiland et al., 1997). Expression of glutamate receptor subunit GluR1 was enhanced after a 7 days immobilization stress paradigm (Schwendt and Jezova, 2000). It was found that the expression of GluR2 was upregulated after chronic stress (Rosa et al., 2002). With respect to GABAa receptors, it was found that α1 and α2 subunits were downregulated in dentate gyrus while β2 was upregulated in all hippocampus region in response to a 10 days corticosterone pellet administration (Orchiniak et al., 1995). Additionally, the expression of the GABAa receptor β2 subunit showed an upregulation in CA1 after chronic stress (Cullinan and Wolfe, 2000). Lately, a group of genes involved in cell differentiation and proliferation was found to be repressed after chronic psychosocial stress, and the repressed expression of those genes e.g. nerve growth factor (NGF) may contribute to the atrophy of CA3 dendrites and restrained cell proliferation (Alfonso et al., 2004). At the PVN level, chronic stress increases expression of CRH and vasopressin (Bartanusz et al., 1993; Herman et al., 1995; Aguilera and Rabdan-Diehl, 2000).
Figure 5 Model of chronic stress related neurological or psychiatric disorders. When an animal is exposed to chronic stress, the GR activation resulting from chronic stress differentially modulates responsive genes at the transcription level. The expression changes give rise to altered neuronal transmission e.g. enhanced glutamate mediated responses (Kole et al., 2002; Karst and Joels, 2003). The expression changes also affect the CA3 neurons, which display atrophy of dendrites, reduction of volume and cell loss. The repressed cell proliferation, altered morphology and enhanced neuronal transmission may mutually affect each other. If altered gene expression, neurotransmission and morphology are maintained for a long period of time, the effects induced by chronic stress will become irreversible. Consequently, learning and memory will be impaired and neurological or psychiatric disorders such as major depression could precipitate.

In view of the actions of corticosteroid receptors on transcriptional regulation, one can expect to see changes in gene expression after chronic stress. This is particularly interesting for the identified dentate granule neurons in which AMPA receptor mediated responses were shown to be enhanced (Karst and Joels, 2003). The enhanced excitation contributed by AMPA components of the synaptic response logically suggests a genomic effect. Therefore we addressed the question which corticosteroid responsive genes are involved in this process in Chapter 4. Apart from the obvious candidates of genes encoding for glutamate receptor subunits, other possible targets could be e.g. GABAa receptor subunits, as enhanced excitation may also be caused by decreased inhibition.

In brief, long term exposure to elevated corticosteroid levels following chronic stress, resulting in prolonged activation of the GR, has considerable impact on hippocampal function, as observed at the molecular, cellular as well as morphological level. The impact of chronic stress at multiple levels would push the animal beyond its physiological limit, thus precipitating pathological states (Holsboer and Barden, 1996; De Kloet et al., 1998).

1.5 Profiling corticosteroid responsive genes

It is clear that corticosteroid receptors are central in mediating the signalling pathway which ultimately results in functional alternations e.g. after ADX or chronic stress. However, corticosteroid responsive genes rather than the corticosteroid receptor itself
eventually implement regulation. Thus, it is essentially necessary to identify corticosteroid responsive genes in order to draw a full picture of the actions of corticosterone.

In the search for genes regulated by corticosterone, classic techniques such as in situ hybridization have been applied to neuronal tissues (see also above). For instance, in the hippocampus, it has been found that corticosterone modulates expression of growth factors and their receptors either in the principal neurons (Hansson et al., 2000) or hippocampal glia (Hansson et al., 2001). The differentially regulated expression of specific growth factors and their receptors may contribute to ADX induced apoptosis.

Recent technological developments, though, now allow expression to be examined for multiple or even many different genes. These technical platforms comprise differential display (DD) (Liang and Pardee, 1992), serial analysis of gene expression (SAGE) (Velculescu et al., 1995; Velculescu et al., 1997) and microarray (genechip) analysis (Schena et al., 1995). With differential display (DD), based on the random PCR amplification approach, the expression of about 6,000 genes has been examined in the hippocampus of ADX rats. In response to kainic acid administration, it turned out that 18 genes were transrepressed by ADX, including the transcription factor KROX-20 (Vreugdenhil et al., 1996a; Vreugdenhil et al., 1996b). Later on, with the same DD method, a novel signalling molecule CaMKLK/CARP was identified (Vreugdenhil et al., 1999). With modified SAGE, or MicroSAGE (Datson et al., 2001), up to 30,000 sequence tags corresponding to about 10,000 genes have been screened in whole rat hippocampi three days after ADX. It was found that genes encoding excitatory glutamatergic neurotransmission were among the most abundantly expressed. Using the same technique, over 200 genes were found to be corticosterone responsive. Interestingly, the majority of identified genes were responsive either to MR or GR. In addition to validating the known corticosteroid responsive genes, a number of novel corticosteroid responsive genes were identified such as FKbp12, N-chimaerin, CF6 and EST Rn5874 (Datson et al., 2001). Recently, gene expression of two types of mice genetically selected based on attack latency has been studied in the hippocampus. The results pointed out a strong correlation between differentially expressed genes and distinct morphology of the hippocampus (Feldker et al., 2003b; Feldker et al., 2003a).

No doubt, large scale expression profiling of identified principal neurons is necessary to understand the actions of corticosterone. However, challenges still remain: Firstly, no matter what expression screening method is applied, a considerable amount of starting mRNA is presently required to be able to perform the experiments. This is not advantageous for complex neuronal tissues, where very often the amount of cells and cell types of interest is very limited. This drawback currently limits the straightforward interpretation of gene expression profiling with modern techniques such as microarray. Secondly, low abundant transcripts were found to be difficult to detect by microarray (Vreugdenhil et al., 2001). Thirdly, due to the sensitivity of current microarray technology, to some extent the results still need to be further confirmed by other techniques such as in situ hybridization or real-time PCR.

Taken together, large scale gene expression screening methods like microarray analysis require a considerable amount of starting mRNA, which can only be retrieved from a region that inevitably includes cell types that are not of interest to the study. To retrieve the required amount of mRNA for expression studies from a target of interest always contains non-specific tissues that considerably mask the results. The advantages and disadvantages of the above mentioned techniques are discussed in Box 1.
Chapter 1

Box 1 Approaches to quantify gene expression in hippocampal cells.

Differential Display (DD): A technique that has been widely applied for measuring multiple gene expression simultaneously (Liang and Pardee, 1992). Using modified oligo (dT) primers to which single nucleotides or dinucleotides are attached (i.e. oligo TTTTTT-CA), mRNA is reversed into cDNA. Subsequently, the cDNA is further amplified by adding second primers containing ~13bp arbitrary sequences. The PCR products are labelled either with isotopes or fluorescence. The labelled PCR products of different samples are displayed next to each other with polyacrylamine gel electrophoresis. Thus, expression changes can be compared based on the sizes of PCR products. The DD is often used to compare expression of small sets of genes between different cell types. It also can identify novel genes and is a rather simple method that does not require sophisticated equipment (Liang, 2002).

Serial analysis of gene expression (SAGE): A method that analyses sequence tags representing unique corresponding transcripts (Velculescu et al., 1995). SAGE provides quantification of transcripts and is able to identify novel genes. To perform SAGE, double-stranded cDNA is synthesized with biotinylated oligo (dT) primers. The cDNA is next digested by a 4 base restriction enzyme (i.e. Nla III), which cuts cDNA at every 256bp. Only 3' ends of the cDNA are harvested by streptavidin beads and further divided into two groups and ligated to two different linkers containing restriction sites for Bsm FI and Nla III. After digestion by these two restriction enzymes, 10bp tags are generated and concatenated into long sequences and subsequently cloned into plasmids. Based on the number of tags after sequencing the clones, the gene expression is estimated.

Microarray (Genechip): A high throughput platform that enables thousands of DNAs (cDNA or genomic DNA) in a genomic scale to be measured simultaneously in one single hybridization (Schena et al., 1995). In general, mRNA (control and experimental group) is isolated and reverse transcribed into cDNA which is labelled with fluorescence i.e. Cy3 and Cy5 which give rise to red and green colours. The labelled cDNAs then hybridize with a large scale of targets (cDNA or oligo) arrayed onto a glass slide (or membrane). Subsequently, the hybridization is scanned and the expression levels are calculated based on the intensity of the fluorescence signals. The applications of microarray technology have been thoroughly reviewed elsewhere (Schena et al., 1995; Blohm and Guiseppe-Elie, 2001; Luo and Geschwind, 2001; Southern, 2001; Barlow and Lockhart, 2002; Heller, 2002).

Real-time quantitative PCR (qPCR): The most accurate and sensitive approach that detects the initial amount of transcripts with a wide range of up to $10^7$-folds. qPCR monitors copies of an amplicon at every PCR cycle. At a certain cycle the fluorescence signal reaches the threshold that can be reliably detected. Then, the number of copies is calculated based on the equation: $N_t=N_0*E^C$. $C$ is the PCR cycle number and $N_0$ means the number of copies at that certain PCR cycle $C$. $N_t$ is the initial number of copies before PCR amplification, and $E$ is the amplification efficiency which is assumed 2 under optimal conditions. When a particular $N_t$ is set as threshold, at which the signal can be reliably detected, the initial $N_0$ can be calculated. There are two common fluorescent labelling methods for qPCR, i.e. the fluorogenic 5' nuclease assay (TaqMan) (Applied Biosystems, Inc.) and SYBR green (Morrison et al., 1998). The Taqman offers a very accurate measurement due to the application of specific probes in addition to two primers, but its high cost limits its broad application. The SYBR green method also is sensitive as it binds to double stranded DNA and thus generates fluorescence. Compared to TaqMan it is less expensive and more broadly applied. Recently, a new technique of real-time competitive PCR was found to increase the accuracy and sensitivity of this approach, more details are discussed elsewhere (Ding and Cantor, 2003a, b).
Table 1 Overview of approaches for gene expression studies. The parameters of throughput, sensitivity, and link to physiology are compared between various techniques discussed above.

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of genes examined</th>
<th>Novel gene</th>
<th>Starting materials</th>
<th>Sensitivity</th>
<th>Links to physiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SAGE</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microarray</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>Q-PCR</td>
<td>-</td>
<td>-</td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>SCA</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1. +: many genes can be studied at a time
2. ++: investigation can reveal unknown sequences
3. ++: investigation can be carried out on a limited amount of biologically relevant tissue
4. ++: low abundant transcripts can be studied
5. ++: expression profile is obtained from cells with known physiological properties

1.6 Single cell approach

In order to understand how gene expression changes contribute to functional alterations at the single cell or network level in response to differential activation of corticosteroid receptors, it will be necessary to profile the expression of large sets of genes in a very small amount of homogenously identified principal hippocampal neurons, or preferably even in single neurons. A bridge connecting the need of large scale profiling and limited RNA material is one of the needs for future. It should be realized that the very low amount of RNA isolated from a small amount of homogenous cell types hampers this effort. It has been shown that subtle changes of starting material or a less homogenous cell type significantly affect the interpretation of expression data (Geschwind, 2000; Sandberg et al., 2000; Carter et al., 2001; Zirlinger et al., 2001; Barlow and Lockhart, 2002; Bonaventure et al., 2002). This drawback is even more serious for neuronal cells, which contain over half of overall transcripts (Colantuoni et al., 2000; Sandberg et al., 2000).

With the introduction of RNA amplification and laser capture microdissection (LCM) the possibility of profiling gene expression in identified neurons became feasible.

Presently, there are two general approaches of RNA amplification: a PCR based method (Jena et al., 1996) and linear amplification (Eberwine et al., 1992). The latter method has been applied in this thesis. Linear RNA amplification could be simplified as T7 promoter mediated in vitro transcription. The T7 promoter attached to an oligo d (T) primer is incorporated into the cDNA. With T7 polymerase antisense RNA is generated (amplified).
Under optimal conditions, two rounds of amplification from a single neuron would enable multiple gene expression to be performed with reverse northern blot (Figure 6).

![Diagram](image)

**Figure 6** Overview of linear antisense RNA amplification. Single cell RNA amplification (SCA) provides a semi-quantitative solution to study gene expression (Eberwine et al., 1992). Single cells can be either collected via the recording electrode just after whole cell recording from alive tissue or by laser capture dissection from fixed slices or tissue. The mRNA is reversed into first strand cDNA with oligo d(T)-T7 primers, followed by doubled stranded cDNA synthesis. In the presence of T7 polymerase the complementary RNA (antisense RNA, aRNA) is generated. With optimal conditions, up to one million folds of original copies of transcripts can be generated in two rounds of amplification. During the second round amplification, radioactive 32P CTP is incorporated in the aRNA. The aRNA is subsequently subjected to examination of multiple genes typically with reverse northern blot, which has been used in this thesis. Applications of Single cell RNA amplification are reviewed elsewhere (Dixon et al., 2000; Eberwine, 2001; Bartlett, 2002; Hinkle and Eberwine, 2003).

The significance of RNA amplification is described in detail elsewhere (Ginsberg and Che, 2002; Shaw, 2002). The reliability and reproducibility of these methods have been thoroughly examined (Jena et al., 1996; Puskas et al., 2002; Jenson et al., 2003; Rajeevan et al., 2003; Wang et al., 2003). It was concluded that the linear RNA amplification method is very well applicable to neuronal tissue. Moreover, the linear amplification method can be extended to the single neuron level (Eberwine et al., 1992).

Another revolutionary technique that greatly facilitates gene expression profiling is the laser capture microscopy (LCM) (Emmert-Buck et al., 1996). This technique opens the possibility of studying expression in specifically identified tissue, which is especially valuable for post-mortem fixed neuronal tissues. LCM enables collection of single cells with high efficiency - up to 1000 single cells can be harvested per day. LCM technology has been widely applied in neuronal gene expression studies (Dolter and Braman, 2001; Jin et al., 2001; Mikulowska-Mennis et al., 2002; Vincent et al., 2002). Presently, the combination of
LCM and RNA amplification, microarray, and quantitative PCR is supposed to be the most effective approach, providing a platform for gene expression profiling in a small amount of identified neuronal tissue. It should be realized, though, that necessary control studies are still not fully provided and that this technically challenging combination is far from routinely applied. One study in this thesis addressed the issue about reliability of the combined techniques (Chapter 5, see below).

1.7 Scope of this thesis

In summary, the stress hormone corticosterone, released from the adrenal gland in response to HPA activation, is a key modulator of hippocampal activity. It achieves its effects by binding to two types of receptors, which serve as transcription factors of responsive genes. Fluctuation of the corticosterone concentration e.g. after ADX and acute or chronic stress profoundly affects hippocampal structure and function, most likely through changes in cellular properties caused by transcriptional regulation of responsive genes. Profiling the expression of corticosteroid responsive genes is the first necessary step towards the discovery of the molecular mechanism underlying changes in physiological functions. A combination of single cell patch clamp recording and single cell RNA amplification allows examining simultaneously the electrophysiological response and gene expression generated from the same synaptically active neurons. This may provide a first impression of putative target genes for corticosterone that contribute to physiological functions in the hippocampus.

Questions:

This thesis aims to answer the following questions:

I. In response to acute stress in vivo or acute application of corticosterone in vitro the calcium current in CA1 pyramidal neurons is enhanced. Are calcium channel subunits a direct target for corticosterone? (Chapter 2)

II. Depletion of endogenous corticosterone by ADX results in apoptosis in part of the dentate gyrus cells 3 days later while neighbouring cells stay healthy. Which molecules in single dentate granule cells potentially contribute to the cell survival versus cell loss under conditions of no activation of MR? (Chapter 3)

III. Chronic stress accompanied by elevated corticosterone levels leading to long-term substantial activation of GRs in addition to MRs enhances AMPA receptor mediated transmission in dentate granule neurons. Do expression changes of putative genes determining neuronal excitation correlate with enhanced excitation in single dentate granule neuron? (Chapter 4)

IV. It is important to extend research to human stress-related neurological and psychiatric diseases. Technically, the possibilities to study large scale gene expression in biologically relevant human tissue are still limited. The only possible solution so far is to study fixed post-mortem material. How reliable is gene expression data retrieved from fixed material? (Chapter 5)
Experimental approaches

All experiments described in this thesis were conducted on tissue from hippocampal slices of the rat (Figure 7). In Chapter 2 the CA1 area was dissected from slices under the microscope. The CA1 tissue was subjected to RNA isolation and further mRNA expression was examined by quantitative PCR (qPCR). Expression of ligand gated ion channel and calcium channels subunits was examined. In Chapter 3 and 4 granule neurons of the dentate gyrus were identified during recording with microscopy, based on their location and shape. Linear RNA amplification was applied to single DG neurons collected just after whole-cell recording. The amplified RNA was subjected to slot-blot hybridization. In Chapter 5 either ethanol or para-formaldehyde fixed dentate granule cells (50 cells) were collected by LCM. The mRNA from fixed cells was isolated and linearly amplified. The expression patterns generated from those granules cells were compared with the expression patterns generated from single dentate granule cells collected just after recording from life tissue.

Figure 7 Schematic overview of the whole cell patch clamp recording method (Hamill et al., 1981) and the hippocampal slice. A. Diagram of a hippocampal slice illustrating the synaptic pathways. Anatomically, the hippocampus is divided into three major subregions, i.e. the CA1, CA3 and dentate gyrus (DG). The principal, granule neurons of the DG receive input from the entorhinal cortex via the first excitatory pathway (perforant pathway). The mossy fiber - axons of dentate granule neurons - project to pyramidal neurons of the CA3 region, which provides the second excitatory pathway (mossy fiber pathway). The dendrites of CA1 neurons at stratum radiatum receive input from Schaffer collaterals, which originate from CA3 neurons, forming the third pathway (Schaffer collateral pathway). B. Simplified diagram of whole cell recording technique. The hippocampal slice with a thickness of ~ 300μm was placed in the recording chamber filled with the recording buffer. Single dentate granule cells were identified under the microscope based on shape and location. Then, a very fine glass pipette approaches the cell membrane to form a gigaseal by applying a negative pressure. Once the gigaseal has been established, the small piece of membrane under the glass pipette is ruptured and thus the whole cell recording configuration is established. The ion current created by ions flowing through the cell membrane can be detected and amplified by an
ultra sensitive amplifier. Parameters such as current amplitude, voltage sensitivity and kinetics were analysed. By application of corticosterone, corticosteroid receptor mediated physiological responses can be investigated. When the whole cell recording is finished, the cell content is collected through the recording pipette for subsequent single cell RNA amplification.

References are in the back of this thesis