Effects of stress and corticosterone on the hippocampus: linking gene transcription to physiology
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Chapter 6

General discussion
6.1. Summary

The research described in this thesis is aimed at unraveling whether and how transcriptional regulation induced by stress and corticosteroids can be linked to functional changes in principal cells of the rodent hippocampus. We used several approaches to study this link between altered physiology and transcriptional changes.

**Study transcriptional regulation to explain known physiological changes**

Acute stress and corticosterone application have been shown to increase 5-HT1A receptor-mediated responses in hippocampal CA1 cells (Hesen and Joels 1996; Joels and de Kloet 1992; Joels et al. 1997). For this increased response, GR-mediated transcriptional changes are necessary (Karst et al. 2000). In contrast, chronic high corticosterone levels resulted in a decrease in 5-HT1A receptor-mediated responses (Karten et al. 1999; van Riel et al. 2003). These changes in 5-HT1A receptor function can not be directly explained by changes in 5-HT1A receptor mRNA expression after acute or chronic corticosterone treatment (Czyrak et al. 2002; Karten et al. 1999; Lopez et al. 1998; Lopez et al. 1999; Meijer and de Kloet 1994; Meijer and de Kloet 1995; van Riel et al. 2003; Watanabe et al. 1993). We here tried to explain the altered 5-HT1A receptor-mediated responses by studying transcriptional regulation of several other candidate genes after acute as well as prolonged exposure to high corticosterone levels.

In **chapter 2**, mRNA expression of two candidate genes was studied by *in situ* hybridization after a single corticosterone injection. RGS4 increases GTPase activity and can thus inhibit G-protein signaling in response to 5-HT1A receptor activation (Berman et al. 1996; Ghavami et al. 2004; Watson et al. 1996). SGK1 stimulates cell surface expression of GIRK channels (Gamper et al. 2002; Lang et al. 2003; Yoo et al. 2003) and can thus enhance 5-HT1A receptor-mediated responses. We hypothesized that either decreased RGS4 or increased SGK1 mRNA expression would be the underlying mechanism for the enhanced 5-HT1A receptor-mediated responses in the hippocampal CA1 area after acute corticosterone application. Our results indicate that mRNA expression of both RGS4 and SGK1 is unaltered in rat hippocampus (CA1, CA3, and DG) 1 hour after a single injection with a high dose of corticosterone. A clear (five-fold) increase in SGK1 expression was seen in the corpus callosum, indicating that within this time-frame it is possible to see marked transcriptional regulation via GRs with respect to the candidate genes tested. However, we concluded that the effect of acute corticosterone application on 5-HT1A receptor-mediated responses is not mediated via either altered RGS4 or SGK1 mRNA expression.

Transcriptional changes underlying the attenuated 5-HT1A response after chronic high corticosterone levels were studied with *in situ* hybridization in **chapter 3**. The same candidate genes as in chapter 2 were studied, i.e. RGS4 and SGK1. Additionally, mRNA expression of NCAM was analyzed. NCAM reduces cell surface expression of the 5-HT1A
receptor-coupled GIRK channel (Delling et al. 2002; Stork et al. 1999). We thus hypothesized that either increased RGS4, decreased SGK1, and / or increased NCAM mRNA expression was responsible for the attenuated 5-HT$_{1A}$ responses found in the CA1 area after 21 days exposure to high corticosterone levels. We found that hippocampal mRNA expression of none of the candidate genes was affected by 21 daily injections with a high dose of corticosterone. Likely, these genes are not involved in the altered physiological 5-HT$_{1A}$ receptor-mediated responses found previously.

Considering that we made a deliberate choice for the most likely candidate genes underlying changes in 5-HT$_{1A}$ receptor function after acute or chronic corticosteroid exposure, we have to conclude that this approach did not help to resolve the molecular mechanism underlying the observed functional changes.

**Study physiological effects of known transcriptional changes**

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. Voltage-dependent calcium currents in the hippocampal CA1 area are highly regulated by corticosterone and stress (Karst et al. 1997a; Karst et al. 1994; Kerr et al. 1992). In dentate granule cells, increased calcium currents were reported 1-2 days after ADX (Karst and Joels 2001), which is accompanied by an increase in relative mRNA expression of the VDCC $\alpha_{1C}$ subunit in single dentate granule cells (Nair et al. 2004). After chronic stress, alterations in relative mRNA expression of VDCC subunits were also found in hippocampal dentate granule cells. These transcriptional changes were only apparent when slices obtained from chronically stressed animals were acutely incubated with corticosterone (Qin et al. 2004), indicating that the history of the animal is important for the corticosterone effect.

In chapter 4, we tested whether these alterations in VDCC subunit mRNA expression in the DG after chronic stress were translated into physiological changes. We thus recorded whole cell calcium currents from dentate granule cells after a 21-days chronic stress paradigm in the presence and absence of acute corticosterone application. Additionally, the effect of 4 days treatment with the GR antagonist mifepristone on calcium currents in control and chronically stressed rats was studied. Our results indicate that chronic stress by itself does not affect whole cell voltage-dependent calcium currents recorded one day after the last stressor. However, calcium current amplitude was significantly enhanced after chronic stress when slices had been treated with a high dose of corticosterone 1-4 hours earlier. After 4 days treatment with mifepristone, this effect was no longer seen. Our results indicate that dentate granule cells from animals with a history of chronic stress are exposed to an increased calcium load after exposure to an acute stressor. Interestingly, the findings on voltage-dependent calcium currents are in line with the previous results on VDCC $\alpha_{1C}$ mRNA expression after chronic stress.
Study physiology, mRNA and protein expression in parallel

The chronic stress study (chapter 4) indicated that there are regional differences in the effects of chronic stress and corticosterone treatment on voltage-dependent calcium currents. Specifically, in the DG calcium currents were only affected when chronic stress was combined with acute application of 100 nM corticosterone 1-4 hours before recording (van Gemert and Joels 2006). In the CA1 area, however, chronic stress by itself already led to a dramatic increase in whole cell calcium currents, while acute corticosterone application only resulted in larger calcium currents in naïve, but not in handled or chronically stressed animals (Karst and Joels 2007).

To further elucidate these subregional differences, we studied the effects of acute corticosterone application in the CA1 area and the DG in chapter 5. We tested these effects at three levels. (i) At the physiological level, we made whole cell patch clamp recordings of voltage-dependent calcium currents 1-4 hours after incubation with 100 nM corticosterone. We replicated the previously reported corticosterone-induced increase in voltage-dependent calcium currents in the CA1 area. In the DG, however, no effect of corticosterone on calcium currents was observed. (ii) At the level of mRNA expression, in situ hybridization of VDCC subunits 1 hour after injection with a high dose of corticosterone was studied. We found that mRNA expression of the β4 subunit was enhanced in corticosterone-injected versus naïve animals, while α1D expression was only enhanced in corticosterone- versus vehicle-injected animals; the expression of the α1C subunit was not changed at all by corticosterone treatment. The observed differences in β4 and α1D subunit expression were found in all hippocampal areas and could thus not explain the differences in physiological effects between CA1 and DG. (iii) At the protein level, we used the Western blot technique to study VDCC subunit β4 and α1C expression in hippocampal material obtained 2-3 hours after corticosterone incubation. We found that corticosterone incubation enhanced β4 as well as α1C protein expression in the CA1 area but not in the DG. Together, results from this study indicate that changes that account for the different effect of corticosterone between the two subfields don’t exist at the (pre)transcriptional, but rather at the posttranscriptional level.

6.2. Experimental design

In this thesis, a choice for specific techniques and models was made. Although we considered the approach most optimal under the given circumstances, inevitably the methods that were selected also have their drawbacks. Some of the considerations for our choices are discussed here.
Technical considerations

Whole cell patch clamp recordings in hippocampal slices

For electrophysiological recordings, we made use of in vitro acute hippocampal slices. This in vitro preparation is often used to test the effects of corticosterone on neuronal function in the hippocampus (e.g. (Joels 2001; Joels et al. 1994)). The hippocampal trisynaptic pathway is largely preserved in transverse slices, which makes it possible to study synaptic contacts in hippocampal slices and properties that depend on intact networks, e.g. LTP (Sarvey et al. 1989). Because of the clear organization of the different cell layers, principal cells within each hippocampal subfield can be easily distinguished on the basis of their location.

In hippocampal slices, it is possible to study physiological properties of single cells with the in situ whole cell patch clamp method (Hamill et al. 1981). A great advantage of this method over e.g. intracellular recording using sharp electrodes is the ability to clamp the membrane voltage of an individual cell at any desired value. Different voltage steps differently activate VDCCs located in the membrane, and the calcium currents flowing through these channels can thus be recorded. Because for this technique a rather large hole has to be made in the cell membrane, the inside of the cell is rapidly dialyzed and replaced by the pipette solution (Pusch and Neher 1988). Although this has the advantage that the intracellular milieu can be controlled, the disadvantage is that the stability of intracellular regulatory and signaling mechanisms can be compromised. For example, in our lab it was found to be difficult to record 5-HT$_{1A}$ receptor mediated K$^+$ currents using the whole cell patch clamp technique (as opposed to intracellular recordings using sharp electrodes), possibly because of the dilution of G-proteins (Van Gemert, N.G., Karst, H. and Joels, M., unpublished results). Voltage-dependent calcium currents, however, do not critically depend on second messenger systems and thus can be reliably recorded with the whole-cell patch clamp technique. It should be realized, though, that neurons recorded under such conditions may exhibit properties (e.g. membrane potential) that differ substantially from those in the intact animal, where principal neurons in the hippocampal subregions receive extensive extra-hippocampal inputs.

In situ hybridization on brain sections of corticosterone-injected animals

Several methods exist to study gene expression changes in response to stress or corticosterone. Profiling gene expression changes on a large scale can be done using (among other techniques) serial analysis of gene expression (SAGE) and microarray. With the SAGE technique (Velculescu et al. 1995), mRNA is transcribed into complementary DNA (cDNA) and from each cDNA 10-14 nucleotide long sequence tags are cut. The tags are then ligated and cloned into plasmids. After sequencing the clones, the number of tags can be used to estimate gene expression per sample. This technique is quite laborious, but has the advantage that unexpected or (so far) unknown transcripts can also be detected. Another method to investigate gene expression at a large scale is
the microarray technique (Schena et al. 1995). With this technique, cDNA from the experimental samples is fluorescently labeled and hybridized to a microarray. This microarray consists of a large number of probes that are printed on a microscopic glass slide or chip. Hybridization signals for each of the transcripts can be quantified. The microarray technique is easier and faster than SAGE, but here no novel genes can be detected. The sensitivity of SAGE and microarray is comparable in brain tissue (Evans et al. 2002).

The advantage of large scale gene profiling is that many genes can be studied simultaneously, and unexpected genes can be found to be regulated by corticosterone. A disadvantage, however, is the sensitivity of these techniques: low abundant genes like ion channels and receptors are often not reliably detected. Specifically, it was estimated that no more than 30% of the hippocampal transcriptome is reliably detected using these large scale techniques (Evans et al. 2002). The importance of this bias is e.g. evident from a microarray study examining transcriptional regulation by GRs, in a protocol that is highly comparable to the one used in this thesis (Morsink et al. 2006). It was found that 1 hour after treatment of hippocampal slices with a high dose of corticosterone exclusively transrepression is seen. Yet, a more sensitive technique (e.g. in situ hybridization, see below) unequivocally shows that transactivation does occur after such a short delay (see chapter 5).

Therefore, when specific genes are thought to be involved in e.g. the physiological effects of stress and corticosterone, a candidate gene approach may be chosen over a large scale expression study. One technique that can be used in this regard is real-time quantitative polymerase chain reaction (qPCR) (Higuchi et al. 1993). Using this technique, cDNA is amplified in many cycles. After each cycle, fluorescence is detected and the threshold cycle is analyzed, after which the number of initial copies is calculated on the basis of a standard curve. Real-time qPCR is a rapid and accurate technique to investigate a limited number of transcripts. The technique used to study gene expression changes in this thesis is in situ hybridization (Larsson 1989). Radioactively labeled probes that recognize specific mRNA sequences are hybridized to tissue sections and exposed to film to calculate grey values. The main advantages of this technique are the sensitivity and the spatial resolution: mRNA expression can be studied at different locations in the brain simultaneously, without the risk to dilute possible effects due to localized expression changes. The main disadvantage of the candidate gene approach is obvious from the current thesis: when a limited number of candidate genes does not respond to GR activation in a manner that can explain the functional phenomena, one can exclude the involvement of these candidate genes but still has no indication about the critical target genes for corticosterone.

In vivo versus in vitro
In this thesis, gene expression changes were studied with in situ hybridization after in vivo injection with a high dose of corticosterone, since in vitro corticosterone application
is not well compatible with *in situ* hybridization. It should be realized that with *in vivo* administration of corticosterone (as opposed to *in vitro* treatment) the hormone can have effects all over the brain and the periphery, thus providing the possibility for e.g. the amygdala to modulate effects on the hippocampus (Nathan et al. 2004). Therefore, it is important to consider to what extent GR-mediated effects in the hippocampus caused by *in vivo* administration of corticosterone are comparable to effects after *in vitro* administration.

One approach to solve this issue is to examine the effect of *in vivo* versus *in vitro* administration of corticosterone on the same functional parameter. This has been done for responses to 5-HT and calcium currents in hippocampal CA1 neurons. It was found that *in vivo* administration of corticosterone to rats has effects on 5-HT₁A responses and voltage-dependent calcium currents that are very similar to the effects of corticosterone application to *in vitro* hippocampal slices (Joels and de Kloet 1992; Joels et al. 1997; Karst et al. 1997a; Kerr et al. 1992), indicating that at least for these cell properties results obtained in *in vitro* hippocampal slices can be largely extrapolated to the *in vivo* situation.

A second approach is to compare the expression of a known set of genes after *in vivo* corticosterone administration with that after *in vitro* corticosterone incubation. Recent experiments support that for these (abundant) genes the effects of *in vivo* corticosterone injection tested with *in situ* hybridization are largely comparable with results obtained from *in vitro* incubation of hippocampal slices when tested with the microarray approach (Morsink et al. 2007). Thus, modulation by e.g. the amygdala does not necessarily have a large influence on gene expression changes in response to corticosterone in the hippocampus, although the differences between *in vivo* and *in vitro* experiments should be kept in mind with every new transcript studied.

**Model systems**

**Stress models**

To test the effect of long-term stress on voltage-dependent calcium currents in dentate granule cells, we used a chronic unpredictable stress model adapted from Herman et al. (Herman et al. 1995). This model was also used in a previous study, where it was found to influence transcription of VDCC subunits in the DG (Qin et al. 2004). The chronic unpredictable stress paradigm was chosen to prevent adaptation to stress in the animals, which is found during e.g. repeated restraint or chronic ethanol stress (Gadek-Michalska and Bugajski 2003; Garcia et al. 2000; Magarinos and McEwen 1995; Marin et al. 2007; Spencer and McEwen 1990; Watanabe et al. 1992). Decreased body weight gain and increased adrenal gland volume were found here as well as in previous studies using this model (Cullinan and Wolfe 2000; Herman et al. 1995; Joels et al. 2004), indicating that this paradigm indeed leads to hyperactivity of the HPA axis. In the current study, plasma corticosterone level measured on day 22 was not significantly increased in chronically stressed animals; this was likely due to large variation. In agreement, corticosterone
levels were found earlier to be quite variable and only significantly upregulated in response to chronic unpredictable stress with very large group sizes (Verkuyl et al. 2004). Although presently no significant elevation of plasma corticosterone level was found after chronic stress in the morning of day 22, differences may of course exist at other time points in the diurnal rhythm, similar to e.g. the flattened circadian cortisol rhythm found in patients suffering from psychotic depression (Keller et al. 2006). Related to this, it would be interesting to study corticosterone levels under basal conditions and in response to acute stress at various time points during the chronic stress paradigm to get a better idea of the development of HPA axis hyperactivity and feedback regulation during the chronic stress protocol.

In our in situ hybridization study, we injected animals with high doses of corticosterone for 21 consecutive days. This resulted in increased corticosterone level (even 1 day after the last injection) and decreased thymus weight. Since in this case exogenous corticosterone was applied, other components of the HPA axis are likely to be suppressed via a negative feedback mechanism. An indication that this is indeed the case comes from the observed decreased adrenal weight in these animals. Thus, this model causes chronic hypercorticism without inducing HPA-axis hyperactivity. Still, the outcome on a physiological and structural level seems very comparable with the chronic unpredictable stress paradigm; in both cases a reduction in 5-HT_{1A} receptor-mediated responses in the hippocampal CA1 area (Karten et al. 1999; van Riel et al. 2003) and decreased neurogenesis in the DG (Mayer et al. 2006; Oomen et al. 2007) is found. These findings support that, at least for these parameters, chronic high corticosterone levels are a major mediator of the effects induced by chronic stress.

Treatment with the GR-antagonist mifepristone during the last four days of the chronic unpredictable stress protocol did not reverse the changes in body or adrenal weight in our study. Similarly, mifepristone treatment did not reverse the effects of corticosterone injections for 21 days on thymus and adrenal weight (Mayer et al. 2006). At the physiological level, however, four days treatment with the GR antagonist reversed the effects of chronic unpredictable stress on voltage-dependent calcium currents and LTP in the CA1 area (Karst and Joels 2007; Krugers et al. 2006b). Also, at the structural level, the decrease in neurogenesis induced by chronic stress or chronic corticosterone injections in the DG was reversed by the drug (Mayer et al. 2006; Oomen et al. 2007). It is tempting to correlate these findings with findings in the clinic, where short term treatment with mifepristone is effective in reducing the cognitive symptoms of psychotic depression (Young et al. 2004a).

Choice of control group
In chapter 2, we examined mRNA expression in animals that had received a single injection with a high dose of corticosterone one hour before decapitation and used a control group that had received an injection with arachide oil; this was comparable to the paradigm with which a corticosterone-induced increase in 5-HT_{1A} receptor-mediated
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responses was found (Hesen and Joels 1996). However, the results from our in situ hybridization study in chapter 5 suggest that the choice of the control group is not always trivial when studying the effects of acute stress. Corticosterone affected mRNA expression of VDCC subunits in the hippocampus differently, depending on the control group that was used for comparison, i.e. naïve or vehicle-injected animals. Since the corticosteroid-induced effects on voltage-dependent calcium currents in the same study were tested in in vitro hippocampal slices from naïve animals, the naïve control group is best comparable to the control group we used for electrophysiological recordings. Still, a single injection with vehicle solution can apparently affect hippocampal mRNA expression of VDCC subunits. Similarly, a single intraperitoneal saline injection was found to rapidly increase Fos expression in several brain regions, including the hippocampus (Sharp et al. 1991). The mechanism behind this effect of a single vehicle injection is unknown. Plasma corticosterone levels were not elevated after the single vehicle injection in our study (chapter 5), and it was previously shown that increased Fos expression after stress is not dependent on hormones secreted by the adrenal glands (Helmreich et al. 1996). Thus, other stress-induced factors like CRH or vasopressin might also play a role in the regulation of other transcripts. Based on our results in chapter 5, it seems important to incorporate both a naïve and a vehicle-injected control group in the study design, to get a more complete picture of the role of corticosterone and other stress-related compounds on gene expression.

In chapter 3, data from animals that received corticosterone injections for 21 days were compared to animals that had received vehicle injections. This way, direct comparisons could be made to the physiological findings on 5-HT1A receptor mediated responses in the CA1 area, for which similar injection paradigms were used (Karten et al. 1999). However, daily vehicle injections for 21 days have been shown to increase spine density in the CA1 area (Seib and Wellman 2003). In retrospect, a naïve control group should have been added in the experimental design, to estimate the influence of the injections per se.

Similarly, in our chronic stress study (chapter 4), we compared voltage-dependent calcium currents in dentate granule cells from animals that had been subjected to a chronic unpredictable stress paradigm to animals that were handled for 3 weeks (to control for handling during the stress paradigm) and received milk via a gastro-oesophageal tube for the last four days (to control for mifepristone treatment). This handling protocol was found to reduce dendritic length of CA1 pyramidal cells when compared to naïve animals (Alfarez et al. 2008). Apparently, repeated handling or vehicle injections by itself are sufficient to alter hippocampal cell properties (also see section 6.3).

In conclusion, handling or vehicle treatment as a control procedure can thus result in a different condition of the animal when compared to an undisturbed control group. This may also affect subsequent stress- and corticosterone-mediated responses (see section 6.3). Therefore, it would be best to include a handled or vehicle-treated as well
as a naïve control group in all future studies examining the effect of (chronic) stress or corticosterone treatment, until regulation of the parameter studied by control treatment is ruled out.

6.3. Context-specific effects of corticosterone

History of the animal
The history of the animal does not only affect various hippocampal cell properties under basal conditions, it also has a large influence on the response to acute stress or corticosterone application later in life. For an overview of some of the parameters affected, see Figure 1.

In this thesis, we show that a history of chronic unpredictable stress does not affect the amplitude of voltage-dependent calcium currents in the hippocampal DG under basal conditions, but results in increased currents when slices were incubated with a high level of corticosterone 1-4 hours earlier. A similar pattern was found when studying mRNA expression of various gene transcripts, including the VDCC α1C subunit and AMPA-receptor subunits (Qin et al. 2004); in accordance with the latter, AMPA-mediated synaptic currents in dentate granule cells were not affected by chronic stress itself, but the history of chronic stress affected the subsequent response to acute corticosterone application (Karst and Joels 2003). LTP, however, was found to be decreased in the DG after chronic stress already under basal conditions (Alfarez et al. 2003).

Also in the hippocampal CA1 region, the history of chronic stress can influence the response to a subsequent acute stressor. For example, acute corticosterone application

![Figure 1: Effects of corticosterone incubation on hippocampal cell properties in naïve (grey bars), handled (white bars), and chronically stressed (black bars) animals. Corticosterone differentially affects voltage-dependent calcium currents in the CA1 area and the DG as well as the length of apical dendrites of CA1 neurons, depending on the history of the animal. The interaction between acute corticosterone application and the history of the animal differentially affects each of the parameters studied. Based on references (Alfarez et al. 2008; Karst and Joels 2007; van Gemert and Joels 2006) and chapter 5 of this thesis.](attachment:image.png)
decreases voltage-dependent calcium currents in CA1 cells of chronically stressed animals, while the same treatment led to increased currents in slices from naïve animals of the same age (Karst and Joels 2007) (see Figure 1). A different picture emerged from studies on LTP, where corticosterone incubation was found to reduce LTP in control handled animals, while no further reduction of LTP by corticosterone was found in slices from chronically stressed animals (Alfarez et al. 2003). At the morphological level, corticosterone did not affect dendritic length of CA1 neurons in naïve animals, but decreased dendritic length in animals that were previously subjected to chronic stress (Alfarez et al. 2008). Chronic stress thus not always results in discernable effects on hippocampal cell properties under basal circumstances when HPA-axis activity is low, but differences can become apparent when corticosterone levels are raised, e.g. due to an acute stressor. Alterations in hippocampal physiology due to chronic stress should therefore not only be studied under basal conditions, but also after in vivo activation of the HPA-axis or in vitro incubation with corticosterone. That way, the full range of effects of chronic stress on hippocampal physiology can be studied.

The lack of effect of chronic stress under basal corticosterone conditions on several parameters in the DG as well as the CA1 area suggests that chronic stress does not dramatically influence the function of the MR (although the potential importance of an altered balance in MR/GR function cannot be ruled out), but rather leads to increased GR availability and / or activity. The finding that some of the effects can be reversed by 4 days treatment with the GR-antagonist mifepristone, further supports that the GR is involved in the development of altered corticosterone-responsiveness after chronic unpredictable stress (Alfarez et al. 2008; Karst and Joels 2007; Krugers et al. 2006b). Previous studies, though, show that chronic unpredictable stress does not affect GR mRNA, hnRNA or protein expression in the hippocampus (Herman and Spencer 1998; Paskitti et al. 2000; van Riel et al. 2003), while other chronic stress paradigms actually decreased hippocampal GR expression (Chen et al. 2008; Raone et al. 2007). Not all physiological parameters are affected to a large extent by corticosterone application after chronic stress. For instance, hippocampal LTP was affected by chronic stress, while subsequent corticosterone application had no additional effect (Alfarez et al. 2003). It thus seems unlikely that general enhanced GR activity in the hippocampus is responsible for the enhanced corticosterone-induced effects found after chronic stress. Other factors influencing GR-mediated transcription, such as the availability of transcriptional coactivators and corepressors might play a role here. Extensive studies concerning GR-mediated transcriptional regulation after chronic stress are warranted to elucidate the mechanisms behind this altered corticosterone responsiveness that is often, but not always, found.

Not only chronic stress, but also long-term handling can alter corticosterone-mediated effects on hippocampal cells. At the morphological level, 3 weeks of handling decreased dendritic length and complexity of CA1 neurons when compared to naïve animals (see Figure 1). Acute incubation with corticosterone led to an increase in
dendritic length in handled animals, while no effect was found in naïve animals (Alfarez et al. 2008). In addition, corticosterone has been repeatedly found to enhance voltage-dependent calcium currents in naïve rodents, while this increase was not found in rats that were handled for 21 days (Karst and Joels 2007).

These effects of handling on corticosterone responsiveness are not unprecedented. Effects of early life handling on responsiveness to stress or corticosterone in adulthood have been repeatedly described (e.g. (Hsu et al. 2003; Lippmann et al. 2007; Meaney 2001)). Similarly, maternal care early in life was found to dramatically influence corticosterone-induced effects on hippocampal LTP in adulthood. Specifically, in animals that had received high levels of maternal care, in vitro corticosterone application reduced hippocampal LTP, while in animals that were given low amounts of care, corticosterone led to an increase in LTP (Champagne, D.L. et al, personal communication). The effects of handling in adulthood, though, have been studied in far less detail. Repeated handling was found to attenuate HPA-axis responsiveness to a subsequent stressor (Gadek-Michalska and Bugajski 2003), indicating that adaptive changes in the HPA-axis occur during the handling procedure. Handling in adulthood might influence corticosteroid receptor activity and thus alter corticosterone responsiveness. More extensive studies on corticosteroid receptor properties in handled rats are necessary to further resolve this issue.

**Cellular context**

A second determinant of context-specific corticosteroid effects is the cellular content. This becomes clear when looking at the different effects of corticosterone in two hippocampal regions, the CA1 area and the DG, as described in chapter 5. Although both regions abundantly express the MR as well as the GR (Han et al. 2005; van Steensel et al. 1996), application of a high dose of corticosterone leads to increased voltage-dependent calcium currents in the CA1 area, but has no effect on these currents in the DG.

In general, corticosterone affects several parameters of CA1 pyramidal cell physiology with a U-shaped or bell-shaped dose dependency (Diamond et al. 1992; Joels 2001; Joels 2006). Thus, adrenalectomy on the one hand and substantial activation of the GR (in addition to MR) on the other hand both result in (i) increased voltage-dependent calcium currents, (ii) increased 5-HT$_{1A}$ receptor-mediated K$^+$ currents, (iii) increased cholinergic responses, and (iv) reduced NMDA-receptor dependent long-term potentiation when compared to a situation where predominantly the MR is occupied. For an overview and references, see Figure 2A.

In the DG, removal of the adrenal glands also affects various properties of the granule cell. In this hippocampal subregion, removal of the adrenal glands results in reduced glutamate transmission and enhanced voltage-dependent calcium currents. GR activation, however, had no effect on glutamate-mediated synaptic responses in handled animals. Similarly, incubation with a high dose of corticosterone did not affect voltage-dependent calcium currents in the DG in long-term handled (chapter 4) or naïve (chapter
5) animals (see Figure 2B for an overview and references). Furthermore, ADX leads to attenuation of long-term potentiation in the DG (Krugers et al. 2007), while effects of high doses of corticosterone on LTP in this region are less clear. When recorded in vitro, incubation of hippocampal slices with corticosterone did not significantly affect LTP (Alfarez et al. 2003; Pu et al. 2007). When recorded in vivo, however, synaptic transmission in the DG was found to be either unaffected by cold stress (Bramham et al. 1998), impaired after restraint stress (Yamada et al. 2003), or enhanced after swim stress (Kavushansky et al. 2006). Apparently, the effects of in vivo stress on LTP in the DG depend on the type and timing of the stressor (Straube et al. 2003), possibly due to the differential involvement of other stress-related hormones like noradrenalin or the presence of modulatory influences by other brain regions (e.g. the amygdala).

While MR mRNA is equally expressed over all hippocampal subfields, MR hnRNA is expressed to a high level in the DG when compared to the CA regions (Herman and Watson 1995; Paskitti et al. 2000). This indicates that processes like mRNA translocation and degradation might be differentially regulated in the various hippocampal subfields.

**Figure 2:** Effects of differential receptor occupation on physiological parameters of CA1 pyramidal and dentate granule cells. (A) In CA1 neurons, a U-shaped corticosterone dose dependency is found for voltage-dependent calcium currents (black circles), 5-HT1A receptor-mediated K+ currents (white triangles), and cholinergic responses (white diamonds). Long-term potentiation in the CA1 area shows a bell-shaped dose dependency (black squares). (B) In the DG, adrenalectomy affects both voltage-dependent calcium currents (black circles) and glutamatergic responses (white squares), while activation of the GR in addition to the MR has no additional effect. Figures are based on references (Joels et al. 2003; Karst and Joels 2001; Karst et al. 2000; Karst et al. 1994; Karst et al. 1997b; Kerr et al. 1992) and chapter 5 of this thesis for voltage-dependent calcium currents; references (Beck et al. 1996; Joels et al. 1991; Karst et al. 2000; Karten et al. 1998) for 5-HT responses; references (Hesen and Joels 1993; Karten et al. 1998) for cholinergic responses; references (Krugers et al. 2005; Pavlides et al. 1996; Wiegert et al. 2005) for LTP; and references (Joels et al. 2001; Karst and Joels 2003) for glutamatergic responses.
(Herman and Spencer 1998). MR mRNA expression has been found to be more susceptible to exposure to chronic stress in the DG than in other hippocampal regions (van Riel et al. 2003). However, the differences in corticosteroid receptor availability between CA regions and DG involve the MR rather than the GR, while the differences in corticosterone responsiveness are more likely to pertain to properties of the GR.

Apart from the differences in MR and GR abundance between CA1 and DG cells, there could be other cell-specific properties which give rise to the discrepancies in corticosterone responsiveness. Clearly, the various cell types in the hippocampus have different morphological and functional properties. In two microarray studies comparing gene expression between hippocampal subfields under basal conditions, large differences between the subfields were found (Datson et al. 2004; Lein et al. 2004). Especially genes involved in signal transduction, regulation of gene expression, calcium homeostasis, general metabolism, and protein biosynthesis were found to be differentially expressed. It is thus well possible that different cell types also differentially express genes that influence corticosterone-induced alterations in cellular physiology.

First, the local availability of corticosterone might be different. The multidrug transporter mdr1b prevents corticosterone from entering the cell and is present in high amounts in the hippocampus. mRNA expression of this transporter was found to be very similar in CA1 and DG (Volk et al. 2004), though. Alternatively, the efficiency of the conversion of corticosterone into its inactive form by the enzyme 11β-HSD-2 might be a source for differences in corticosterone-induced effects. However, 11β-HSD-2 is expressed to a rather low level in adult hippocampus, and no obvious differences in expression between CA1 area and DG are found (Robson et al. 1998; Zhou et al. 1995). The enzyme 11β-HSD-1, which catalyzes the reverse reaction, is also expressed to comparable levels in both hippocampal subfields (Moisan et al. 1990; Wan et al. 2005), indicating that subregional differences in corticosterone availability are not due to differences in local corticosterone availability. Still, protein levels or activity of mdr1b or one of the corticosterone-converting enzymes might of course differ between the hippocampal subfields, even though mRNA levels are very similar.

A second possible regulator of corticosterone-induced transcriptional regulation is the expression or posttranslational modification of different GR isoforms which have been shown to differ in their transcriptional activity (Lu and Cidlowski 2005; 2006; Oakley et al. 1999). Although expression of some of these GR isoforms was found to be tissue specific (Lu and Cidlowski 2005), there is to date no information on expression of different isoforms in the different hippocampal subregions.

Third, differences in the availability of various cofactors might play a role in the differential effects. In experiments with splice variants of the coactivator SRC-1, corticosteroid-induced effects on transcription were found to depend on the interaction between the GRE composition of the promoter and the available cofactors (Meijer et al. 2005). Thus, different genes with different promoter compositions display different glucocorticoid-induced transcriptional responses, depending on the intracellular
availability of cofactors. Furthermore, the ratio between corepressors and coactivators affects the dose-response curve of GR-dependent gene expression (Szapary et al. 1999; Wang et al. 2004). A wide variety of cofactors has been identified, and probably many at present unidentified cofactors exist, providing a large target for putative cell-specific regulation of GR-induced gene transcription.

Of course, a final possible explanation of the differential effects of corticosterone in different cell types is that similar transcriptional changes take place, but posttranscriptional regulation differs depending on the cellular context. This possibility is discussed in more detail in section 6.4; also see Figure 3.

Since corticosteroids and stress thus differentially affect cell properties in two hippocampal subfields, the question arises how stress and corticosterone affect the functioning of the hippocampus as a whole. At this moment, further investigation on the effects of corticosterone on cellular properties in the various hippocampal subfields and the involvement of the different receptor types is needed. These results can be combined with studies examining the effect of corticosterone on behavior that relies specifically on the CA1 area versus the DG (Gilbert et al. 2001; Leutgeb et al. 2007). This way, more information can be obtained regarding the effects of corticosterone on hippocampal functioning at a behavioral level.

![Corticosterone affects calcium currents and VDCC subunit expression differently in the CA1 area (light grey bars) and the DG (dark grey bars). While voltage-dependent calcium currents are differently affected by corticosterone in both regions, VDCC subunit mRNA expression is regulated similarly in both regions. Protein expression of VDCC subunits, though, is again differently regulated by corticosterone in CA1 area versus DG. The figure is based on data obtained in chapter 5.](image-url)

**Figure 3:** Corticosterone affects calcium currents and VDCC subunit expression differently in the CA1 area (light grey bars) and the DG (dark grey bars). While voltage-dependent calcium currents are differently affected by corticosterone in both regions, VDCC subunit mRNA expression is regulated similarly in both regions. Protein expression of VDCC subunits, though, is again differently regulated by corticosterone in CA1 area versus DG. The figure is based on data obtained in chapter 5.
6.4. Link between transcription and physiology

Findings in this thesis
Corticosterone application leads to altered hippocampal physiology via a GR-mediated genomic mechanism, for which de novo protein synthesis is a prerequisite (Karst and Joels 1991; Karst et al. 2000; Kerr et al. 1992). In this thesis, we tried to link specific transcriptional changes to alterations in physiology in principal cells of the hippocampus.

Since corticosterone-induced alterations in 5-HT1A receptor-mediated responses are not accompanied by alterations in 5-HT1A mRNA expression that could explain the altered physiology (Czyrak et al. 2002; Lopez et al. 1999; Meijer and de Kloet 1994; Meijer and de Kloet 1995), we studied mRNA expression of other likely candidate genes that might underlie the effects on serotonergic transmission. None of the genes we hypothesized to be involved in altered 5-HT1A responses after acute or chronic corticosterone application, i.e. SGK1, RGS4, and NCAM, were found to be transcriptionally regulated by corticosterone (chapters 2 and 3). Thus, we found no direct link between the GR-mediated effects of corticosterone on 5-HT1A receptor-mediated K+ currents in the CA1 area and the regulation of transcripts that might underlie these changes.

We next focused on the effects of corticosterone on voltage-dependent calcium currents. Altered mRNA expression of the VDCC α1C subunit after chronic stress in combination with corticosterone treatment was reflected in altered voltage-dependent calcium currents in the dentate gyrus. Since the α1C subunit is one of the subunits responsible for the L-type calcium current, and this type of current is most affected by corticosterone in the CA1 area (Chameau et al. 2007), this correlation between enhanced mRNA expression and larger currents suggests that in this case a link can be made between mRNA expression and physiology. However, when studying the effects of corticosterone incubation on voltage-dependent calcium currents in CA1 area versus DG at the level of mRNA expression, protein expression, and physiology simultaneously, this link was found to be not as clear as expected. We found that in the CA1 area and the DG very similar alterations in VDCC subunit mRNA expression occur in response to corticosterone, while corticosterone affected calcium currents only in the CA1 area and not in the DG. Protein expression seemed to fit more closely with the physiological data than did mRNA expression. Differential regulation of posttranscriptional mechanisms might explain this discrepancy.

Possible mechanisms
In general, corticosterone-induced physiological alterations are often not in parallel with alterations in mRNA expression of obvious candidate genes, i.e. ion channel or receptor subunits (Figure 3). Other corticosterone-induced changes must thus be responsible for
the effects on hippocampal physiology. Corticosterone might affect cellular physiology by posttranscriptional regulation of the proteins involved in altered physiology (Figure 4).

**Regulation of protein levels**

Apart from affecting mRNA expression, corticosterone might also regulate protein levels by influencing protein biosynthesis or the rate of protein degradation. When looking at protein regulation in the whole body or in muscle cells, it was found that corticosterone indeed affects both protein synthesis as well as degradation (Quan and Walser 1991; Savary et al. 1998; Tomas et al. 1984).

Also in the hippocampus, 3 hours after injection with a high level of corticosterone many corticosterone-responsive genes were found that are associated with protein biosynthesis and turnover (Datson et al. 2001). Also when hippocampal slices were incubated *in vitro* with corticosterone, mRNA expression of ribosomal and other translation-associated proteins was found to be regulated in a time-specific manner: 1 hour after corticosterone treatment, mRNA expression was downregulated, while up- as well as downregulated genes were found after 3 hours (Morsink et al. 2006). In addition, many genes involved in protein synthesis are differentially expressed in the different hippocampal subfields already under basal conditions (Datson et al. 2004; Lein et al. 2004), which might explain some of the regional differences in corticosterone-induced effects.

**Figure 4**: Schematic representation of possible posttranscriptional regulation of transcripts or proteins involved in altered hippocampal cell physiology: [1] protein synthesis, [2] protein degradation, [3] trafficking to and from the membrane, [4] posttranslational modification of membrane receptors or ion channels.
How these effects of corticosterone on general factors associated with protein biosynthesis result in specific translational regulation of some membrane receptors and ion channels, is at present unknown. Corticosterone was shown to affect translation - and not transcription - of the glutamate transporter EAAT2 in mouse astrocytes in vitro and in vivo (Tian et al. 2007). The mechanism behind this translational regulation and the specificity for the EAAT1 gene needs to be elucidated, but the length of the 3’ untranslated region of the EAAT2 mRNA is proposed to play a role (Willis 1999).

As indicated, corticosterone can also affect protein breakdown, at least in muscle cells. Whether corticosterone also specifically influences breakdown of VDCC subunits or 5-HT1A receptors in hippocampal neurons is presently unknown. These putative effects of corticosterone on protein breakdown of transmembrane proteins might be tested with pulse-chase experiments, in which the outer part of transmembrane proteins is labeled and the amount of labeled protein is subsequently monitored in time.

**Trafficking**

After membrane receptor and ion channel proteins are synthesized, they have to be transported from the endoplasmic reticulum to the cell surface. Although many factors can affect trafficking to the membrane and subsequent endocytosis of G protein-coupled receptors (GPCRs) such as the 5-HT1A receptor, a lot about the exact mechanisms regulating this transport of GPCRs is still unknown (Drake et al. 2006; Xu et al. 2007).

When the 5-HT1A receptor has reached the membrane, though, palmitoylation of the receptor targets the receptor to lipid rafts in the cell membrane (Papoucheva et al. 2004; Renner et al. 2007), and it can be subsequently transported across the membrane via lateral diffusion in a G protein-dependent manner (Pucadyil and Chattopadhyay 2007). This lateral diffusion might provide an additional mechanism for rapid changes in 5-HT1A receptor-mediated effects by transporting the receptors into and out of the area of the synapse. Interestingly, recent experiments from our laboratory indicate that in primary hippocampal cultures trafficking of AMPA receptor GluR2 subunits to or from the synaptic density is also regulated in a slow, gene-mediated GR dependent fashion (Wiegert et al. 2006).

Trafficking of voltage-dependent calcium channels is well-studied (for review see (Jarvis and Zamponi 2007)). Apart from the auxiliary β, α2-δ and γ subunits, various other factors influence calcium channel targeting to the plasma membrane. For instance, members of the RGK family of small GTP-binding proteins affect L-type, but not N-type channel trafficking to the membrane by interacting with the associated VDCC β subunit (Chen et al. 2005; Kelly 2005). Although calcium channel internalization has been shown to occur, to date relatively little is known about mechanisms by which VDCCs are removed from the membrane (Jarvis and Zamponi 2007).

Aside from specific factors involved in regulation of the GluR2, and possibly also VDCC or 5-HT1A, receptor transport to and from the cell membrane, more general factors that are involved in trafficking and / or endocytosis might also be regulated by
corticosterone and stress. An interesting candidate in this respect is caveolin-1, a protein associated with lipid rafts in the membrane and involved in endocytosis of various membrane proteins (Cohen et al. 2004). G protein-coupled receptors as well as voltage-dependent calcium channels, along with many other membrane proteins, are associated with caveolin (Daniel et al. 2006; Ostrom and Insel 2004). Caveolin-1 mRNA expression was found to be affected by corticosterone in various peripheral organs, e.g. lung, spleen and kidney (Meijer, O.C. et al. personal communication). Similarly, GR-mediated induction of caveolin-1 expression in pulmonary epithelial cells has been described (Barar et al. 2007). Caveolin-1 is expressed in the hippocampus as well and this expression is upregulated in aged rats (Bu et al. 2003; Kang et al. 2006). Possibly, corticosterone similarly affects caveolin-1 expression in hippocampal neurons, thus regulating trafficking and endocytosis of various membrane proteins.

Posttranslational modifications
Various posttranslational modifications of proteins might influence hippocampal cell physiology. As indicated, palmitoylation of the 5-HT$_{1A}$ receptor is essential for localization of the receptor in lipid microdomains and affects receptor-mediated signaling (Papoucheva et al. 2004; Renner et al. 2007). The most interesting form of posttranslational modification, though, is phosphorylation of proteins associated with regulation of cell physiology.

Phosphorylation of proteins is an ubiquitous mechanism by which the activity of various ion channels is regulated (Levitan 1994). 5-HT$_{1A}$ receptor-mediated responses can be influenced by phosphorylation at multiple levels. First, the 5-HT$_{1A}$ receptor itself can be phosphorylated at various sites (Pucadyil et al. 2005; Raymond 1991; Raymond and Olsen 1994). Second, G-proteins that are coupled to the 5-HT$_{1A}$ receptor can be subject to phosphorylation (Hensler 2003; Lohse 1993), and, third, the 5-HT$_{1A}$ receptor-associated K$_v$3.1 channel contains multiple phosphorylation sites (Doupnik et al. 1995). Finally, several G protein-coupled receptor kinases, which modulate G protein coupled receptor signaling, can be phosphorylated (Penela et al. 2003). Interestingly, activation of protein kinase C (PKC) was found to reduce 5-HT$_{1A}$ receptor-mediated K$^+$ currents in hippocampal neurons (Andrade et al. 1986), indicating that phosphorylation can indeed influence serotonergic responses. Which exact protein(s) need(s) to be phosphorylated by PKC in order to affect 5-HT$_{1A}$ receptor-mediated responses is presently unknown and awaits further studies.

Many VDCC α1 and β subunits can be phosphorylated by protein kinase A (PKA), leading to increased calcium currents (Bunemann et al. 1999; Hell et al. 1995; Nunoki et al. 1989). Interestingly, in aged rats, which are hypothesized to be exposed to corticosteroids more than young animals (Hibberd et al. 2000), enhanced L-type calcium currents are found in the hippocampal CA1 area (Campbell et al. 1996; Thibault and Landfield 1996), which can be explained by a dramatic age-related increase in phosphorylation of the α1C subunit at the serine 1928 site (Davare and Hell 2003).
Phosphorylation of this residue increases the open probability of the channel (Trautwein and Hescheler 1990; Yue et al. 1990), thus giving rise to enlarged voltage-dependent calcium currents. It seems unlikely, though, that this process explains the GR-dependent enhancement of calcium current amplitude in CA1 neurons, since it was found that the number rather than single channel conductance of L-type calcium channels is increased by the hormone (Chameau et al. 2007).

It is possible that corticosterone regulates expression and / or activity of one of the various players that control the activity of PKA or PKC. Unpredictable stress was indeed found to increase PKA activity in the nucleus accumbens (Araujo et al. 2003), and it is thus possible that corticosterone similarly leads to enhanced PKA-mediated phosphorylation of various receptors or ion channels in (subfields of) the hippocampus. Similarly, hippocampal PKC protein expression was found to be downregulated - although not in all mouse strains tested - after acute stress (McNamara and Lenox 2004), which might provide an explanation for the enhanced 5-HT\textsubscript{1A} receptor-mediated responses found in response to acute stress or corticosterone application.

6.5. Conclusion

When the research described in this thesis was started, corticosterone was known to affect many physiological properties in hippocampal cells via GR-mediated transcriptional regulation. The exact link between transcriptional regulation of specific genes and specific physiological parameters, however, was not clear.

In this thesis, we studied (i) possible transcriptional changes underlying alterations in 5-HT\textsubscript{1A} receptor-mediated responses in the CA1 area after acute or prolonged elevations of corticosterone levels, (ii) the physiological effects of altered VDCC subunit mRNA expression after chronic stress in the dentate gyrus, and (iii) the effects of corticosterone on voltage-dependent calcium currents, VDCC mRNA expression and VDCC protein expression in the CA1 and the DG. We found that the mRNA levels observed in hippocampal cells after stress or corticosterone treatment do not always correlate well with the physiological outcome, while protein levels (chapter 5) seem to correlate better with the physiological outcome than the level of mRNA expression.

We thus propose the possibility that corticosterone does not affect the genes directly linked to the functional parameter(s) but rather targets other genes influencing translational processes, trafficking, or posttranslational modifications of the receptors or channels involved. Instead of regulating mRNA expression of (among other things) many ion channels and membrane receptors simultaneously, corticosteroids could in this way efficiently target a (few) gene(s) that is / are able to affect a wide range of physiological parameters.