Glucocorticoid signature in a neuronal genomic context

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Chapter Six

General Discussion

6.1 Summary of main conclusions

In this thesis, we have reported studies in which the primary response to glucocorticoids was examined in a neuronal context by analyzing the DNA-targets bound to and genes regulated by the activated glucocorticoid receptor (GR). The neuronal context was either the widely used PC12 neuronal pheochromocytoma cell line or the hippocampus of the rat brain. DNA sequences with GR binding motifs were identified either with an *in silico* approach or with next generation sequencing of DNA samples obtained by chromatin immunoprecipitation (ChIP-seq). In addition, DNA microarray analysis of discrete hippocampal subregions isolated with laser microdissection was performed to identify genes regulated by the endogenous glucocorticoid hormone in the rat, corticosterone (CORT). The thesis is concluded with the study of the effect of chronic stress on one of the identified CORT-responsive gene networks, the mammalian target of rapamycin (mTOR) pathway.

Our results showed that we were able to successfully validate *in silico* predicted GRE-containing GR-binding sites (GBS). In the rat hippocampus these GBS were located near genes previously found to be regulated by stress and CORT (*Chapter 2*). This suggests functionality of these evolutionary conserved GREs. We subsequently applied ChIP-seq to identify genomic binding sites of GR in two different neuronal substrates: neuronal-like PC-12 and rat hippocampal tissue (*Chapter 3 and 4*). At the time of publication, these studies were the first to demonstrate the application of the ChIP-seq technique in a neuronal context.

Using ChIP-seq we identified thousands of GBS of which the majority is novel. In order to validate these findings we analyzed the extent and pattern of GR binding for a selection of the identified GBS in rat hippocampus after administration of different amounts of CORT (*Chapter 4*). Our findings imply that, depending on the amount of CORT, different sets of GR-target genes are activated in the hippocampus. In addition, we were able to measure binding of MR to a majority of this GBS selection. In almost all cases, MR binding was already apparent at lower CORT concentrations than GR binding (*Chapter 4*), which is in line with the ten-fold higher affinity to CORT displayed by MR as compared to GR (Reul and de Kloet, 1985).

We subsequently screened the GBS that were identified in the ChIP-seq studies for motifs that resemble known binding sites of GR and other transcription factors. As expected, a motif strongly resembling the canonical GRE consensus sequence was the most prevalent motif identified. In neuronal PC12 cells 58 % of GBS contained a canonical GRE sequence (*Chapter 3*) and this figure nearly approached 100 % in hippocampus (*Chapter 4*). In addition to the GRE, other motifs were identified that resemble sequences of possible transactivation and transrepression partners of GR. These include Maz1, SP1, Zbtb3, Gabpa, Prrx2, Zfp281, Gata1, Zfp740,

Sox12, Sox4, Srf and GM397 or Zscan4c, several of which had not yet been linked to GR function and may be important factors for GR signaling in a neuronal context

6.2 Methodology

The main method used in this thesis was ChIP-seq, which was a relatively new method still subject to technological improvements. *In vivo* studies performed in brain tissue were scarce and we therefore choose to first apply the technique *in vitro* in neuronal PC12 cells. This allowed us to get more acquainted with the technique and to obtain the first ChIP-seq data in a neuronal setting. Subsequently, we were able to develop our own methods and to apply the ChIP-seq technique successfully in rat hippocampus. In neuronal PC12 cells as well as in the rat hippocampal tissue we identified thousands of new GBS. Since a consensus in ChIP-seq analysis is lacking in literature, we considered validation of our methods using RT-qPCR to be essential. The validation provided the evidence that our methods and the cut-offs we applied in both Chapter 3 and 4 were appropriate. It is clear that ChIP-seq has contributed tremendously to a better understanding of the interaction of transcription factors with the genome (Mundade et al., 2014). Recently, special guidelines and practices of the ENCODE consortium have been published allowing standardization of ChIP experiments (Landt et al., 2012; Mundade et al., 2014).

The *in silico* approach GenSig, that we have developed (*Chapter 2*), proved to be a suitable method to screen known CORT-responsive genes for GRE-like sequences. We were able to show GR-binding to 47 % of the predicted and selected GREs, which is a high success rate. We believe that this high percentage is due to the fact that GenSig takes evolutionary conservation into account. For Estrogen Receptor binding sites, it was recently confirmed that the higher affinity estrogen response elements display a higher degree of evolutionary conservation in comparison to their flanking sites (Gertz et al., 2013), which supports our *in silico* GenSig approach. Other factors contributing to the predictive success rate of GenSig are the fact that the GRE consensus motif was based on validated GREs and only genes known to be responsive to CORT were included in the analysis. In conclusion, the methods used in this thesis were suitable to investigate our aim which was to identify primary GR targets in neuronal-like cells and hippocampal tissue.

6.3 Chapter 3: discussion

Findings

In *Chapter 3*, a genome-wide analysis of GR-binding sites in neuronal PC12 cells was presented. Where previously knowledge regarding GR-mediated action of glucocorticoids had come from studies on peripheral tissues, we were now able to show

data in a neuronal context. This is important, since it was already apparent that GR-binding is highly cell type-specific with minimal overlap in GBS between different cell types (John et al., 2011; Pan et al., 2011; Reddy et al., 2009; Yu et al., 2010).

The raw ChIP-seq data was analyzed using the software of CLC genomics workbench, resulting in 1,183 GBS that we considered to be significant (FDR cut off 5 %). When these GBS were compared with other non-neuronal studies, it turned out that 87 percent of these sites were unique to the neuronal PC12 cells (Figure 6.1). Interestingly, the majority of these PC12-unique GBS were located nearby genes with a known neuronal function, such as axonogenesis, neuronal differentiation and neuronal development. In terms of genomic location, almost one third of the GBS were located within genes and mostly within intronic regions, which is consistent with other GR ChIP-seq studies (Reddy et al., 2009; Yu et al., 2010). The functionality of GR-binding to an intron was demonstrated in an *in vitro* study investigating the effect of DEX stimulation of cultured Beas-2B airway epithelial cells on the expression of, amongst others, the anti-inflammatory target tumor necrosis factor, alpha-induced protein 3 (TNFAIP3) (Altonsy et al., 2014). It became evident that GR-binding to an intronic GBS in the TNFAIP3gene was required to enhance its transcription. At the time we did our experiments, an *in vivo* study demonstrating the functional relevance of intronic GR binding was lacking.

The GBS were screened for DNA-motifs which are known to bind certain proteins. This resulted in the identification of motifs for GR (the canonical GRE motif), Gabpa, Prrx2, Zfp281, Gata1 and *Zbtb3*. The GRE motif was similar to the motif identified by others and also had a comparable prevalence. The GRE was the most prevalent motif identified in our study, indicating that direct GR binding to specific sites in the DNA via transactivation is an important mechanism GR uses to regulate gene expression in a neuronal context. Zbtb3 was exclusively found in non-GRE containing GBS and was the most frequently observed non-GRE motif with a frequency of 80 % within this group. This suggested that Zbtb3 might be a new transrepression partner of GR.

In neuronal PC12 cells the genes that were associated with a GRE-GBS were involved in general cell functions and processes, i.e. cell motility, vascular processes and protein dimerization activity. In contrast, genes near a non-GRE GBS had a clear role in neuronal processes such as neurogenesis, plasticity and growth, synap-

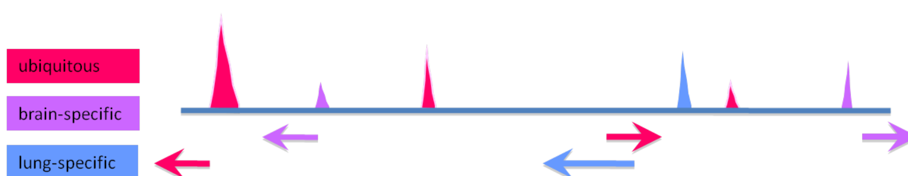


Figure 6.1: GBS are cell-type specific. Whereas the GBS that are shared between different experimental models seem to be more ubiquitous expressed, the cell-specific genes are located nearby genes with a known neuronal function.

tic transmission and neurotransmitter biosynthetic processes. This suggests that the Zbtb3 transcription factor is a novel crosstalk partner of GR that tethers GR to DNA sites in a transrepressive mode of action in order to regulate neuronal gene expression in neuronal PC12 cells upon GR activation.

Update of Chapter 3: findings since publication

Since our publication appeared, other ChIP-seq studies have been published on GR-binding *in vitro* in cell lines *(Gertz et al., 2013; He et al., 2013; Paakinaho et al., 2014)*. The cell type specificity of GBS has also been found *in vitro* in A549 cells, a lung carcinoma cell line, and ECC-1 cells, an endometrial cancer cell line, where only 7.7 % shared GBS were identified between those two cell types (Gertz et al., 2013). In addition, it was found that these shared GBS were significantly enriched for GRE's, suggesting that cell-specific and shared GBS have distinct underlying DNA sequence patterns. Interestingly, 75 % of the GRE-GBS only became accessible after DEX-treatment which is in contrast with nonGRE-GBS where 67 % was found in open chromatin prior to treatment. This would imply that DEX activation of GR facilitates chromatin accessibility of GRE-regions, enabling modulation of gene transcription. This has previously been found by others as well who in addition had found that the GRE-composition could be linked to the degree of chromatin accessibility prior to hormone treatment (John et al., 2011). Zbtb3 is essential for the growth of cancer cells involved in human melanoma, lung carcinoma and breast carcinoma (Lim, 2014). Since the PC12 cells originate from tumor cells, we cannot exclude that our finding is not neuronal but rather carcinoma-specific. However, in Chapter 4 binding motifs of Zbtb3 were also frequently observed in hippocampal tissue. In contrast to the neuronal PC12 cells where all identified Zbtb3 motifs occurred in non-GRE containing GBS, Zbtb3 was found in the hippocampal GBS that did contain a GRE, suggesting they may function as GR tethering sites. Interestingly, in a study focusing on combinatorial interactions among transcription factors, it was found *in vitro* that GR and Zbtb3 proteins are able to interact (Ravasi et al., 2010). Since this was only one of more than 700 interactions that were studied, no additional information was provided regarding the GR-Zbtb3 interaction. It is clear that the combination of GR and Zbtb3 in a neuronal setting requires further investigation. Regarding the other proteins Gabpa, Prrx2, Zfp281 and Gata1, no new information in relation to glucocorticoids in a neuronal setting has been found since publication of Chapter 3.

To conclude, the study in Chapter 3 has provided insight into new aspects of GR-mediated action of glucocorticoids in the neuronal PC12 cells. Even though we have not been able to validate all the discovered GBS and the newly hypothesized transactivation and transrepression partners, they provide a valuable inventory for new investigations into GR action in a neuronal context.

6.4 Chapter 4: discussion

Findings

Similar to Chapter 3, the aim of this chapter was to identify GBS within a neuronal context. Instead of a cell line, rat hippocampal tissue was used for ChIP-seq, resulting in an inventory of 2,460 significant GBS. The analysis was designed to compare the GR binding profile at different doses of CORT (ranging from 3–3,000 μg/kg) at 1 hour after administration to ADX rats. In addition to GR also MR-binding to a selection of GBS was examined. It appeared that the binding pattern of GR to its genomic targets is dependent on the concentration of CORT. Whereas some of the DNA-targets are more sensitive and did bind GR at the lower CORT dose (30 μg/kg and higher), others required higher CORT (300–3,000 μg/kg) doses. Our results showed the existence of 2 populations of GBS in the rat hippocampal genome that can be distinguished by their binding at different CORT concentrations.

The population of GBS identified under low dose conditions suggests that GR is already active during basal levels of circulating CORT. The genes activated by low CORT levels may therefore be involved in the permissive actions of CORT that operate during synchronization and coordination of daily and sleep-related events. The other population of GBS that becomes occupied during the higher CORT concentrations as present during stress, likely is involved in stress adaptation, learning and memory processes and recovery. The current inventory provides an important source of information to dissect the function of these GBS in different contexts.

We were interested in whether the GBS are also bound by MR, because MR and GR have DNA-binding domains that are 94 % identical and may form heterodimers. Both receptors are activated by CORT, with the only difference that MR has a much higher affinity for CORT and, consequently, is activated at lower CORT levels in comparison with GR. We were able to show significant MR-binding to 10 out of 13 GBS. However, in contrast to GR, a plateau of MR binding capacity was reached at 300 μg/kg CORT, while for GR a sharp increase in binding was observed at 3,000 μg/kg CORT. A hypothesis that would need further investigation is that at lower CORT concentrations MR may predominantly form homodimers, whereas at higher concentrations mimicking stressful conditions when GR activation becomes more abundant, the incidence of heterodimerization with GR and ultimately GR homodimerization increases, with differential consequences for the repertoire of bound GBS.

Similar to in neuronal PC-12 cells, intragenic GBS were highly represented (39 %) in rat hippocampus, the majority of which was located within introns. It was striking that except for 14 GBS, all the other GBS contained a GRE-like motif. In addition to the GRE, the 500 most significant GBS contained motifs resembling binding sites of transcription factor Zbtb3, Zfp740, Sox12, Sox4, Srf and Zscan4c. Zbtb3, of which the motif is present in 58 % of the GRE-GBS, is of particular interest, since these results in rat hippocampus contradict our observations in PC12 cells,where motifs for

Zbtb3 binding were exclusively observed in non-GRE containing GBS. Within the rat hippocampus, a combination of GRE and Zbtb3 motifs were present in GBS associated with genes involved in regulation of apoptosis, regulation of transcription, regulation of macromolecule metabolic processes and the insulin receptor signaling pathway. Interestingly, these processes are connected to the mTOR pathway (Figure 1.7, Chapter 1). The 14 GBS that did not contain a GRE, all contained 2 motifs resembling the binding motif of the protein CUP. In Drosophila, CUP is an eukaryotic translation initiation factor 4E (EIF4E)-binding protein that represses the expression of specific maternal mRNAs. Since EIF4E is a downstream target of the mTOR pathway this might imply that GR operates at several levels affecting regulators as well as targets of the mTOR pathway.

Update of Chapter 4: findings since publication

The fact that we were able to perform a ChIP experiment with MR *in vivo* was at that time very new and exciting. Since then ChIP-seq for MR has been performed by others *in vitro* in a murine distal convoluted tubular epithelial cell-line (mDCT) that was stimulated with 10^{-7} M aldosterone (Ueda et al., 2014). Sgk1, Fkbp5, Rasl12, Tns1 and Tsc22d3 (Gilz) were identified by ChIP-seq and validated as direct target genes of MR by quantitative RT-qPCR and ChIP-qPCR. MR binding regions adjacent to Ctgf and Serpine1 were also validated. Interestingly, with the exception of Rasl12, we have found GBS near all the above mentioned genes. To what extent these regions overlap with the GBS identified in rat hippocampus is unknown and beyond the scope of the current thesis. It would be of interest for a future study to investigate this further.

Dose-dependent GR-binding has also been described by others, *e.g.* in A549 cells where a distinction was made between hypersensitive (bound at 0.5 nM DEX after 60 min), medium sensitive (bound at 5 nM DEX after 60 min) and low sensitive GBS (bound at 50 nM DEX after 60 min). The hypersensitive GBS had overall stronger binding signals which is similar to our observations in the rat hippocampus in the low-CORT group. In addition it was found that dose-dependency of GR binding is not driven by a specific version of the GRE (Reddy et al., 2012). Instead chromatin accessibility appeared to be a determinant of GR binding, predominantly to the hypersensitive sites. The sequences that surround the GBS may affect chromatin accessibility by recruiting proteins that increase or decrease this accessibility or that aid in stabilization of GR-DNA interactions. This may be one of the molecular causes for differences in affinity of GR to its GBS within one tissue-or cell-type as well as potentially underlying the cell-specificity of GBS.

Interestingly PER1, of which one of its GBS's is the most sensitive in our selection of validated GBS, was found by others to be uniquely sensitive to low doses of glucocorticoids. In this in vitro study in A549 cells, 50 % of the PER1 expression response occurred at 0.47 nM DEX which was accompanied by GR-binding at an upstream GBS at the same DEX-concentration and time-point. This is in contrast

to the other GBS and genes investigated where GR-binding and differential gene expression were not measured until approximately 8 nM of DEX was used for stimulation with (Reddy et al., 2012). .

Another source of diminished chromatin accessibility can be methylation signatures present within the GBS which prevent GR binding to the GBS in one cell type in comparison to another. DNA methylation predominanty occurs at CpG dinucleotides in the human genome, but recently evidence has been found showing that adult human brain tissue is among the tissues with the highest number of methylated non-CpG cytosines (Varley et al., 2013). Methylated non-CpG cytosines might explain why GBS that have been identified in other non-neuronal studies were not identified in our hippocampal dataset. The presence of methylated non-CpG cytosines in the brain was confirmed in mouse frontal cortex (Xie et al., 2012).

In our analysis we have found motifs resembling the binding site of Zbtb3, Sox4 and Srf. New findings regarding Zbtb3 have been described in the previous section regarding Chapter 3. Since publication of this data, it has been found that Sox4, a neurogenesis-related transcription factor, has a crucial role in regulating hippocampal neurogenesis in mice (Miller et al., 2013). Srf, an important regulator of cell growth and differentiation, appears to be involved in an alternative cellular mechanism for the regulation of cell death in hippocampal CA1 neurons. Since five of the ten most enriched functional GO clusters in Chapter 4 are “Apoptosis” and “regulation of programmed cell death”, “neurite projection” and “neuron differentiation” and “positive regulation of transcription”, Srf and Sox4 transcription factors appear to be good candidates to further explore as a transactivation or tethering partner of GR, in addition to Zbtb3 (Chang and Chao, 2013).

As a reference genome Rattus Norvegicus 4 (rn4) was used to align the isolated DNA tags (7). However, since then two new versions have been published and Rn6 contains a new, partially assembled Y chromosome as well as improvements to other regions of the genome. If one would continue with the outcome of the experiments of this chapter, aligning the reads against the newest version of the rat genome should be performed.

6.5 Chapter 5: Discussion

Findings

In this study we showed that in the rat hippocampus CORT directly regulates the mTOR signalling pathway, which plays a central role in translational control and has long-lasting effects on the plasticity of specific brain circuits. We demonstrated that rats with a history of chronic stress have higher basal hippocampal mTOR protein levels in comparison to control animals. Interestingly, mTOR protein was decreased when chronically stressed animals received an acute CORT challenge. This

is in contrast with the non-stressed controls which did not show an effect on mTOR protein.

Using microarray expression analysis, we identified three regulators of the mTOR protein (DDIT4, FKBP51 and DDIT4L) as well as a downstream target (DDIT3), to be differentially expressed in response to a CORT-injection. Interestingly, this expression differed between the hippocampal subregions CA3 and DG, suggesting a key role of the mTOR pathway in the differential plasticity of these subregions in response to acute CORT exposure. If the animals had experienced CRS, DDIT4 and DDIT3 were no longer differentially expressed in the rat DG, which was accompanied by higher mTOR protein levels in whole hippocampus. Interestingly, using ChIP-seq in a separate experiment, GREs were found near the mTOR regulators DDIT4, DDIT4L, FKBP51 as well as near DDIT3, which were validated in the case of DDIT4 and FKBP51.

Update of Chapter 5: findings since publication

In chapter 5, we demonstrated that the action of glucocorticoids on the expression of mTOR pathway members as well as on hippocampal mTOR protein levels is context-dependent and is highly sensitive to chronic stress. In addition, we proposed that direct regulation of the mTOR pathway by CORT represents an important mechanism underlying CORT-effects on neuroplasticity in the brain, with different outcomes depending on prior stress history. The sensitivity of mTOR for environmental stressors has been demonstrated recently by others as well, showing that chronic restraint stress in rats (10-days, plastic restrainer, 6 h daily) leads to increased mTOR mRNA expression, which is in line with the increased protein levels that we have found (Orlovsky et al., 2014).

Several studies have demonstrated that extracellular signal-regulated kinase (ERK) levels are decreased in the hippocampus in animal models of chronic stress and chronic CORT exposure (First et al., 2011; Gourley et al., 2008). ERK1/2 plays a crucial role in synaptic and structural plasticity and operates upstream of the mTOR pathway. There are indications that the decreased ERK level in the aforementioned animal models is specifically present within the dentate gyrus (First et al., 2011; Gourley et al., 2008). Since a decrease in ERK 1/2 leads to a reduced inhibitory action on the mTOR pathway, it is to be expected that mTOR expression would be increased, which is consistent with our findings.

The fact that CRS affects gene transcription has been observed in another study as well where 21 days of chronic restraint stress resulted in an increased basal gene expression level when measured one day later in the hippocampus of Male C57/bl6 mice (Gray et al., 2014). Even though expression of most genes recovered after a r period of 3 weeks upon cessation of the chronic stressor, many other genes remained altered and did not return to baseline including glutamate transporter EAAT2 (Slc1a2), Histone deacetylase 8 (Hdac8) and Period circadian clock 2 (Per2). Interestingly it has been found that different stress paradigms induce distinct tran-

scriptional profiles (Gray et al., 2014; Orlovsky et al., 2014). This might explain some of the conflicting results obtained in different studies. Whereas CRS rats (10-days, plastic restrainer, 6 h daily) showed an increase in GR and a decrease in MR mRNA levels in the hippocampus, GR mRNA was decreased in another study performed in Male C57/bl6 that experienced CRS (21-days, conical tubes, 2 h daily), of which GR was elevated again after a recovery period of 22 days. Even though the results are not consistent, collectively these studies support our finding that GR-mediated gene transcription is affected by CRS.

Similar to our results, it was found in other studies that some of the hippocampal changes induced by chronic stress can only be observed if GR is activated acutely by stress-induced or injected CORT (Datson et al., 2013; Gray et al., 2014). Naturally, immediate early genes always were induced after 1 hour by the forced swim exposure, independent of the CRS background, and this group included besides c-fos, also Per1 and Sgk1. However, the study by Gray et al (Gray et al., 2014) also showed that CRS + forced swim resulted after one hour in an enormous increase in the amount of differential expressed genes, which increased from 1,298 to 3,999 genes. Many of these responsive genes are involved in chromatin modification, epigenetics and the cytokine/NFκB pathways. Interestingly, similar cytokine/NFκB genomic changes were observed after repeated social defeat (Feldker et al., 2006). The responsive network showed overlap with the genomic response to CORT applied to rats with a similar stress history (*e.g.* the Ddit4 pathway), in this case restricted to the dentate gyrus only (Datson et al., 2013). Allowing the animal to recover brought the number of differentially expressed genes back to the level observed in naïve FST exposed animals, being 1,251 genes. However, the overlap of this gene pattern between the recovery and the naïve groep exposed to forced swim was low. The stress-induced change in some of the genes of the CRS group, such as GR and BDNF, persisted for several weeks.

The findings illustrate that chronic stress creates a profoundly altered state of transcriptional reactivity to a novel stressor. The altered gene expression response is likely to be the result of local chromatin remodeling induced by CRS resulting in altered chromatin accessibility for transcription factors such as GR. Within the rodent DG, genes that are involved in chromatin structure and epigenetic processes have been found to be differentially expressed after CRS, which supports this hypothesis (Datson et al., 2013). This new information is in line with our finding that a history of CRS affects chromatin accessibility and consequently the ability of GR to bind to its genetic targets.

To summarize, it is evident that chronic stress affects the genomic response within rodent hippocampus. This becomes evident when the animal is subsequently exposed to an acute CORT-injection or novel stressor, the response to which is shaped by the chromatin accessibility caused by CRS. It has been suggested that the altered transcriptional response at least partially underlies the enhanced vulnerability to stress-related disorders like depression that can be caused by chronic stress

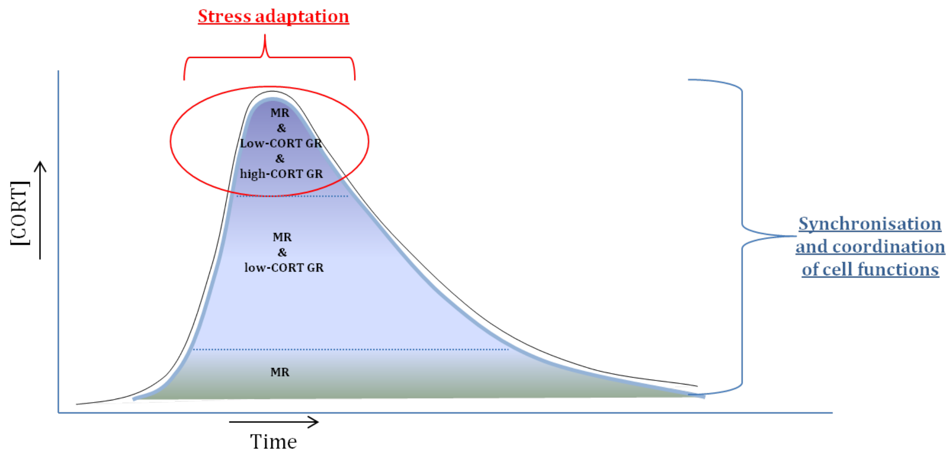


Figure 6.2: A model that illustrates the stress-response which is divided into three parts based on the CORT-receptors that are active.

(Datson et al., 2013). One of the molecular pathways affected is mTOR for which evidence has been found in our experiments and is supported by others (Orlovsky et al., 2014). While in our study we did not focus on GR and MR expression, neither mRNA nor protein based on new findings in literature this would be a very relevant topic to add. In addition, it would be of added value if mTOR protein would be measured in the DG separately.

6.6 Proposed Models

The results obtained in Chapters 2–5 and the studied literature has led to the formulation of two hypothetical models described below. Model 1 focuses on the functional implication of targeted GBS-groups that are subjected to various CORT-concentrations, following the stress response. Model 2 elaborates on the hypothesized regulatory role that CORT has in maintaining an optimal balance of the mTOR pathway and how this balance is impaired by CRS.

Model 1: stress adaptation involves the activation of a distinct GBS population

In our hippocampus model, GR binds to its genomic targets in accessible chromatin. There are two distinct populations of GBS, namely the Low-CORT and High-CORT populations. In the High-CORT group, MR predominantly binds the GBS and it is not until the CORT concentration becomes very high that GR-binding takes over this dominant role (Chapter 4). We propose a model in which a stress-response is divided into three parts based on the CORT-receptors that are active (see also (de

Kloet et al., 2005; de Kloet, 2014) (Figure 6.2)). The MR-group is activated at low levels of CORT (between 3 and 30 μg/kg) and remains to be active at higher CORT as well. The high-CORT GR group is only activated at high CORT concentrations (3,000 μg/kg) and the low-CORT group is activated somewhere in between depending on the GBS investigated. The group of genes that are associated with GBS can be divided into two groups. The genes that are targeted by MR as well as GR during lower and higher [CORT] are involved in general cell processes such as coordination of daily and sleep-related events involving genes like Per1, MT2a, Ddit4, Klf9. The genes that are bound by GR only during high [CORT] are involved in the cell specific processes required for stress adaptation such as energy metabolism, neuronal plasticity and recovery ultimately leading to memory formation, recovery and in preparation for coping with a new stressor. In the hippocampus, we have identified the following genes for this group: Lyst, Cacna2d3, Arpc2, Serp2, Slc7a6, St3gal3, Ndnl2, Nrxn1.

Model 2: History of chronic stress modulates mTOR regulation: possible implications

We have observed that GR binding to its targets after an acute CORT challenge is affected by the stress history of the rats. When rats are challenged with CORT and do not have a history of chronic stress, GR binds to thousands of GBS in the hippocampus, which in almost all cases contain a GRE-like sequence (Chapter 4). This list of GBS includes several mTOR pathway-members, which are differentially expressed in the rat Dentate Gyrus, being either up (FKBP51 and DDIT4) or down-regulated (DDIT4L and downstream target DDIT3) (Chapter 5). Both DDIT4 and FKBP51 contain a GRE-like sequence to which GR binds which implies that these mTOR regulators are upregulated after an acute CORT challenge via direct interaction of GR with a GRE. At the level of mTOR protein expression, acute CORT does cause a minor increase but this is not significant.

Animals that have experienced a history of CRS have significantly higher mTOR protein levels in the hippocampus during basal conditions, which is dramatically decreased when the animals experience a subsequent CORT challenge. DDIT4 and mTOR downstream target DDIT3 are not differentially expressed in these animals. Apparently, the accessibility of the GRE of DDIT4 for GR-binding is compromised, thus inhibiting the regulatory function of DDIT4 in the mTOR pathway. These findings demonstrate that the hippocampal mTOR protein is sensitive to a history of chronic restraint stress in rats.

It has become clear that mTOR activity is very sensitive to stimulation by CORT in changing environments. Whether a low or high mTOR activity is better for neuronal functioning, is unclear and depends on the context and timing of such stressful stimuli. As suggested in Chapter 5, an optimal balance of the mTOR pathway would promote LTP and memory formation, while at the same time promoting

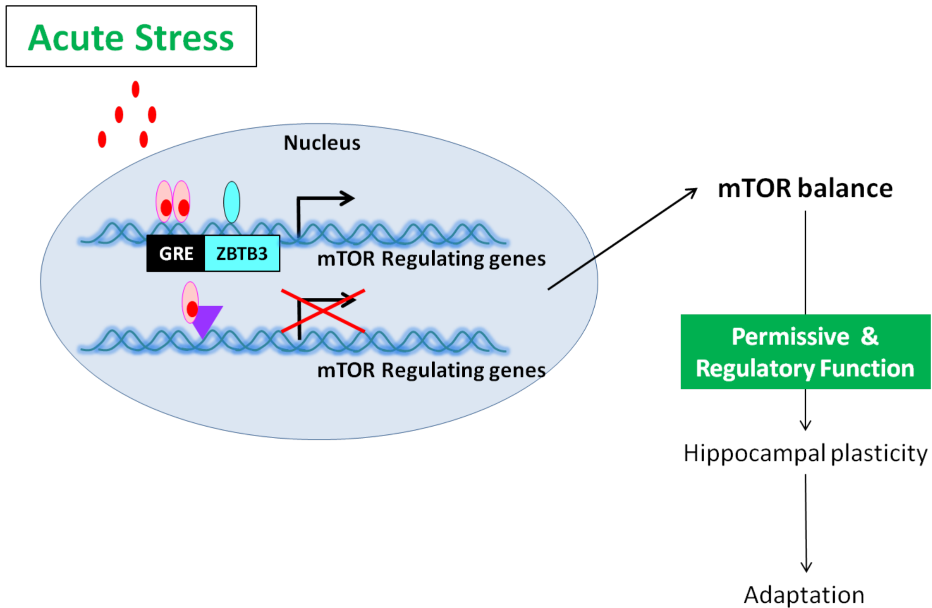
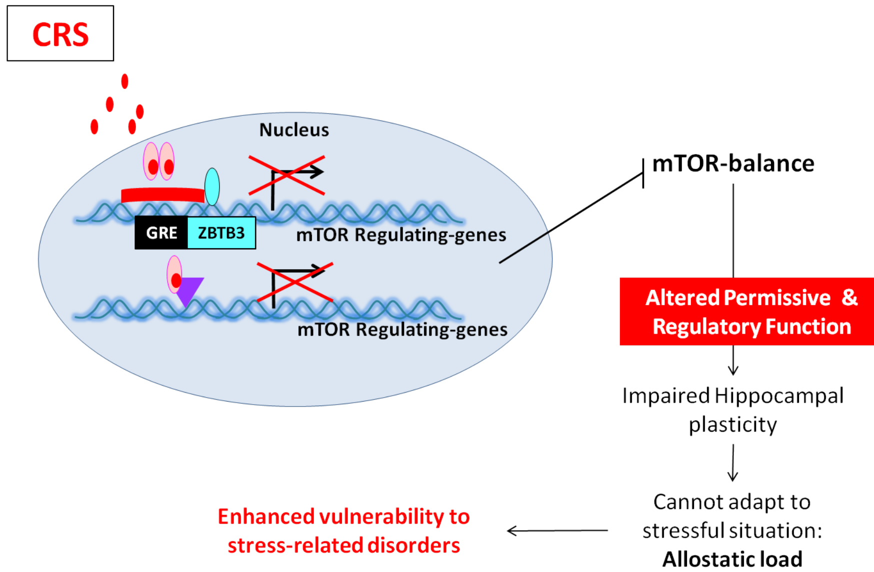


Figure 6.3: A model that illustrates the regulatory role that CORT has in maintaining an optimal balance of the mTOR pathway, allowing the organism to adapt to the situation.

cell survival and resilience. CORT is proposed to be a regulatory component of the mTOR balance in the hippocampus (Figure 6.3).

Chronic stress affects the chromatin accessibility and poses an extra regulatory level to CORT action. Ultimately this is reflected in an impaired hippocampal synaptic plasticity and enhanced vulnerability to stress-related disorders (Figure 6.4). CORT functioning is regulated in the context of chronic stress as well by chromatin remodeling that affects the capability of CORT-receptor GR to bind to its genetic targets, including mTOR regulators. This illustrates the complexity of regulation of the mTOR pathway by external and internal factors. If mTOR regulation is not well balanced, than this might result in affected LTP/LTD and resilience/survival pathways ultimately leading to enhanced vulnerability and to the development of stress-related neuropsychiatric disorders such as major depressive disorder or post traumatic stress syndrome.

Putting our findings into a translational perspective it would be of interest to examine in future studies to what extent the stress-CORT-mTOR interplay plays a role in the proliferation, migration and positioning of newborn neurons in the hippocampal circuitry. Such studies would help to understand to what extent the mTOR pathway is implicated in regulating neuronal plasticity, a process which underlies hippocampal-dependent learning and memory.



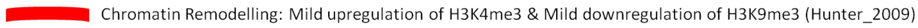


Figure 6.4: A model that illustrates the inhibiting effect of CRS on the mTOR balance that can result in impaired hippocampal plasticity which might contribute to an enhanced vulnerability to stress-related disorders.

6.7 General Conclusion

In this thesis the primary genomic targets of GR have been analysed within a neuronal context. Using ChIP-sequencing thousands of GBS were identified in neuronal PC12 cells and in the rat hippocampus. New transactivation and transrespression partners that enable GR potentially to generate neuronal-specific gene transcription were proposed. Two populations of GR were observed that have different sensitivities to their genetic targets depending on the concentration of CORT. Furthermore, MR was found to be capable of binding to identified GBS. Finally, a direct suppression of the mTOR pathway by CORT within the rat hippocampus was revealed if the animals previously had experienced chronic stress. Taken together, these findings contribute to a better insight into the interaction of GR with the genome in a neuronal setting and point to the pathways that are under control of GR during stress exposure and recovery. The mTOR regulation within the rat hippocampus which is clearly affected by the stress history calls for further research.