Glucocorticoid signature in a neuronal genomic context

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Chapter Seven

Summary

In daily life, the human body is faced with internal and external stimuli (also referred to as stressors) that challenge homeostasis. The body responds to these stimuli by turning on a stress response, which consists of activation of the sympathetic nervous system and the hypothamus-pituitary-adrenal (HPA) axis and their end products adrenaline and corticosteroid hormone respectively, that are released from the adrenal gland. CORT readily penetrates the brain and gives feedback to precisely on those structures that have initially produced the stress response. One important target is the hippocampus, a limbic brain structure which assigns a context, time and place to the experience of a stressor. In the hippocampus CORT promotes learning and memory processes. This effect exerted by CORT is mediated by the Glucocorticoid Receptor (GR) and the Mineralocorticoid Receptor (MR), after which the receptors migrate into the nucleus, affecting the transcription of CORT-responsive genes. While MR is involved in maintenance of basal activity of the stress system, the regulation of appraisal processes and the onset of the stress reaction, GR activation results in recovery from stress and behavioral adaptation. Ultimately, their balanced activation is an important determinant of neuronal excitability, neuronal health and stress responsiveness.

Since GR has an important role in the normalization of the homeostasis after the occurrence of a stressor, this protein has received a lot of attention in research in relation to the hippocampus. This has led to an impressive amount of information that has been gathered throughout the years regarding CORT-responsive genes. However, these inventories are a mix of primary GR-targets as well as downstream targets and a differentiation between these groups was not possible. Due to innovative technologies, resulting in the first ChIP-seq studies, it became possible to perform genome-wide identification of transcription factor binding sites. Aim of this thesis was to investigate the genome-wide targets of GR within a neuronal context and the possible functional implications that these binding sites might have on a pathway involved in neuronal plasticity, the mTOR pathway. For this purpose four aims were specified which are addressed in **Chapters 2** till **5**.

AIM I: "To use an *in silico* approach with the goal to predict neuronal-specific GREs in the genome followed by their experimental validation. For this purpose we have developed the Position Specific Scoring Matrix (PSSM) GenSig."

GR is able to dimerize and as such can recognize and bind glucocorticoid response elements (GREs) in the DNA by which it can regulate the expression of target genes. GRE-dependent processes are important in the brain, because mice in which GR is not able to bind to GREs, due to a mutation that inhibits GR homodimerization, hippocampal excitability and spatial memory were impaired. The GREs responsible for the action of GR *in vivo* in the brain are largely unknown which makes it

very difficult to predict them. Furthermore, GR-binding to a GRE-like sequence may not always lead to a functional effect. In **Chapter 2** the application is reported of a position-specific scoring matrix from 44 GREs described in literature that is used to predict evolutionary-conserved GREs. Using this matrix, large genomic regions were scanned surrounding CORT-responsive genes that have been identified in a neuronal context. Fifteen out of 32 predicted GREs were identified that are bound by GR in the rat hippocampus of which at least 10 are novel. Furthermore GC-box associated motifs were discovered that are present in the GRE-flanking sequences. This characteristic of binding was found to be absent in another dataset with GR-binding and GR-nonbinding sites, suggesting a mechanism for tissue-specific CORT signaling that may determine GRE usage in the hippocampus. In conclusion, our current finding can be considered a first step towards understanding the direct downstream pathways of GR signaling in the brain.

AIM II: "To identify genome-wide GR-binding sites (GBS) in vitro in neuronal PC12 cells and in vivo in rat hippocampus using ChIP-seq and to identify genes located in the vicinity of these GBS that are activated/repressed by GR in a neuronal-specific context."

GR is able to bind directly to genomic glucocorticoid response elements (GREs) or indirectly to the genome via interactions with bound transcription factors. These two modes of action, respectively called transactivation and transrepression, result in the regulation of a wide variety of genes important for neuronal function. Despite the fact that a lot of knowledge was already available regarding differential expressed genes after the activation of GR, its primary targets were not investigated yet in a genome-wide neuronal context. In **Chapter 3**, a genome-wide analysis is presented of GR-binding sites (GBS) in neuronal PC12 cells. At the time of publication, this was the first genome-wide discovery of GR-binding sites in a neuronal context. Interestingly, the majority of the PC12 GBS that were uniquely identified in this model system were located nearby genes with a known neuronal function. In **Chapter 4**, a genome-wide analysis of GBS in rat hippocampus is reported after administration of an acute CORT pulse to the rats. Both chapters revealed a high prevalence of intragenic GBS of which the majority was located within introns. In both **Chapter 3** and **4** motif screening revealed that the GRE was the most prevalent motif, 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. In addition, in both **Chapter 3** and **4** a Zbtb3-binding motif in the GBS was discovered. In neuronal PC12 cells, Zbtb3 is reported to be exclusively found in non-GRE containing GBS (Chapter 3), suggesting that it might be a new transrepression partner of GR in the neuronal context of this cell line. In contrast, in the rat Zbtb3 was only identified in GRE-containing GBS (**Chapter 4**), implying it may be a new transactivation partner of GR. Within the rat hippocampus, a combina-

tion of GRE and Zbtb3 motifs was found to be present in GBS that are associated with genes involved in regulation of apoptosis, regulation of transcription, and regulation of macromolecular metabolic processes and the insulin receptor signaling pathway. Interestingly, these processes are all connected to the mTOR pathway. To conclude, the studies in **Chapters 3** and **4** have provided insight into new aspects of GR-mediated action of glucocorticoids in a neuronal context. 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. Understanding GR-signalling in a neuronal context is important given the profound effects of glucocorticoids on neuronal plasticity and consequently on brain function.

AIM III: "To investigate whether MR binds to the same GBS as GR in the hippocampus and to measure binding of both receptors to these DNA sites in response to different concentrations of ligand."

In the brain, CORT binds to MR and GR. Whereas GR is abundantly expressed throughout the brain, MR has a much more restricted expression in predominantly limbic brain structures. Both receptors differ in their affinity for CORT, where GR has a relatively low affinity for CORT and MR a relatively high affinity. As a consequence, GR is activated when circulating CORT increases, during stress, and MR is already activated under basal nonstress conditions. Their balanced activation is an important determinant of neuronal excitability, neuronal integrity, and stress responsiveness. For GR it is known that it can bind as a dimer to GREs that are present in the DNA and consequently induces transactivation. Furthermore, GR is able to bind via other Transcription Factors indirectly to the genome resulting in transrepression of these stress-induced pathways.

Knowledge on interaction of MR and GR with the genome is sparse, in particular in the brain. In **Chapter 4**, we investigated GR-binding to hippocampal GBS identified in male adrenalectomized rats that were challenged with increasing doses of the GR agonist CORT ranging from 3 to $3{,}000\ \mu g/kg$. Furthermore, we analyzed the potential of MR to bind to these GBS under the same conditions. We have shown that under varying CORT concentrations, 2 groups of different binding sites are recruited. The low-CORT group shows GR binding to GBS at $30\ \mu g/kg$ CORT which increases with higher CORT concentrations. In the high-CORT group GR binding to the GBS is evident after injecting $3{,}000\ \mu g/kg$ but not at lower concentrations. MR-binding at both groups is apparent at $30\ \mu g/kg$ and in most cases stabilizes thereafter, indicating a saturation of MR in both situations.

In conclusion, our results highlight the existence of 2 distinct populations of GBS in the rat hippocampal genome that can be discriminated by the extent of CORT binding. Furthermore, we have shown that MR is able to bind to a selection of GBS which contributes to the knowledge on the primary genomic targets of MR.

The simultaneous binding of GR and MR to the same GRE-containing GBS might imply heterodimerization of GR and MR.

AIM IV: "To translate the genome-wide knowledge regarding GBS into a functional application by investigating how chronic stress affects GR-mediated action of acute glucocorticoid exposure to the mTOR pathway as a novel mechanism involved in the regulation of brain plasticity."

The mTOR pathway plays a central role in translational control and has long-lasting effects on the plasticity of specific brain circuits. Whether low or high mTOR activity is better for neuronal functioning, is unclear and depends on the context and timing of stressful stimulation this pathway operates. An optimal balance of the mTOR pathway would promote LTP and memory formation, while at the same time promoting cell survival and resilience. The mTOR pathway is known to be activated by a wide variety of extracellular stimuli and also by hormones such as CORT. However, this knowledge is based mainly on peripheral tissues and has been studied less well in the brain.

In **Chapter 5**, we showed that CORT directly regulates the mTOR signalling pathway in the dentate gyrus of the rat hippocampus. Furthermore, 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 to the non-stressed controls where CORT did not show an effect on mTOR protein. We have observed that regulators of the mTOR pathway are differentially expressed after an acute CORT pulse, which was affected by a history of chronic stress. Interestingly, direct GR-binding has been validated in the case of mTOR regulators DDIT4 and FKBP51. In conclusion, we propose that direct regulation of the mTOR pathway by CORT represents an important mechanism regulating neuronal plasticity in the rat hippocampal dentate gyrus, which changes after exposure to chronic stress.

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.