Contextual glucocorticoid signaling in-vivo: a molecular perspective

Buurstede, J.C.

Chapter 1

General introduction

Stress was, is and will always be part of life. This was the case for our prehistoric ancestors, currently is the case for modern man and will most likely remain to be so for many coming generations (1). While the predominant type of daily stressor may have shifted from being of a physical to a more psychological nature, our bodies still need to properly respond to the factors that bring about stress. In fact, all mammals need to overcome and cope with a large variety of stressors that each challenge their acute and future survival and/or well-being. Over time our "stress-system" – in the widest definition of the term – has evolved to do so rather effectively. Yet, the incidence of stress-related disorders (often resulting from chronic or excessive stressors) is a growing societal problem. Stress-related disorders affect the overall well-being and health of people worldwide and thereby also negatively impact various aspects of our society, ranging from our healthcare system to our economy (2-4). Therefore, stress is not merely an interesting topic to study, but one with a clear relevance for our fast-paced and complex society. A better understanding of the stress-system can aid the prevention and treatment of stress-related disorders and thereby relieve the pressure that it puts on our society and its participants. In this thesis I focus specifically on the molecular signaling of one major class of stress hormones, the glucocorticoids, and on the effects that they have on gene transcription in a diversity of cell types and organs in the mammalian body. This is a small, but crucial cog in the large machinery of the stress-system.

THE ADAPTIVE VALUE OF THE STRESS SYSTEM

Stress, stressors and the stress-system

We all know what it is like to feel stressed, but it is hard to define what stress is. The World Health Organization defines it as "any type of change that causes physical, emotional or psychological strain" (5). Arguably, this rather defines a *stressor*. What we collectively call "stress" is how we experience our body's reactions to these stressors, the state that follows upon exposure to a stressor (6). Stressors exist in many forms, ranging from worrying about your next career step (psychological stress) to being attacked by an angry hippopotamus (physical stress) and anything in between.

While the number of conceivable stressors approaches infinity, they all elicit a similar response by our bodies (7). This response is optimally shaped to deal with acute physical stressors, but is very similar (although sustained) in the case of chronic stressors. The reaction to stressors is coordinated by the stress-system, a complex system that includes feedback and -forward loops (8). The stress response is (by and large[a]) orchestrated by two types of stress mediators: (nor)adrenaline and glucocorticoids (9-12). Of these, the neurotransmitter noradrenaline and the hormone adrenaline are the first responders. They enable our initial fight/flight response by increasing our heart rate, enhancing blood flow to our muscles, dilating our bronchi for higher oxygen intake and mobilizing energy to support the strenuous effort. These rapid responses of the sympathoadrenal system are aimed at ensuring survival through activity (6).

The second phase of the acute response to a stressor involves the adrenal glucocorticoid hormones. This phase predominantly aims at restoring our bodies when the threat has passed (returning to homeostasis) and subsequently storing information regarding the threat to properly respond to future similar stressors (13). However, multiple acute stressors or exposure to chronic stressors can result in a sustained stress response, which can become maladaptive over time (14). Both acute and chronic glucocorticoid responses depend on activation of the hypothalamus-pituitary-adrenal (HPA)-axis (**Figure 1**; 15). Hypothalamic cells located in the paraventricular nucleus signal to the pituitary by releasing corticotropin-releasing hormone. The anterior pituitary in turn releases adrenocorticotropic hormone, to which the adrenal glands respond by producing and secreting glucocorticoid hormones (predominantly cortisol in humans and corticosterone in rodents). It are these glucocorticoid hormones that enable us to cope with stressors by modulating and adjusting internal processes (16).

a While (nor)adrenaline and glucocorticoids are the main effectors in the body, many more hormones and neurotransmitters are at play in the brain. This includes a clear role for the corticotropin-releasing hormone.

Before addressing the action and effects of glucocorticoids in more detail, it is important to note that while glucocorticoid exposure is beneficial in the short term as a response to an acute stressor, glucocorticoids mediate many of the maladaptive effects following chronic stress (17). These chronically elevated hormone levels can increase vulnerability to stress-related disorders, such as depression or anxiety (13, 18-20). In addition, chronic stress – via glucocorticoids – has many effects outside of the brain, for instance on the cardiovascular, immune and metabolic system (21, 22). Moreover, excessive exposure to glucocorticoids *per se* (even in the absence of stress, as is the case with Cushing's disease) can result in symptoms like weight gain, high blood sugar (hyperglycemia) and serious psychiatric disease such as depression (23).

If the "maladaptive" aspects of the stress response are predominantly mediated by glucocorticoids, one could wonder why we do not simply block the production of these hormones to prevent these negative effects altogether. Indeed, there are approaches to reduce their synthesis (24), or block their activity (25-27). Yet, while glucocorticoids are indeed "stress hormones", they are also actually crucial for normal "adaptive" functioning of our body in non-stressed conditions.

On a daily basis, glucocorticoid levels rise before the active period and thereby function as a messenger of the biological clock, informing the brain and peripheral tissues to prepare for the day (28). An example is the role of glucocorticoids in increasing blood sugar levels via glucose metabolism: after fasting during the rest period (night for humans, daytime for rodents), there should be sufficient fuel at the onset of the active period (29). This effect accounts for the term *gluco*corticoids that is used for this class of hormones. Absence of sufficient presence of glucocorticoids, as is the case in humans with Addison's disease, will result in a myriad of symptoms including extreme fatigue, low blood sugar (hypoglycemia) and depression (30). Therefore, there needs to be a balance between homeostatic support and stress adaptation on the one hand and prevention of excess signaling on the other hand (31). To understand why both a lack of *and* over-exposure to glucocorticoids have so many and severe negative effects we need to understand where they exert their function. From here onwards I will mainly focus on corticosterone, the endogenous glucocorticoid of rodents on which the data in this thesis is based.

MECHANISTIC UNDERPINNING OF GLUCOCORTICOID ACTIONS

Mineralo- and glucocorticoid receptors

Corticosterone is the endogenous ligand for two types of receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (32). These receptors are widely expressed throughout the body, and their activation (or lack thereof) will therefore affect the functioning of almost all organs to a varying degree (33). Perhaps most familiar to the general public are the anti-inflammatory effects mediated by the GR, known because of the often-prescribed synthetic glucocorticoids for asthma, skin conditions or allergies (34, 35). Besides peripheral effects, glucocorticoids strongly affect the brain where both receptor types are expressed, modulating behavior and regulating processes such as learning and memory consolidation (36). Although the receptors were already described in 1985 there is still much research with respect to their exact distribution and roles in both neuronal and non-neuronal cells.

After a stressor, activation of the HPA-axis results in corticosterone levels that are higher than the daily peak prior to the active period. The effects of corticosterone at the onset of the stress response (or even preceding it) are regulated by the MR, because its affinity for corticosterone is high enough to sense even minor deviations in basal hormone levels. The subsequent long-term adaptation and normalization of the response is predominantly facilitated by GR, which has a lower affinity for corticosterone and therefore becomes activated particularly by increased hormone levels during the circadian peak and after a stressor (37). Increased corticosterone levels usually enhance memory consolidation, which is highly adaptive since it helps to remember a threatening situation (38). Excessively high levels can however result in overgeneralization of the memory, as is often seen in post-traumatic stress disorder (39, 40). On the other hand, retrieval of memories is impaired by stress and glucocorticoids (41). The above once more exemplifies the importance of an appropriate balance of glucocorticoid actions and timing thereof: we cannot properly function without, nor with too much of them. To understand how corticosterone exerts its effects we need to focus on how glucocorticoid signaling mechanistically works.

Glucocorticoid receptors: activation, modulation and outcome

GR and MR are both ligand-activated members of the nuclear receptor family of transcription factors. However, also rapid non-genomic effects have been described for both receptors (42, 43). Regardless, transcriptional regulation remains their main (at least mostly studied) mode of action, particularly in relation to their long-term effects. Besides sharing glucocorticoids as a ligand, GR and MR are structurally similar, with

particularly large overlap in their DNA- and ligand-binding domains (DBD and LBD) (44). In comparison, the unstructured N-terminal domains (NTD) of the receptors strongly differ, and their mode of functioning is less clear. Despite the predominant structural similarities, the same endogenous ligand and partly overlapping expression of both receptors, the outcome of GR and MR activation differs and this indicates that the molecular workings of glucocorticoid signaling are intricate: there are many details that need a better understanding.

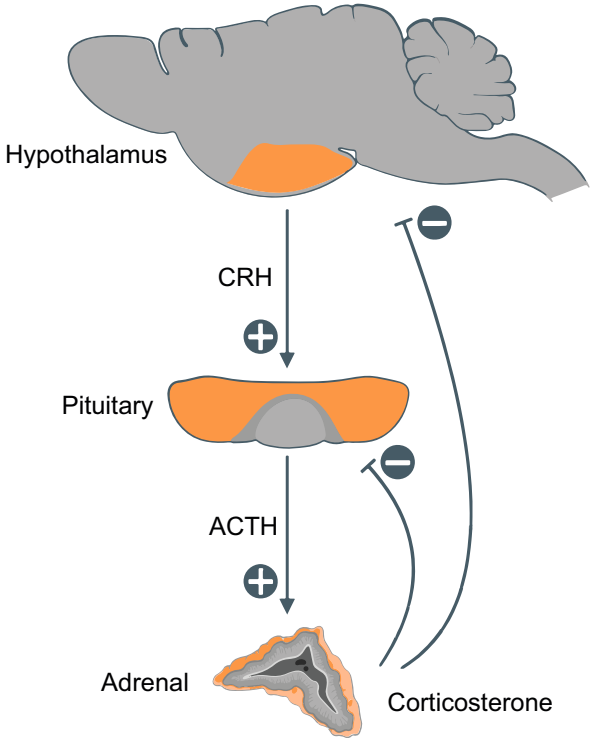

Figure 1: Schematic representation of the murine Hypothalamus-Pituitary-Adrenal (HPA) axis. ACTH: adrenocorticotropic hormone, CRH: corticotropin-releasing hormone.

The classical principle of glucocorticoid transcriptional regulation is simple (**Figure 1**). Glucocorticoids enter the cell and bind the LBD of GR/MR in the cytosol[b] (45), which then translocate to the nucleus (46). Ligand-bound GR- or MR-dimers subsequently bind to a specific sequence in the DNA guided by their DBD. This motif in the DNA is called the glucocorticoid response element (GRE) and consists of a palindromic repeat of six base pairs with a three base-pair spacer (variations on AGAACAnnnTGTTCT).

b Chaperone proteins interact with and guide GR/MR from ligand binding up to DNA binding. This constitutes a research field on its own and is out of scope for this thesis.

GREs are located near the transcription start site of genes, but also often present at intergenic enhancer regions of the genome. These GREs enable binding of dimerized receptors (GR-GR, MR-MR or GR-MR) activating or repressing transcription of the target gene(s) associated with the GRE. The transcriptional outcome of GR binding to the DNA can be highly variable. It depends on the activity of different types of proteins in a single cell (of a specific organ) at a certain time, and even on the precise DNA sequences flanking individual GREs (47). Besides the classical model (**Figure 2**) there is a substantial number of alternative mechanisms via which nuclear receptors such as GR and MR can act (48), and specialists in the field still argue about the relevance of some of these. As a result of transcriptional regulation, exposure to corticosterone leads to changes in expression of a large number of genes, that differs for each organ or individual cell type (49). At least four levels that influence the eventual transcriptional outcome of GR/MR activation are covered in this thesis: I) receptor levels, II) ligand availability, III) expression of coregulators and IV) the chromatin landscape.

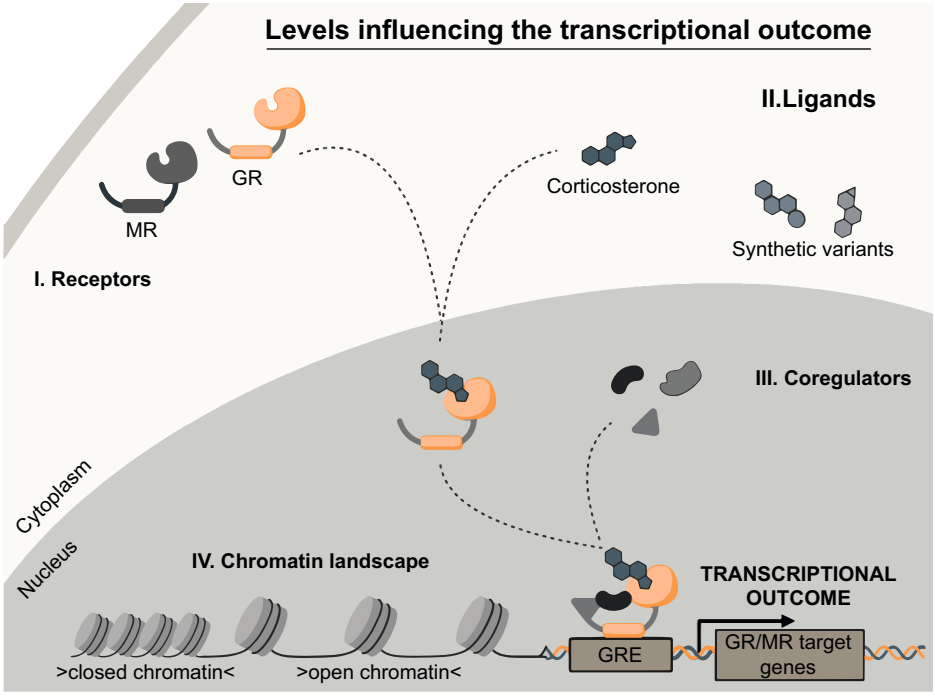

Figure 2: Schematic representation of GR's and MR's main mode of action, indicating the four levels of influence covered in the introduction affecting the transcriptional outcome of glucocorticoid activation. I. Receptor levels, II. Ligand levels: endogenous and synthetic variants, III. Expression of coregulators and IV. Chromatin landscape. GR: glucocorticoid receptor, GRE: glucocorticoid response element, MR: mineralocorticoid receptor.

I) Receptor levels

In the brain, GR and MR are both strongly (yet differentially) expressed in the limbic areas, most notably in the hippocampus (32), a brain region that is crucial for memory formation (50). Single cell gene expression analysis revealed that there are populations of hippocampal cells that specifically or predominantly express either GR (*e.g.* microglia) or MR (*e.g.* CA2 neurons) (51) and the transcriptional outcome after glucocorticoid exposure can differ per cell (52). Besides the absolute presence or absence of the receptor(s), the level of receptor expression is relevant for their functioning. Expression of GR and MR is regulated by a negative feedback loop, *e.g.* high levels of corticosterone activate GR, which in turn downregulates its own expression to moderate the responsiveness in a process known as homologous downregulation (53). This loop contributes to the prevention of long-term overactivation of the stress-system. Nevertheless, even in Cushing's patients (which suffer from excessive levels of endogenous glucocorticoids), this "homologous downregulation" does not lead to a full desensitization to glucocorticoids. Next to receptor expression levels, activity of the GR and MR after corticosterone binding depends on phosphorylation and many other post-translational modifications (54, 55).

Multiple factors influence GR and MR expression levels and their activity, and this "receptor-status" determines the sensitivity of cells to glucocorticoids.

II) Ligands: endogenous and synthetic

In order for expressed GR and MR to be activated, ligand has to be available in a sufficient amount and in an active form. The main determinant of corticosterone levels in the blood is the activity of the HPA-axis. However, availability of corticosterone can also be regulated locally, for example by the two related enzymes HSD11b2 and HSD11b1 (56). The former can inactivate corticosterone, rendering cells largely insensitive to corticosterone. The latter enzyme does the opposite, and reactivates corticosterone form its inactivated form, leading to a locally higher concentration.

Besides the endogenous ligands, synthetic variants are often administered in clinical practice or research settings. These encompass strong agonists such as dexamethasone that are potently anti-inflammatory and immunosuppressive. At the other end of the spectrum are antagonists such as the GR antagonist RU486 that can alter the transcriptional outcome by competing with / preventing binding of the endogenous ligand. As such, RU486 is used in the USA to treat patients with Cushing's disease, but

not without side effects (57). In addition, selective receptor modulators – compounds that can combine agonistic and antagonistic effects – have been developed, all of which specifically alter the conformation of the receptor and thereby its transcriptional outcome (58).

Of note, ligand availability in the brain also depends on the characteristics of the ligand. While endogenous corticosterone readily passes the blood-brain-barrier due to its lipophilic nature, the penetration of synthetic variants differs per ligand. Dexamethasone for instance poorly reaches the brain as it is actively removed by a drug transporter protein (59). In this manner the blood-brain-barrier can lead to periphery-specific effects of synthetic glucocorticoids.

The type and concentration of the ligand leads to activation or inhibition of GR/MR signaling (or a mix thereof).

III) Expression of coregulators

To exert their role as transcriptional regulators, GR and MR collaborate with other regulatory proteins. These interactions occur via specific regions located at the LBD and NTD of the receptors. As the NTD differs substantially between GR and MR, it enables interactions with a different set of coregulators. This leads to unique receptor associated complexes of coregulators (60), making the NTD a logical contributor to receptor specific effects in cells with a similar cellular environment, *e.g.* expressing the same coregulators. In comparison, the coregulators that interact with the LBD seem to be largely shared between GR and MR (61), and therefore contribute less to the receptor specificity.

Two main groups of coregulatory proteins exist: co-activators and co-repressors. As one would intuitively assume, agonists of the GR will predominantly recruit co-activators while some "active" antagonists – like RU486 – favor co-repressor recruitment. However, it is not always that straightforward, for instance at "negative GREs" where GRs suppress gene transcription via corepressor recruitment by GR agonists (62). The interaction between coregulatory proteins and selective modulators does not show such a clear distinction and can be seemingly random, resulting in the specific – and also hard to predict – outcomes (63). As is the case for GR and MR, these coregulators are also expressed in a tissue- and cell-specific manner (64), providing an additional level of complexity that can fine-tune the transcriptional outcome of endogenous glucocorticoids or synthetic ligands of both GR and/or MR.

The conformation of GR/MR – depending on the ligand – influences the interaction with coregulators, and in turn these strongly affect the transcriptional machinery.

IV) Chromatin landscape

If we assume that the expression level of the receptor, the availability and type of the ligand and the expression of coregulators are identical in two cells, the transcriptional outcome can still differ. By definition, different cell types express different genes, and this is based on chromatin structure that determines the accessibility of regulatory elements on the DNA (65). These include the GREs, that dictate the predominant mode of interaction with GRs and MRs. The accessibility of the DNA (open or closed chromatin) is determined by various types of modifications of histone proteins and epigenetic marks. In combination, these determine whether a region is relatively "open or closed", and this defines the chromatin landscape (66). Histone modifications and epigenetic marks are regulated by the previously discussed coregulatory proteins. While interaction of GR and MR with these proteins is in some instances able to open inaccessible regions (pioneering), GR and MR mainly act on regions that are already accessible (67). Of note, this are regions where other transcription factors can also bind. The chromatin accessibility again differs per tissue and cell-type, explaining why the transcriptional outcome can still differ in the situation introduced at the beginning of this paragraph.

Chromatin accessibility determines where GR and MR complexes can bind to the DNA – a prerequisite to regulated the expression of their target genes.

SOME UNRESOLVED QUESTIONS ABOUT THE MECHANISTIC UNDERPINNING

Current status

We currently have a very good understanding of the general working mechanism of glucocorticoids (33). However, a large portion of these insights is based on *in-vitro* studies using various cellular models, *e.g.* performed in a test tube or in cultured cells instead of in an intact organism (*in-vivo*). While those *in-vitro* models proved to be very informative and are the basis for the entire research field, they are also rather far

from actual *in-vivo* glucocorticoid biology. None of the four levels introduced previously are truly accurate in an *in-vitro* cellular model. These models often result in receptor expression levels that are either too low or too high and ligand availability and dynamics are artificial. In addition, cellular models are often immortalized (so they can be used in the laboratory for prolonged periods of time) which affects coregulator expression and chromatin structure.

As glucocorticoids have such pleiotropic effects (simultaneously serving different goals in various tissues/cells), the transcriptional outcome needs to differ accordingly in each setting. A recent review of the transcriptome changes after glucocorticoid exposure highlighted this. Including both *in-vitro* and *in-vivo* data, the review showed that the overlap in target genes between brain-focused studies is limited. Not a single gene was differentially expressed in all 17 studies included and most consistently affected by glucocorticoids was the expression of two genes (detected in 9 out of 17 studies). Overall, 88 genes were deemed consistently regulated by the authors, albeit only reported in at least 4 out of 17 studies (68). These numbers are small in comparison with the longlists (often hundreds or thousands) of differentially expressed genes that are often obtained after glucocorticoid treatment in a single experiment.

Clearly, any generic model of glucocorticoid signaling is missing "something". The *in-vitro* models provided a good starting point, but if one really aspires to understand glucocorticoid signaling one needs to study – at least – the organ involved in the biological question under investigation. However, simply comparing different *in-vivo* studies will likely result in even bigger discrepancies, so *in-vivo* is not *per se* the component missing in the generic model. We believe this "something" is relevant context, as the effect of glucocorticoids are tailored specifically for the encountered scenario. Therefore, we set out to study GR signaling in various *in-vivo* experiments in which we varied "context" in a controlled manner to assess multiple aspects that in our eyes required some elucidation.

Five aspects of context

The context of transcriptional regulation is immensely broad as it encompasses all factors and variables that can directly and indirectly affect the outcome measure, and therefore one can never fully "deconstruct" it. We studied five "aspects of context" of which we felt they address important gaps of knowledge or current misconceptions (realizing that there are more contextual aspects worth investigating). These aspects are introduced below and form the foundation on which the studies presented in the subsequent chapters of this thesis were build.

1: Are MR-mediated effects indeed saturated at higher corticosterone levels?
GR and MR together mediate the responses to glucocorticoids in the hippocampus, the brain structure crucially involved in memory formation. The long-standing assumption of glucocorticoid transcription is that MR is the transcription factor responsible for the effects at basal or low glucocorticoid levels and GR is responsive to higher levels of glucocorticoids (at the circadian peak and after a stressor). This concept originates from cellular studies which showed that MRs have a 10-fold higher binding-affinity in comparison to GRs (32). Therefore, MRs would already be fully occupied at low to intermediate corticosterone levels. We questioned whether this notion holds true for transcriptional effects in an *in-vivo* setting with administration of exogenous corticosterone and assessing hippocampal gene expression.

2: Does duration of corticosterone exposure affect the transcriptional outcome?
Transient glucocorticoid exposure in the context of acute stress is mostly adaptive and beneficial. However, chronically elevated levels are known to pose a risk factor for many diseases. Whether or not a gene is a "target" for GR is often stated irrespective of duration of exposure. While this might hold true for well-established target genes as *Fkbp5* and *Per1*, this is definitely not the case for all genes. Are the effects of acute and chronic exposure comparable, or does the duration affect the context and therefore the eventual transcriptional outcome? We investigated this by assessing and comparing the GR-dependent transcriptome in the mouse liver after acute and chronic corticosterone.

3: Dependence of corticosterone-bound GR on other transcription factors?
There are no genes that are exclusively regulated via just GR and MR, and so they are not the only transcription factors at play in the chromatin landscape. Other transcription factors are active at the same time and they may act in the vicinity of the GR and MR binding sites on the DNA. This is for instance the case in the context of stressful learning, where glucocorticoid-enhancement of memory consolidation depends on the arousal-induced release of noradrenaline (which via its receptors leads to activation of the transcription factor CREB by phosphorylation; 69). Together this leads to the question whether or not – and if so to which extent – corticosterone-bound GR depends for its functioning on other transcription factors. Does GR DNA-binding and subsequent transcription differ with/without arousal, or does the dependence originate elsewhere? We determined GR and pCREB DNA-binding and subsequent transcriptome changes in the hippocampus to address the question.

4: Does a period of stress during early life lastingly affect the developing brain?
Stress is part of life and despite its negative connotation it is not necessarily a "bad" thing. However, strong acute or chronic stress – especially during early age – increases

the risk for stress-related disorders later in life (70). How does early life stress affect later life mental-health? To this end stress researchers have developed various rodent models to investigate the effects of stress in the context of a developing brain that are still apparent in adulthood. The extent of these changes and potential curative reversibility thereof are so far unclear and require further investigation. We studied multiple cohorts of early life stress animals and in addition assessed the effects of RU486 (a GR antagonist) intervention to increase the understanding.

5: Can the typical longlist of GR-target genes be shortened to pinpoint the genes relevant for the process under investigation?

Activation of the GR – as will become evident in the next chapters – typically results in a longlist of genes with altered expression levels. These will in part be directly regulated by GR, while the expression of other genes is altered in an indirect manner. When studying a specific process, it currently is (unfeasibly) challenging to pinpoint the regulated genes that are causal to that process from the longlist of all regulated genes. This often results in researchers following up on the genes most significantly altered or those with the largest change in expression, but this by no means guarantees any functional relevance. In an attempt to improve the process of identifying functionally relevant GR-target genes, we conceptualized the approach of "pharmacological filtering". For this we utilized multiple GR-ligands including selective glucocorticoid receptor modulators. This approach filters the obtained longlist of GR-target genes based on the functional and context-dependent outcome per ligand with the associated transcriptome changes, condensing the longlist to a shortlist of functional GR-target genes. We showcased this approach using auditory fear conditioning, a behavioral paradigm susceptible to glucocorticoid modulation, for which the important GR-target genes were so far unknown.

THESIS OUTLINE

In this thesis we investigated GR signaling in relevant and functional *in-vivo* contexts. We first showed that MR is responsive to increases in corticosterone levels and identified MR-specific target genes (**Chapter 2**). We next assessed how changes in context affected the transcriptional outcome of GR activation by exogenous corticosterone. Specifically, we did this by adjusting the duration of corticosterone exposure (acute vs. chronic) before assessing the effect on the hepatic transcriptome (**Chapter 3**) and by investigating DNA-binding of GR and subsequent transcription in the hippocampus with and without arousing object location training (**Chapter 4**). Next, we investigated the long-lasting effects of early life GR activation on hippocampal chromatin accessibility and the transcriptome, specifically assessing the reproducibility of the effects on the

latter (**Chapter 5**). As all the previous studies resulted in longlists, we applied our pharmacological filtering approach in a setting of a well-characterized GR-dependent effect. By utilizing selective glucocorticoid receptor modulators in combination with the context of auditory fear conditioning behavior we attempted to shorten the longlist of GR-target genes (**Chapter 6**). To conclude, an overall reflection on the obtained results and key lessons learned in the process are discussed (**Chapter 7**).

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