Contextual glucocorticoid signaling in-vivo: a molecular perspective

Buurstede, J.C.

Chapter 7:

General discussion

In the work described in this thesis, we studied genomic GR signaling in various *in-vivo* experiments. We varied the biological context in a controlled manner to assess five aspects that in our eyes required further elucidation. In brief, the selected aspects were: the role of the MR, the duration of glucocorticoid exposure, interactions with other transcription factors, the age at glucocorticoid exposure and the type of GR-ligand (**Figure 1**). The common denominator of the selected aspects is the fact that they affect GR signaling, but are often not (explicitly) addressed in experimental studies. Especially when interpreting, comparing and extrapolating the outcome of glucocorticoid activation, we argue that it is important to pay attention to these aspects of GR signaling. Failure to do so may lead to "inconsistent" results – simply because the experimental basis differs – and contribute to what is known as the replication crisis (1, 2). This chapter will first highlight our most important findings per aspect studied and then discuss the three main limitations of the studies conducted. Subsequently, we will present some of the general lessons and overarching concept learned, followed by concluding remarks.

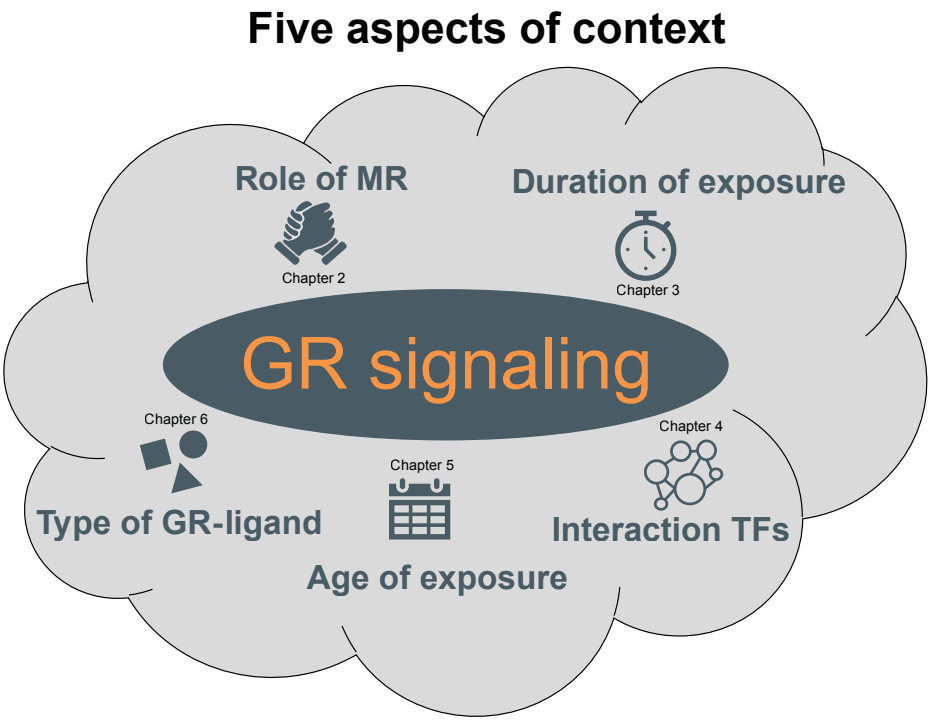

Figure 1: Visualization of the five aspects of context studied in this thesis with a reference to the associated chapter. The cloud represents the dynamic context that surrounds GR signaling and thereby influences the outcome. NB: In this thesis we specifically focused on early age and young adulthood. GR: glucocorticoid receptor, MR: mineralocorticoid receptor, TFs: transcription factors.

MAIN FINDINGS

The role of the MR

We previously found that exogenous administration of high (0.3mg/kg) and very high (3mg/kg) doses of corticosterone resulted in differential MR DNA-binding in the rat hippocampus in a dose-specific manner (3). In **chapter 2** we demonstrated that MR-specific DNA-binding is conserved between species (rat - mice). Using a forebrain MR knockout model (4), we subsequently confirmed that the MR affects the expression of a subset of the predicted MR-target genes. Forebrain-specific MR knockout reduced the basal hippocampal expression of *Jdp2*, *Nos1ap* and *Supv3l1*.

Of note, absence of the MR also reduced the expression of the canonical "GR-target" gene *Fkbp5*. This effect on *Fkbp5* is a striking example that the contribution of the MR is often overlooked. Not only is it evident that *Fkbp5* is an MR- (and AR) target gene (5, 6), but it also has consequences for GR signaling. The MR-induced FKBP5 protein limits GR functionality, because FKBP5 dampens the sensitivity of the GR to glucocorticoids (5). This finding is a good example of GR signaling being different in two contexts – cells that express the MR and cells that do not.

The MR did not only affect gene expression levels under basal, non-stressed conditions, but also regulated the transcription of one of its specific target genes in response to increased corticosterone levels. This was demonstrated using a restraint stress model, in which increased hippocampal expression of *Jdp2* was detected after the stressor-induced increase in endogenous corticosterone, although it should be noted that the experimental condition was also associated with different levels of other stress mediators.

Further studies are required to identify all MR-target genes and reveal the full extent of genomic MR signaling after increased corticosterone levels. Of note, genomic MR-responsiveness could be specific to certain cells, for instance those in which MR and GR are co-expressed, or to a hippocampal region, for instance the CA2 area in which MR is essential and GR virtually absent (7). The contextual dependence of the MR and GR goes in two directions. For instance, epidermal MR requires the GR and vice versa for a proper glucocorticoid response (8). Even though we so far lack the full picture, we can confidently conclude that we cannot simply ignore the transcriptional contribution of the MR in the context of stress-responses and GR function, in contrast to the existing notion of MR saturation at basal corticosterone levels (9).

The duration of glucocorticoid exposure

It intuitively makes sense that prolonged exposure to elevated corticosterone levels more extensively alters gene expression in comparison to acute exposure. In **chapter 3** we describe this in our RNA-seq data from mouse livers: chronically elevated glucocorticoid levels resulted in more than five times the number of differentially expressed genes (DEGs) in comparison to acute exposure (3109 vs. 543 respectively). Other differential transcriptome alterations between acute vs. chronic glucocorticoid exposure were previously described. For instance in the eye, which similarly revealed more extensive changes after chronic exposure (10).

Perhaps more surprising, duration also affected the identity ("targetness") of the altered genes: we observed genes with altered expression only after either acute or chronic exposure. The dependence of the transcriptional outcome on the duration of exposure is likely relevant for the notion that the glucocorticoid response after an acute stressor is beneficial, while chronic stress can lead to maladaptation, posing a risk factor for many diseases. The more extensive transcriptome alterations observed after chronic exposure might therefore hold a clue to what underlies this increased risk.

Of note, the observed differences might not be fully driven by chronically elevated hormone levels per se, and may be in part dependent on the loss of glucocorticoid rhythm as a result of the constant exposure (11). It would be of interest to investigate whether overall elevated – but still rhythmic – glucocorticoid exposure would lead to a similarly changed transcriptome. In addition, misalignment of glucocorticoid exposure with the circadian rhythm may be harmful (12). If a loss of a proper glucocorticoid rhythm is the true culprit, experimental study designs might need to be adjusted to better adhere to the natural rhythm of glucocorticoids. Mimicking the rhythm is also clinically advised, for instance for the glucocorticoid treatment regimen of (primary) adrenal insufficiency (13, 14).

Which genes are differentially regulated by corticosterone elevations may be related to changes in where the GR binds to chromatin. However, we discovered that at least a substantial part of the chronic, but not acute, hepatic transcriptome was dependent on active androgen signaling (**Chapter 3**; 15). Our data indicates that GR itself likely created the androgen-dependency by binding to the promotor region of the androgen receptor (AR). Given that the AR is also a transcription factor, this finding alone might explain regulation of an additional set of target genes. However, the *loss* of "targetness" is more difficult to explain. The upregulation of AR also hints towards an interaction between GR and AR. While we did not detect a direct protein-protein interaction between GR and AR, multiple alternative models of crosstalk could be

occurring (16). The biological relevance of this mechanism so far remains enigmatic. It raises the question whether similar sorts of adaptation occur in other organs after chronic glucocorticoid exposure. It also calls for evaluation of chronic corticosterone exposure in female animals.

Irrespective of what drives the androgen-dependency, it is so far unknown in which cell type(s) it developed in our study, as we used "bulk" RNA-seq analysis. Human single-cell transcriptome data (17) revealed that the GR and AR are only co-expressed in hepatocytes and their progenitors, providing a lead for follow-up studies in for example organoids (**Chapter 3**). However, receptor expression might differ in mice, and "basal" expression may not be predictive for the context of chronic corticosterone exposure, as there is the – remote – possibility of *de novo* AR expression in cell types that basally do not express the receptor. In this setting – as in many others – it would be informative to perform a dedicated single-cell RNA-seq experiment comparing acute vs. chronic treatment over time. Besides identifying the cell-type(s) that are differentially affected by duration of exposure, a more in-depth analysis could also provide insight into how the androgen-dependency comes about *in-vivo* over time (18).

As GR and AR are close family members, evidence for overlapping effects and/or functional interactions are not surprising *per se*. The DNA-binding domains of GR and AR are very similar, and – similar to what we reported for the GR and MR *in-vivo* (**Chapter 2**) (3) – they have overlapping as well as receptor-specific DNA-binding sites and target genes (19). For the GR and AR, the specificity in terms of target genes was found to be driven primarily by their respective dependence on accessible chromatin. Therefore, it would be of interest to assess the hepatic chromatin landscape, as chronic corticosterone exposure might increase accessibility at hepatic AR-target genes and pave the way for androgens and the AR to exert their effects. Whether or not cell-specific effects and chromatin accessibility fully account for the extensive difference between the chronic and acute effects and whether these indeed underlie adverse effects of long-term glucocorticoid treatment remains to be determined.

Altogether, our findings provide some insight how maladaptive effects of chronic glucocorticoid exposure might evolve, and these effects are likely not restricted to the liver. In addition, it is highly plausible that "self-created dependencies" are not unique to GR, but also occur for the other members of the steroid receptor family. The alterations that occur during chronic exposure likely happen in a step-by-step manner and assessment at multiple timepoints is required to understand how the androgen-dependence develops. In the bigger picture, these results emphasize the importance of carefully considering exposure duration when designing an experiment, but also act

as a reminder of the importance of experimental (contextual) details when comparing data from different studies, and adhering the label "target gene".

Interactions with other transcription factors

Next to studying interactions of GR and other steroid receptors, we also studied its putative relationship with pCREB. In our work on glucocorticoid-enhancement of memory consolidation we found no evidence of any interaction between GR and pCREB (**Chapter 4**). Our rationale to investigate a potential genomic interaction between GR and pCREB in the hippocampus was two-fold: 1) corticosterone enhancement of memory consolidation depends on the transcriptional capabilities of the GR (20) and 2) likely involves pCREB as downstream mediator of noradrenaline, which in turn is essential for this process (21, 22). We found that, even though memory consolidation was successfully enhanced, arousal induced by object location training did not lead to detectable changes in pCREB activation, and only minimally affected the GR cistrome and the resulting transcriptional changes after corticosterone. As discussed in **chapter 4**, the lack of a GR-pCREB interaction may be related to a dilution effect caused by investigating multiple cell types in "bulk" while only a small subset of these cells is actively involved in the interaction. Alternatively we simply tested an incorrect hypothesis. For example, direct interactions between GR and pCREB may only take place in the amygdala (and therefore not in the presently investigated hippocampal tissue), or noradrenalin and the GR simply affect different brain areas in the neuronal network.

It is probably safe to say that there are no genes that are exclusively regulated by one single transcription factor. In addition, this is well-established from studies investigating crosstalk between nuclear receptors, highlighting that interacting transcription factors play an important role in a multitude of processes (16). For GR signaling, this means that the GR can interact with – and perhaps depend on – other transcription factors to exert its effects. Depending on the context, the presence and activity of other transcription factors could thus prevent, aid or enable GR genomic signaling. Thus, there is good evidence for direct interactions between MR and GR, in part via hetero-, di- or multimerization, (23, 24). Yet, we found in our *in vivo* studies no evidence for physical interactions between GR and AR, and GR and pCREB (**Chapter 2-4**).

Our work was inspired by the power of describing DNA loci where several transcription factors can bind, continuing on the work of van Weert et al. (3). Our data emphasize that interaction between glucocorticoids and other signaling pathways can also occur further up- or downstream. This is for example the case for the interaction between GR and BDNF, in which BDNF alters the level of GR phosphorylation and thereby

GR's transcriptional activity (25). Next to the AR upregulation by GR described in liver (**Chapter 3**), a reciprocal interaction was observed in white adipose tissue where androgens induced expression of the enzyme that regenerates corticosterone from its inactive precursor (15). The induction of *Fkbp5* via MR is yet another example of crosstalk. Further studies at a cellular resolution are required to pinpoint whether the many interactions that involve GR occur within a particular cell type. If so, more targeted studies can be performed to reveal the actual level and mechanism of interaction.

The age at glucocorticoid exposure

The effects of glucocorticoids have been studied from embryonic development to old age. In particular, it has been consistently shown that exposure to stressors during early life is a risk factor associated to stress-related disorders (26). Glucocorticoids, as potent stress hormones that can act on the brain, are often implied as mediators of the long term "programming" effects of early life stress (ELS). However, the developing brain differs in comparison to a matured brain as it still displays a high degree of plasticity, *e.g.* neuronal networks are being constructed, strengthened and programmed (27). Likely therefore the brain is differentially susceptible to the maladaptive effects of glucocorticoids depending on age of exposure.

Our work in **chapter 5** showed that ELS can alter the hippocampal transcriptome, but that it did not strongly impact hippocampal chromatin accessibility. The hippocampal alterations we revealed in adulthood were in accordance with the concept of ELS- and age-dependent sensitivity to glucocorticoids, but we found these alterations to be inconsistent. We found convincing effects on the hippocampal transcriptome in one cohort, but we observed other or no alterations at all in different cohorts. Although the vastly different outcome might suggest otherwise, all animals endured ELS, as inferred from a bodyweight reduction at postnatal day 9. This transcriptional inconsistency is insightful and worrisome at the same time, as plenty of studies investigating ELS do not replicate each other's findings.

The lack of replicability may of course be caused by experimental variation – our own work included experiments that were performed in the Netherlands and Brazil. Yet, we also saw minimal overlap in gene expression that was assessed quite early after the stressor in two replication cohorts that followed the exact same protocol, in the same laboratory and by the same researcher – virtually eliminating all controllable sources of variation. The varying extent of the changes and therefore detectability thereof make them difficult to study. Again, this is certainly the case for bulk genome-wide approaches as other studies show cell-type specific ELS effects, for instance in microglia (28, 29). No matter how the inconsistency is interpreted, it so far remains

unclear why the hippocampal transcriptome is differentially affected after a seemingly identical experience.

Our interpretation – or operational definition – of this inconsistency is that transcriptional changes by ELS are stochastic. This would make the ELS-associated risk not only dependent on genetic background and later life circumstances, but also simply on chance processes during stress exposure.

Reversal of earlier stress effects by RU486 treatment weeks to months after the stressor was proven to be effective in multiple studies (30-37). We found no hippocampal transcriptome effects of ELS that could be reversed. The normalization at the behavioral level seems to be a real effect, but is likely accomplished in a different brain region or via a different mechanism, *e.g.* not "simply" normalizing previously altered expression levels of a set of ELS-target genes. RU486 intervention could occur via a more general adaptation instead of normalization, *e.g.* at the level of the HPA-axis (38) or even by affecting waste clearance from the brain (39). More comprehensive approaches are required to gain insight in the underlying mechanism.

Type of GR-ligand

It is very challenging to identify from a longlist of DEGs the true GR-target genes that are causal to any observed glucocorticoid effect. We conceptualized and applied a pharmacological filtering approach (**Chapter 6**) which enabled pinpointing likely direct GR-target genes using a combination of transcriptomes of multiple GR-ligands and their effect on a functional outcome measure. Auditory fear conditioning – a process that is in part GR driven – altered the hippocampal expression of over a thousand genes. Using our pharmacological filtering approach we reduced this longlist to a shortlist of 13 GR-target genes, a feasible number for further follow-up research. Usage of multiple types of GR-ligands – and especially the addition of selective glucocorticoid receptor modulators – increased the power of the approach and significantly contributed to the reduction of the longlist. Interestingly, our shortlist pointed to a possible role of microglia, and this echoes the data from **chapter 4** where we found that the induction of long-term memory consolidation in rats was paralleled by expression changes in non-neuronal cells – most likely microglia.

Our study, together with other published work (40-42), showed that selective glucocorticoid receptor modulators are a useful tool to better understand GR signaling. Moreover, our proof-of-concept study showed that the conceptualized approach of pharmacological filtering is effective and easily applicable in a vast majority of research projects. Of note, we found that pharmacological interventions (as used in **Chapters**

2-4 & 6) result in "tighter" mRNA expression data, in large contrast to the variable effects of more natural stressors such as ELS (**Chapter 5**). Besides transcriptome filtering, the approach could – in theory – also be applied to other genome-wide approaches after GR activation, from identifying functional GR DNA-binding sites with ChIP-seq to pinpointing protein interactions with GR-targeted proteomics. Lastly, the concept is not limited to the GR, and can be applied to various receptors for which multiple – sufficiently varying – ligands are available.

CONTEXT MATTERS

Throughout the thesis we investigated and discussed a large set of variables effecting GR signaling, including tissue (hippocampus and liver), dosage (0.3mg/kg and 3.0mg/kg of corticosterone), (in)direct interaction of GR with other transcription factors (AR, MR and pCREB), various behavioral paradigms (auditory fear conditioning and object location training) and in part the effect of age of exposure (perinatal period, adolescence and adulthood). Visualization of part of the data from three acute-treatment projects (**Chapter 3, 4 & 6**) highlights that the effects of GR signaling on the transcriptome are extensive, yet very diverse per experimental setting (and possibly partly due to stochastic processes) with rather limited overlap (only eight DEGs, **Figure 2**). Even though one might say context dependence is not extremely surprising, the results provide food for thought. "GR targetness" seems to hardly exists as an invariant characteristic. Yet, we use the concept, for example when looking for enrichment of target genes in new datasets (43). Decades of generic experiments on GR and MR function have helped us to understand basic mechanisms. However, we will simply have to ask very well-defined questions to advance our understanding of specific basic or translational aspects. Understanding GR signaling as an important part of the "stress-system" as a whole will therefore take more time and efforts.

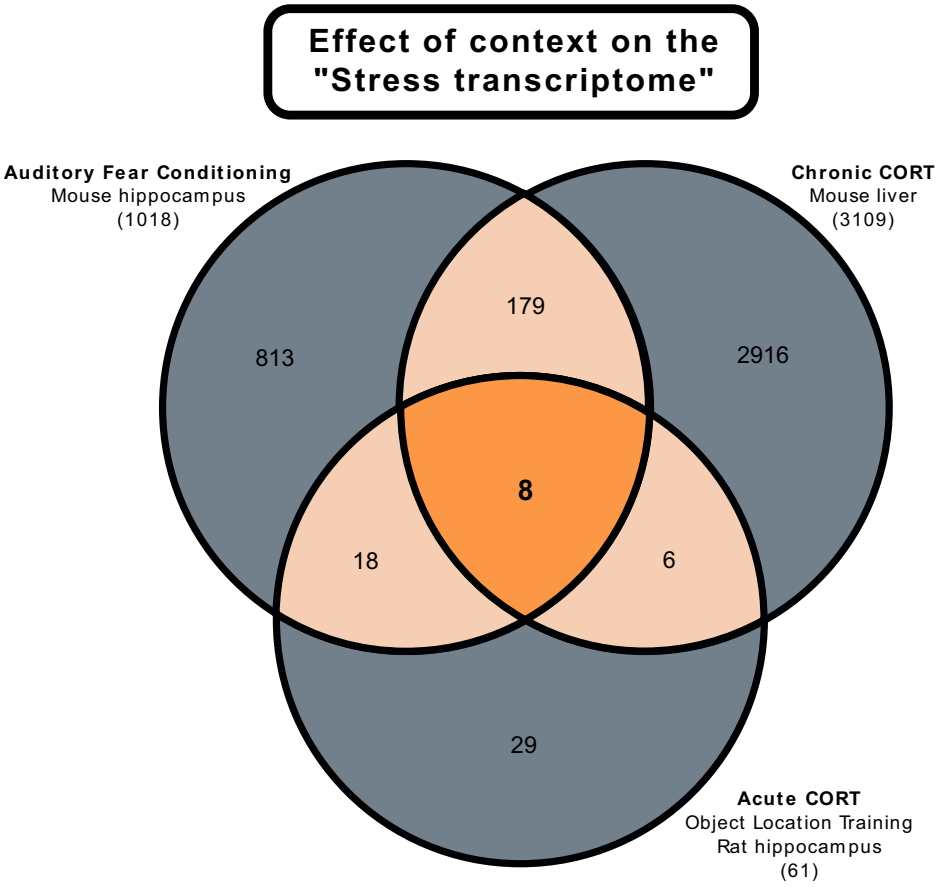

Figure 2: Venn diagram highlighting a limited overlap (eight DEGs) in significant transcriptome alterations after acute GR signaling – the "stress transcriptome" – in varying contexts: auditory fear conditioning (Chapter 6), chronic CORT (Chapter 3) and acute CORT (Chapter 4). CORT: corticosterone, DEGs: differentially expressed genes.

ACKNOWLEDGEMENT OF LIMITATIONS

We selected various questions on context in functional *in-vivo* experiments, and we made a number of important experimental decisions. Nevertheless, our studies were subject to a number limitations of which the three main ones are discussed.

Ignoring sex

First, we rather bluntly disregarded a major contextual factor in biology, as we did not include mice/rats of the female sex. Two of our studies (**Chapter 3 & 5**) follow-up on male-specific effects that were not observed in females (15, 34, 35), and we therefore exclusively included animals of the male sex. Our other studies did not have such a substantiated reason for not including females. For example, including both sexes was not required to proof the concept of pharmacological filtering (**Chapter 6**).

The induction of AR in the livers of male mice (**Chapter 3**) already suggests that sex will affect the outcome of GR activation. In addition, there are also many differences between the HPA-axis of males and females (44, 45). Brain and liver transcriptomes are programmed through sex steroids in early life (46, 47), and multiple studies have shown that the hippocampal transcriptome differs between sexes (48-52). Closer to the work in this thesis, we ourselves previously showed that glucocorticoids differentially affect male vs. female skeletal muscle (53). Of note, while long term functional outcome in the latter study differed between the sexes, the acute transcriptomic response to glucocorticoids was in fact similar. Sexual dimorphic responses exist, but are not default. Overall, while none of our studies set out to understand a specific sexually-dimorphic process or disease, we fully acknowledge it is a key limitation as it would have added valuable information to better understand *in-vivo* glucocorticoid biology.

Bulk tissue analyses

A recurring limitation is that we performed all unbiased genome-wide techniques (RNA-seq, ChIP-seq and ATAC-seq) on bulk tissue samples, *e.g.* whole (dorsal) hippocampus or liver. The heterogeneity of these tissues has been long known, and the extent in which this is the case became even more evident from published single-cell expression data (17, 54). In relation to **chapters 2, 4, 5 and 6**, detailed single-cell RNA-seq analysis of the hippocampus revealed extensive differences in cell-specific expression of GR, MR and of many factors (other transcription factors and co-regulators) that they do interact with to regulate transcription (55). The lack of single cell information complicates decisions on accurate follow-up. For example, should one focus on GR- and MR-expressing CA1 neurons, or rather on the dentate gyrus? In relation to memory, should we indeed direct attention to GR expressing microglia?

Available single-cell expression data can however be used to gain insights on cellular expression patterns. For instance, the use of dot plots may predict in which cell-type a particular alteration in gene expression occurred. Cell-type prediction for a downregulated gene is relatively straightforward as it can only occur in cells that already express the gene under basal conditions. Upregulation is less certain to deduce from basal expression pattern, but is often also more plausible in cells that already express the gene in comparison to *de novo* expression. In our work, available single-cell expression data highlighted a potential role of GR in non-neuronal cells for memory consolidation related processes (**Chapter 4 & 6**) and identified hepatocytes and their progenitors as likely candidates of GR-AR crosstalk (**Chapter 3**).

Bulk analysis not only complicates interpretation of data, but also comes with the risk of dilution. Altered gene expression in a (small) subset of cells can remain undetected in the bulk "summary" signal, *e.g.* the dilution effect, and therefore not be labeled as differentially expressed. This is more likely to occur for genes that are highly expressed in multiple cell-types, and less likely for those expressed in a cell-type specific manner. These limitations of bulk tissue analysis – which also apply to ChIP- and ATAC-seq – affected all our studies, and likely impacted the GR-pCREB interaction project (**Chapter 4**) and ELS accessibility study (**Chapter 5**) the most due to described cell-type diversity and expected small and varying effects respectively. Thus, in relation to memory formation, Arc reporter mice studies showed that only a small subset of hippocampal neurons in which pCREB-driven transcriptomic changes were identified is activated by fear conditioning (56). It is plausible that a potential genomic interaction between GR and pCREB would also occur specifically in these cells, and that we missed the pCREB differential DNA-binding and transcriptome changes due to our bulk tissue approach. Similarly, a dilution effect could have also contributed to the lack of significant effects after ELS on the hippocampal chromatin accessibility, although in that model the assumption that the hippocampus is the most affected brain region is weaker than in the case of memory formation.

While bulk analysis has some obvious limitations, its relative low cost enabled the application of genome-wide techniques on a more frequent basis. For instance, the comparison of multiple ELS cohorts using bulk RNA-seq (**Chapter 5**) revealed an inconsistent effect of ELS on the hippocampal transcriptome, which would not have been found had we performed a single-cell RNA-seq on merely one cohort.

The context of GR-action that is posed by the cell type is yet another layer of complexity. Whether we include it in our *in-vivo* experiments is not so much a matter of experimental design, but rather of resources, time and the research question. Overall, we need to

be aware of the pitfalls and opportunities of both bulk and single-cell approaches and aim to utilize the most suited option on a project to project basis.

Target gene identification

Throughout the experiments we described extensive transcriptome alterations after manipulation of GR signaling. At times, we could reduce longlists of GR-target genes to a shortlist, either by combining gene expression and DNA-binding data (**Chapter 4**) or pharmacological filtering (**Chapter 6**). Despite successfully obtaining manageable lists of putative GR-target genes in these projects, we did not follow-up and actually validate these genes at the protein level, nor as direct GR targets, nor did we prove any causality to memory consolidation.

Knockout of a specific target gene is a potential manner to establish whether or not the gene under investigation is involved in a certain process, but this does not inform on the importance of its "GR-targetness". In fact, a mutated GR DNA-binding sites in the regulatory region of a putative GR-target gene would be required to properly validate the importance of a GR target gene. Proof of principle for this approach was shown by using a naturally occurring genomic deletion near GR-target gene *Per2 (57)*. A Crispr-Cas9 based technology has been used for this purpose in cell lines, assessing the function of specific OCT4 DNA-binding sites (58). Similar technique should make such an approach more feasible for future *in-vivo* studies.

The approach of "GRE knockout" requires the identification of DNA-binding events to regulation of the associated target gene. Transcription factor binding within the promoter region can be associated with reasonable confidence to the nearest gene. In fact, arbitrary insertion of a GR DNA-binding site in the promoter region of a gene can make it responsive to glucocorticoids (59). Yet, GR often binds distant from its target gene, in intergenic regions as far as 100.000bp removed from the nearest gene. Interestingly, promoter-proximal GR DNA-binding is linked to GR-target genes conserved in multiple cell types, indicating intergenic GR DNA-binding loci are of importance for cell-type and thereby perhaps more process-specific effects (60). As GR shows little pioneering activity and predominantly binds pre-accessible chromatin (61), overlapping cell-type specific accessibility data with intergenic GR DNA-binding sites might be useful to determine in which cell types the binding events that are identified in bulk analysis occur. Cell-type specific chromatin looping data could subsequently be used to pinpoint the associated target gene in a more informed manner. While this approach works well in theory, it depends on the availability of datasets that match the project to a sufficient degree: the cellular trait and state contexts matter. This information might become more common in the future with the current speed of dataset generation and public availability

thereof. In general, improved approaches to link DNA-binding events to their associated gene(s) will strongly contribute to target gene identification and actual validation thereof.

While studying the involvement of a specific GR-target gene in a process is difficult, assessing the involvement of GR in a specific cell-type is a more attainable goal. Our results on the topic of glucocorticoid-enhanced memory consolidation indicated that GR in non-neuronal cells plays a potentially prominent role, especially GR in microglia (**Chapter 4** and **Chapter 6**). This finding is in line with a recent increase in interest in non-neuronal cells in stress and behavior (62-64). To investigate the role of microglial GR in memory consolidation we initiated auditory fear conditioning studies with a tamoxifen-inducible, microglia-specific GR knockout model (65). This approach will allow to assess the role of GR specifically in microglia, under the hypothesis that glucocorticoid-enhancement of memory consolidation will be impaired if microglial GR is missing. While not yet included in this thesis, the functional validation following our studies of more exploratory nature is underway and looks promising.

HOW WOULD OUR ANIMALS HAVE BEHAVED?

The design of the study influences its outcome. This adagium implies that every experimental design has its own pitfalls, and that failure to recognize these diminishes the value of the obtained results. This also means that one of the strengths of our approach – to vary context and study GR signaling *in-vivo* – simultaneously is one of the toughest challenges.

An intrinsic limitation lies in the fact that we used different individual animals for behavioral testing and molecular analysis. We were interested in comprehending the acute transcriptional effects of GR after glucocorticoid-enhancement of memory consolidation (**Chapter 6**). In case of auditory fear conditioning, the outcome of glucocorticoid administration (did the treatment enhance freezing in response to the tone, or not?) can only be determined 24 hours later, at retrieval testing. Because transcriptional changes by definition are initiated before the behavior can manifest itself, and because transcriptional changes tend to be transient, it is necessary to first investigate the molecular mechanism and then behavior. However, the current methods to probe the molecular pathways involved cannot be carried out *in-vivo*, ruling out subsequent behavioral testing. Therefore, it is simply not possible to assess the relevant transcriptional and behavioral effects in the same animal. This precludes correlating behavior directly to the "molecular" outcome per animal, and use the inherent biological variation to separate behavioral "responders" versus "non-responders" as an additional filter for the identification of relevant target genes.

A similar challenge presented itself in studying the long-lasting molecular changes after ELS that might render an animal susceptible to later life stress-related disorders in the first place (**Chapter 5**). To determine the state that defines "responsiveness", the molecular analyses need to be performed in an unstressed and unchallenged cohort. First assessing the behavior might be a "second hit", altering the state of the animal under investigation. The limited bedding and nesting model for ELS is – by now – a notoriously variable and sensitive model, and we may in fact have analyzed the brains of "non-responders" alongside that of "responders". Evidently, background, exposure, behavior, molecular alterations etc. are all connected, and affecting one is likely to (in) directly affect all the other factors. Therefore, results obtained are more often than desired only applicable to the context in which they were gathered, and we cannot automatically extrapolate them to vastly different situations.

For lack of direct comparison between gene expression and the functional/behavioral readout, future studies may need to use larger group sizes when studying variable effects. In addition, alternative statistical approaches might prove to be better equipped to handle variation, *a.o.* by using prior knowledge to increase the power (66).

CONCLUDING REMARKS

An overarching aim of our research was to identify GR/MR-target genes (**Chapter 2**) that are related to a specific process, *e.g* the hepatic glucocorticoid response (**Chapter 3**), memory consolidation (**Chapter 4,6**) or long-lasting early life stress effects (**Chapter 5**). This provides an improved understanding of GR-dependent processes in particular contexts and possibly aid treatment of GR-related diseases in the future.

While focused on condensing longlists to shortlists, it is becoming more evident that single (target) gene orchestrated processes are rare at the organism level – although in some cases they may occur. In order to truly comprehend a complex process – such as memory consolidation – we will need to shift towards more integrative approaches that combine data from multiple levels. This may actually require fewer and fewer actual experiments, as the amount of publicly available genome-wide data is rapidly growing. Transcription factor DNA-binding sites are available for many tissues, and so are accessibility profiles of many cell types and the transcriptome, proteome and interactome of many diseases / interventions. This certainly will help formulating hypotheses that are more unbiased then in "old school" research (for better or worse).

However, we need to learn how to gather, combine and interpret such vast amounts of data: a new competence all future scientist will need to develop. The other point is

more fundamental than learning how to write code and applying artificial intelligence approaches: the findings discussed in this thesis underline that the (*in-vivo*) context in which all this data is gathered is crucial for what one will observe, in which – most likely – a role of chance cannot even be excluded. And so, while combining massive amounts of available data holds tremendous potential, the conditions under which such data was obtained remains essential. Thus, final evidence for a role of molecules in any given process will still need solid, well-planned and executed experiments that approach real-life conditions as much as possible.

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