Contextual glucocorticoid signaling in-vivo: a molecular perspective

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Chapter 6:

Application of a pharmacological filter identifies a shortlist of mouse glucocorticoid receptor target genes associated with memory consolidation.

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ABSTRACT

Glucocorticoids regulate memory consolidation, facilitating long-term storage of relevant information to adequately respond to future stressors in similar conditions. This effect of glucocorticoids is well-established and is observed in multiple types of behaviour that depend on various brain regions. By and large, higher glucocorticoid levels strengthen event-related memory, while inhibition of glucocorticoid signalling impairs consolidation. The mechanism underlying this glucocorticoid effect remains unclear, but it likely involves the transcriptional effects of the glucocorticoid receptor (GR). We here used a powerful paradigm to investigate the transcriptional effects of GR in the dorsal hippocampus of mice after training in an auditory fear conditioning task, aiming to identify a shortlist of GR target genes associated to memory consolidation. Therefore, we utilized the properties of selective GR modulators (CORT108297 and CORT118335), alongside the endogenous agonist corticosterone and the classical (non-selective GR) antagonist RU486, to pinpoint GR-dependent transcriptional changes. First, we confirmed that glucocorticoids can modulate memory strength via GR activation. Subsequently, by assessing the specific effects of the available GR-ligands on memory strength, we established a pharmacological filter which we imposed on the hippocampal transcriptome data. This identified a manageable shortlist of nine genes by which glucocorticoids may modulate memory consolidation, warranting in-depth follow-up. Overall, we showcase the strength of the concept of pharmacological filtering, which can be readily applied to other research topics with an established role of glucocorticoids.

INTRODUCTION

Stressors result in increased glucocorticoid secretion from the adrenal glands. Glucocorticoids in turn can bind to two receptor types which act as transcription factors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (1). Via these receptors glucocorticoids affect a multitude of processes throughout the body to cope with the stressor, such as redirecting energy supplies, restoring homeostasis in the aftermath of the stress and memory formation (2). By the latter, glucocorticoids enable long-term consolidation of relevant information so that an individual can adequately respond when exposed to similar conditions in the future (3, 4). This role of glucocorticoids on memory pertains to a multitude of different behaviours that each involve specific brain regions (5). While the modulation of memory consolidation by glucocorticoids is well-established, the underlying molecular mechanism has remained largely elusive, except for a clear involvement of GRs.

For instance, pharmacological inhibition of glucocorticoid signalling by the GR antagonist RU486 prevents long-term memory consolidation, whereas it has been reported that immediate conditioning was not impaired (6, 7). This indicates that the slower transcriptional effects of GR are involved, which is in line with the memory-impairments observed in GR dimerization mutant mice that have impaired GR transcriptional activity (8). GR's transcriptional effects depend on the coregulators in the transcription complex, of which the recruitment in turn depends on the ligand bound to GR (9). Therefore, the outcome of GR's transcriptional activity can be altered by selective glucocorticoid receptor modulators (SGRMs), which combine agonistic and antagonistic properties in a tissue- and cell-type specific manner (10). The compounds CORT108297 and CORT118335 were designed as selective GR antagonists, but both compounds were subsequently proven to act as SGRMs in both brain and liver based on their combined agonistic and antagonistic effects (11-15). These SGRMs can be used as tools to further dissect the effects of glucocorticoids, both at the behavioural and the molecular level. For modulation of memory consolidation, CORT108297 was found to resemble corticosterone (without displaying any affinity for MR as corticosterone in rodents does) – promoting consolidation (15), while CORT118335 impaired memory consolidation (14). The characteristics of CORT118335 are of special interest as this compound can antagonise both GR and MR, while RU486 does not affect MR signalling but has well established progesterone receptor affinity (16-18). The combination of these compounds therefore enables distinction between GR's and MR's involvement in the behavioural outcome and allows further dissection at a transcriptional level.

We set out to utilise the properties of these SGRMs, alongside the endogenous agonist corticosterone and the classical GR antagonist RU486, to reduce the often obtained longlist of GR-target genes that is intrinsic to transcriptomic approaches, and thereby further dissect the role of glucocorticoids in memory consolidation. To this end, we used auditory fear conditioning (AFC), a behavioural paradigm susceptible to glucocorticoid modulation and involving – among several areas – the hippocampus (19, 20), to assess the effects these GR-ligands have on memory strength and on the hippocampal transcriptome. Based on the behavioural outcomes we established pharmacological filters which we imposed on the transcriptome data to identify a shortlist of GR-target genes implicated in the modulation of memory consolidation by glucocorticoids.

MATERIAL AND METHODS

Animals

All animal studies were approved by the ethical committee of the University of Utrecht and the CCD (nr. AVD115002016644) and were in accordance with EU Directive 2010/63/EU for animal experiments. Adult (eight weeks old) male C57Bl6 mice were obtained from Envigo (the Netherlands) and left undisturbed for 1 week with *ad libitum* food and normal day/light cycle. Males were used based on the well-established effects of corticosterone on memory consolidation in literature (14, 15, 20, 21). All animals were individually housed seven days before fear conditioning in the morning. One cohort of animals was used for behavioural analyses (n = 10 per group) and another cohort for molecular analyses (n = 4-5 per group). Animals were killed by decapitation 1.5 or 3 hours after the training for molecular analysis.. Animals in the basal control group were taken directly from their cage and killed without any training or treatment.

Auditory Fear Conditioning

Each mouse was individually placed into a chamber (30 cm x 24 cm x 26 cm) without stripes on the walls (context A). The floor consisted of a metal grid for foot shock application. During the first 3 minutes, mice were allowed to explore the chamber freely, then three tones (2.8 kHz, 78 dB) were played for 30 seconds and during the last 2 seconds of each tone a foot shock (0.2 mA) was applied (20). Thirty seconds after the last shock, mice were removed from the chamber, treatment was administered and the mice were placed back in their home cage. After each trial the box was cleaned with acetic acid (1%).

Retrieval

Twenty-four hours after training memory retrieval of animals in the behavioural cohort was tested in context B (a 30 cm x 24 cm x 26 cm chamber with vertical stripes on the

walls, except for the room facing wall which was made of transparent Plexiglas). Testing trials lasted 11.5 min: In the first 3 min, mice were again allowed to freely explore the chamber. Then, the tone was played 6 times for 30 seconds with intervals of 1 minute in between. After the last tone there was a 30 second interval after which the mice were returned to their home cages (22). After each trial the box was cleaned with ethanol (70%). The experiment was recorded and freezing behaviour was later manually scored through Observer Software (Noldus, the Netherlands).

Treatment

Immediately after the training session mice were injected subcutaneously with either 3 mg/kg corticosterone, 20 mg/kg CORT108297 (C108), 80 mg/kg CORT118335 (C118), 40 mg/kg RU486 or vehicle (100% DMSO) and placed back into their cage. The doses applied were based on the studies that previously characterised the effects of the CORT108297 and CORT118335 on behaviour (14, 15), and investigated 3.0mg/kg corticosterone in AFC (23). CORT108297 (20 mg/kg) was shown to reach the brain and result in occupancy of brain GRs comparable to 3 mg/kg corticosterone (15). This was not shown in such a direct manner for CORT118335, yet multiple central effects have been described, including antagonism of exogenously administered corticosterone at present dose (14, 24).

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Corticosterone measurements

Corticosterone levels were measured in separate cohorts 1.5 and 3 hours after AFC in trunk blood using a high sensitivity EIA kit (AC-15F1, Immunodiagnostic Systems: limit of detection is 0.17 ng/ml).

RNA sequencing

For RNA-sequencing (RNAseq) total RNA was isolated from dorsal hippocampal tissue 3 hours after AFC, the timepoint at which glucocorticoids most extensively affect the hippocampal transcriptome even though plasma levels are already normalized (25). Snap-frozen tissue was homogenized using a tissue homogenizer in lysis buffer of the NucleoSpin RNA kit (Macherey-Nagel). Total RNA was isolated according to the manufacturer's protocol and samples were sent for transcriptome sequencing at BGI Genomics. RNA quality of all samples was assessed using the RNA 6000 Nano kit on a Bioanalyzer (Agilent) and all samples passed the quality criteria for sequencing (RNA Integrity Number >7.0 and 28/18s ratio > 1.0). Stranded mRNA libraries were constructed and 100 bp paired-end sequencing was performed on the DNBseq platform resulting in >20 million reads per samples. RNAseq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE202236.

RNAseq analysis

The Gentrap pipeline, published as part of Bio Pipeline Execution Toolkit (Biopet, https://biopet-docs.readthedocs.io), was used for read quality control, alignment and quantification. Quality control was performed using FastQC and MultiQC. Reads were aligned to mm10 using GSnap aligner (version 2017-09-11). Gene-read quantification was performed using HTSeq-count (version 0.6.1) based on Ensembl release 88 of mm10. HTSeq-count output files were merged into a count matrix as input for differential gene expression analysis.

DEseq2 (version 1.29.4) was used for normalization of the data (median of ratio's method) and identification of differentially expressed genes (26). For the differential expression analysis, we selected all genes which were expressed in a minimum of four replicates with >20 normalized counts, resulting in 15.193 genes in the analysis. One sample was identified as an outlier (sample 10 of the vehicle group) and removed from further analysis. Groups were analysed in pair-wise comparisons and a FDR adjusted p-value of 0.05 was used as a cut-off to determine differentially expressed genes unless stated differently (**Sup. Table 1**).

Gene ontology enrichment analysis was performed on all genes differentially expressed after AFC with the ViSEAGO package (version 1.4.0), using fisher's exact test with 0.01 as a significance cut-off (27). Cytoscape (version 3.9.1) generated a connectivity-network based on reported protein-protein interaction (28). Proteins encoded by all AFC-genes (adjusted p-value < 0.05) and of the genes most robustly differentially expressed after AFC (adjusted p-value < 0.01 and a log2 fold change of at least 0.5 were analysed. Densely connected sub-networks were subsequently detected using the MCODE algorithm (29) and the associated biological processes were determined using Cytoscape's functional enrichment analysis.

GR-binding analysis

Publicly available chromatin immunoprecipitation data of GR was utilized to determine to which extent GR contributed to the differentially expressed genes after AFC. Data of three separate studies were downloaded, investigating GR DNA-binding in the rat hippocampus after corticosterone with or without adrenalectomy and after forced swim stress (21, 30, 31). All genes associated to GR DNA-binding in at least one of the datasets were extracted and the percentage of AFC-genes associated to *in-vivo* GR DNA-binding was calculated.

qPCR validations

cDNA was synthesized from 1.000 ng of RNA using random hexamers and M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol. Real-time quantitative PCR (RT-qCPR) was performed using GoTaq® qPCR Master Mix (Promega) with a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). mRNA expression levels were normalized to housekeeping gene Rplp0 using the $2^{-\Delta\Delta CT}$ method. Used primer sequences were included as a supplementary table (**Sup. Table 2**).

Single-cell expression data

Single-cell mouse hippocampal expression data of the Allen Institute for Brain Science (32) was used to visualize the genes identified by the pharmacological transcriptome filter by dotplot using Seurat's visualization tools with standard settings (version 3.1.5) as previously published (33). These public data indicate the gene expression levels under basal conditions as the mice were not exposed to any behavioural training or treatment.

Statistics

One-way ANOVAs were used for the analyses of plasma corticosterone levels Dunnet's multiple comparison tests were used as post-hoc. Freezing data were analysed by an ANOVA with treatment * tone as factors and predefined contrasts: corticosterone vs. Vehicle, CORT108297 vs. corticosterone, RU486 vs. Vehicle and CORT118335 vs. RU486. Analysis was subsequently repeated for all contrasts to expand the pharmacological transcriptome filter (6 treatment levels, and six consecutive tones). Gene expression data were analysed using one-way ANOVA following Šidák multiple comparison tests with predefined contrasts: CORT10927 vs. Veh, CORT118335 vs. CORT and RU486 vs. CORT. Statistical analyses of corticosterone levels and gene expression data were performed with GraphPad Prism 7 software (GraphPad Inc.) and freezing data were analysed in R (version 4.0.0).

RESULTS

The experimental design for the pharmacological transcriptome filtering approach

Upon secretion endogenous glucocorticoids reach virtually all organs of which the vast majority expresses either GR, MR or both. The resulting transcriptional effects are extensive, often identifying a longlist of hundreds or thousands of (in)direct target genes, which hampers the identification of genes that are directly relevant for the studied biological process. We here introduce the concept of pharmacological transcriptome filtering using SGRMs to obtain a short- instead of a longlist and illustrate the feasibility of the approach in male mice after AFC (**Fig. 1**). The essence of this approach is to use

several ligands for one receptor type that all have different molecular effects on gene expression. Only those genes that consistently correlate with the behavioural effects are then considered as potentially causal.

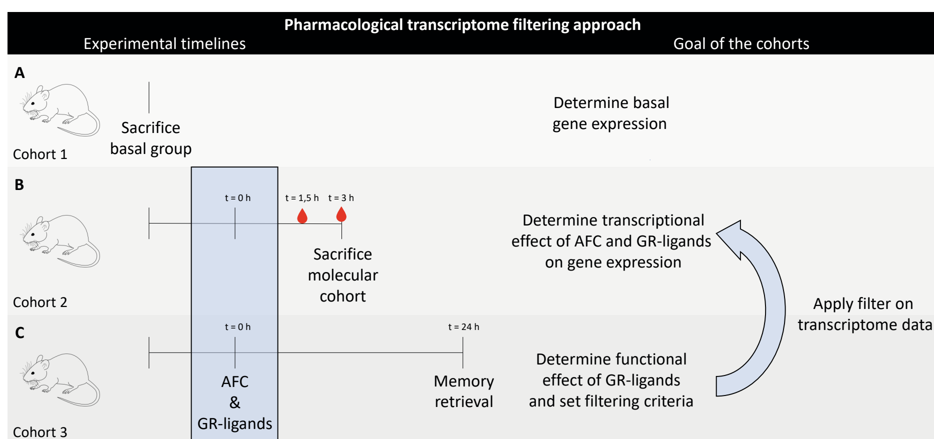

Figure 1. Experimental design of pharmacological filtering approach.
Three separate cohorts were used to apply the pharmacological transcriptome filter based on Auditory Fear Conditioning (AFC) on the dorsal hippocampal transcriptome. A) Cohort 1: Untrained and untreated cohort to determine basal hippocampal gene expression. B) Cohort 2: AFC trained animals with post-training injection with GR-ligands to determine the subsequent transcriptional effects after 3 hours. Corticosterone levels were determined at 1.5 and 3 hours after AFC and treatment. C) Cohort 3: AFC trained animals with post-training injection with GR-ligands to determine behavioural effects by memory retrieval after 24 hours.

In the current study we used three separate cohorts. An untrained and untreated group of animals was used to determine baseline hippocampal gene expression (Cohort 1, **Fig. 1A**). A separate molecular cohort of animals was exposed to AFC and subsequent treatment with a specific set of GR-ligands to determine GR's molecular effects after 3 hour (Cohort 2, **Fig. 1B**). Lastly, a behavioural cohort was ran in parallel to the molecular cohort to determine GR's role in AFC memory consolidation (Cohort 3, **Fig. 1C**). Based on the behavioural outcome of cohort 3 we selected suitable contrasts to impose on the transcriptome data obtained from cohort 2, enabling the identification of genes which expression pattern correspond to the functional outcome studied.

Glucocorticoid receptor activity regulates fear memory consolidation

Mice were subjected to AFC training. Corticosterone levels were significantly higher 1.5 hours after AFC in the corticosterone and RU486 groups compared to vehicle group ($F(4,20) = 33.08$; p-value < 0.0001, **Fig. 2A**). Corticosterone levels after treatment with SGRMs CORT108297 and CORT118335 did not differ, indicating that HPA-axis feedback was not affected by these compounds. No differences in corticosterone levels were

observed 3 hours after AFC (F(4,20) = 1.763; p-value = 0.1760, **Fig. 2B**). Memory retrieval was determined 24 hours after AFC by assessing freezing to six subsequent tones in another context. Initial freezing to the first tone and the level of freezing to subsequent tones differed per group (**Fig. 2C**). The average freezing percentage of all tones over time was used as a summary measure of memory strength, which was significantly affected by post-AFC treatment (F(29,270) = 7.025; p-value < 0.0001, **Fig. 2D**). Corticosterone administration resulted in overall higher freezing, while treatment with the classic GR antagonist RU486 and the SGRM CORT118335 significantly reduced overall freezing to similar extent. The latter points to impaired memory consolidation compared to the vehicle control group, where secretion of endogenous corticosterone forms an intrinsic part of the acquisition (and consolidation) phase. Average freezing levels of mice treated with the SGRM CORT108297 were significantly lower compared to corticosterone treated animals yet comparable to the vehicle group, indicating that it did not affect the consolidation of this type of memory. Altogether, application of the pharmacological filter on the behavioural data confirmed GR as regulator of memory consolidation and identified contrasts to be applied in subsequent steps of the transcriptome analysis, with corticosterone as an agonist, CORT118335 and RU486 as antagonists and CORT108297 as an inactive compound in this paradigm.

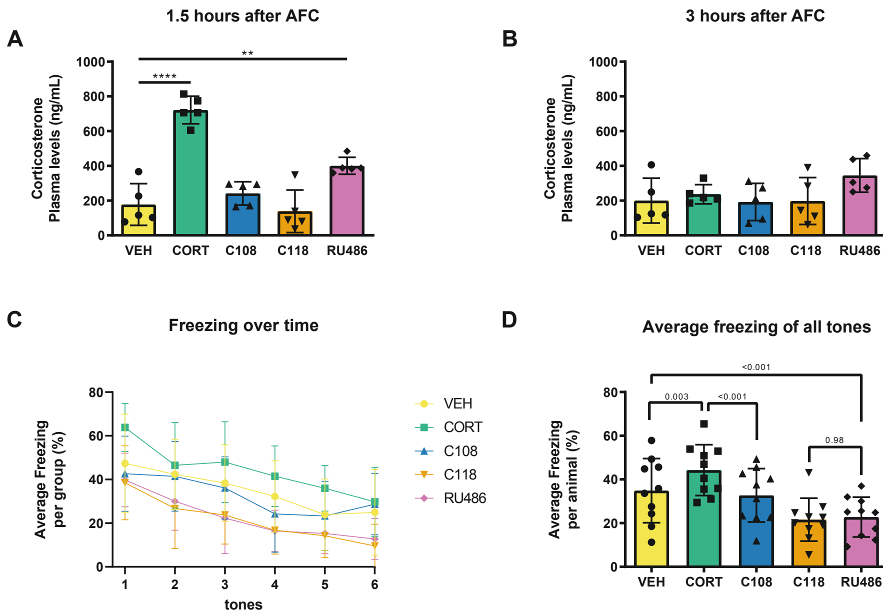

Figure 2. Pharmacological modulation of GR activity affects memory consolidation.
Plasma corticosterone levels 1.5 hours (A) and 3 hours (B) after Auditory Fear Conditioning (AFC) (n = 5 per group). C) Average freezing over time per group and D) average freezing of all six tones per animals at memory retrieval testing 24 hours after AFC (n = 10 per group). VEH: vehicle, CORT: corticosterone, C108: CORT108297, C118: CORT118335.

Auditory fear conditioning strongly affects the hippocampal transcriptome

AFC affected the hippocampal transcriptome 3 hours after training as evident from the separation between conditioned vehicle and basal control (untrained and untreated) animals in the principal component analysis (PCA, **Fig. 3A**). Differential gene expression analysis revealed the effect was extensive and identified 1.018 differentially expressed genes (DEGs) (455 up- and 563 down-regulated, **Fig. 3B**). Gene ontology analysis of these AFC-genes identified 110 enriched biological processes, 17 molecular functions and 16 cellular components (**Sup. Table 3**). Top terms included synapse-related processes (**Fig. 3C**) and transcription-related functions (**Fig. 3D**). Network analysis of all AFC-genes resulted in a large network with 27 densely connected subnetworks, of which the top three were related to kinase signalling, regulation of proliferation and glycosaminoglycans (**Fig. 3E and Sup. Table 4**). Analysis of the most robustly regulated genes (adjusted p-value < 0.01 and log2 fold change > 0.5) identified ten densely connected subnetworks with only the top hit indicating a specific process: neurogenesis (**Fig. 3F** and **Sup. Table 4**). The contribution of endogenous glucocorticoids to the effect of AFC was evident as the top six DEGs included five established glucocorticoid responsive genes (*Dpf1*, *Fkbp5*, *Hif3a*, *Plin4* and *Tsc22d3*, **Fig. 3G**). Of all genes differentially expressed after AFC, 32.7% was associated to *in-vivo* GR DNA-binding based on three separate studies, once more confirming the role of hippocampal GR in AFC (**Fig. 3H**) (21, 30, 31).

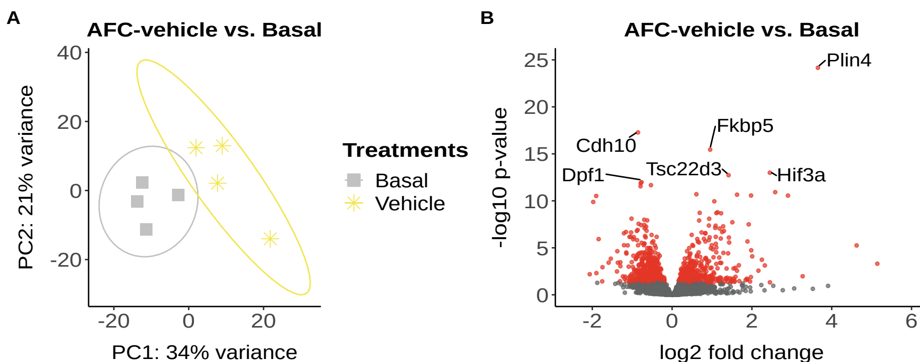

Figure 3. Glucocorticoids drive the hippocampal transcriptome changes after auditory fear conditioning. A) Principal component analysis plot visualising separation between vehicle-injected auditory fear conditioned (AFC) and basal control (untrained and untreated) animals based on the hippocampal transcriptome determined 3 hours after training (n = 4 per group). B) Volcano plot visualising differentially expressed genes (DEGs) between vehicle-injected AFC and basal animals. Red dots represent DEGs (AFC-genes) at adjusted p-value 0.05, the six most significant genes are labelled. Top 10 of the GO term enrichment analyses on all AFC-genes for biological processes (C) and molecular functions (D). Top 3 dense networks revealed by the MCODE algorithm based on all AFC-genes (E) and AFC-genes with an adjusted p-value < 0.01 and log2 fold change > 0.5 (F). G) Normalized RNAseq count plots of the top six significantly DEGs (n = 4 per group). H) Venn-diagram visualizing overlap between all AFC-genes and genes with associated proximal GR DNA-binding in the hippocampus based on three separate studies (Polman et al., Mifsud et al. and Buurstede et al.).

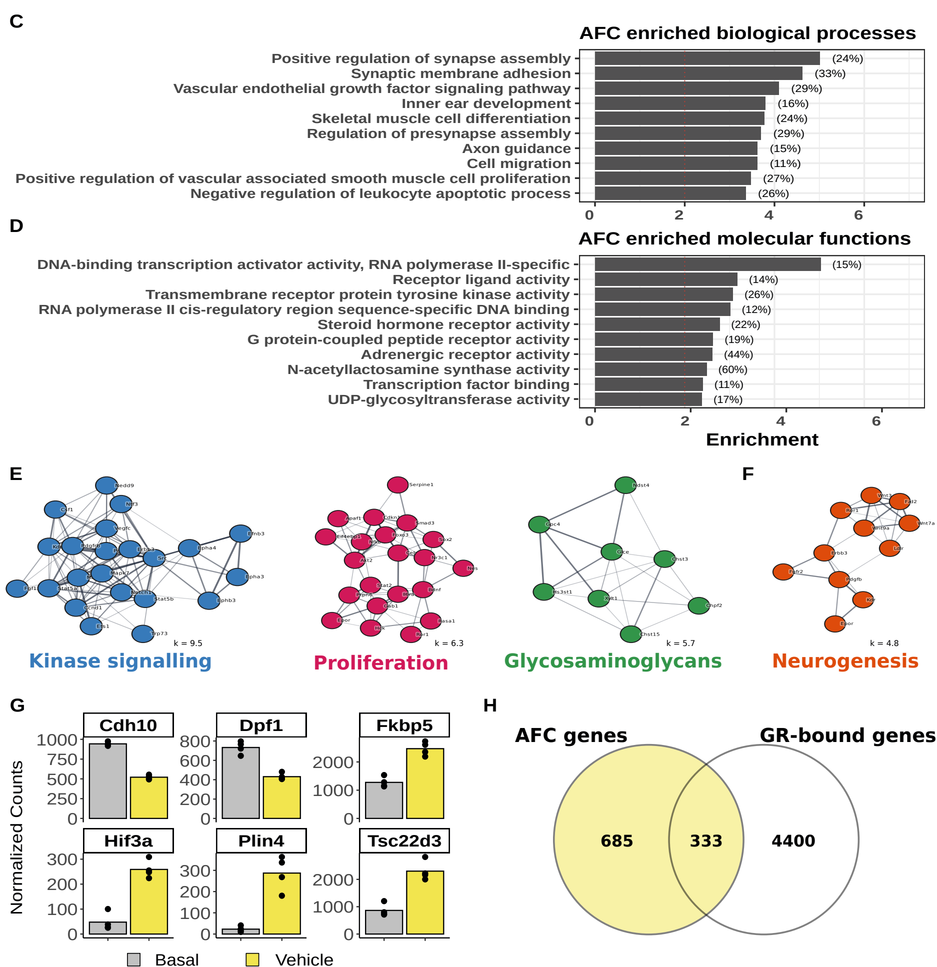

Figure 3. Continued.

Differential modulation of the hippocampal transcriptome by GR-ligands

Next, we assessed how modulation of GR activity by each GR-ligand affected the hippocampal transcriptome three hours after AFC training in comparison to AFC vehicle-injected animals, where elevation of corticosterone forms an intrinsic part of the test situation. Administration of exogenous corticosterone resulted in 84 DEGs (47 up- and 37 down-regulated, **Fig. 4A**). The SGRM CORT108297 most strongly affected the hippocampal transcriptome with 298 DEGs (154 up- and 144 down-regulated, **Fig. 4B**). Antagonist RU486 significantly affected the expression of 45 genes (5 up- and 40 down-regulated relative to AFC-vehicle, **Fig. 4C**) and the SGRM CORT118335 resulted in 46 DEGs (3 up – and 43 down-regulated, **Fig. 4D**). In previous characterizations of

the SGRMs in relation to memory consolidation, CORT108297 predominantly acted as an agonist while CORT118335 acted more as a GR antagonist (14, 15). Therefore, both SGRMs were also directly compared to corticosterone and RU486 respectively. Despite the difference in total DEGs between CORT108297 and corticosterone compared to vehicle, only 4 genes were significantly different between these compounds (**Fig. 4E**). Direct comparison of RU486 and CORT118335 did not reveal any DEGs (**Fig. 4F**), indicating the effects of these compounds on hippocampal genes expression were highly comparable. Overall, the effects of pharmacological modulation of GR activity after AFC were subtle, as was also evident from the lack of separation between the different GR-ligand-treated groups in the PCA plot (**Fig. 4G**).

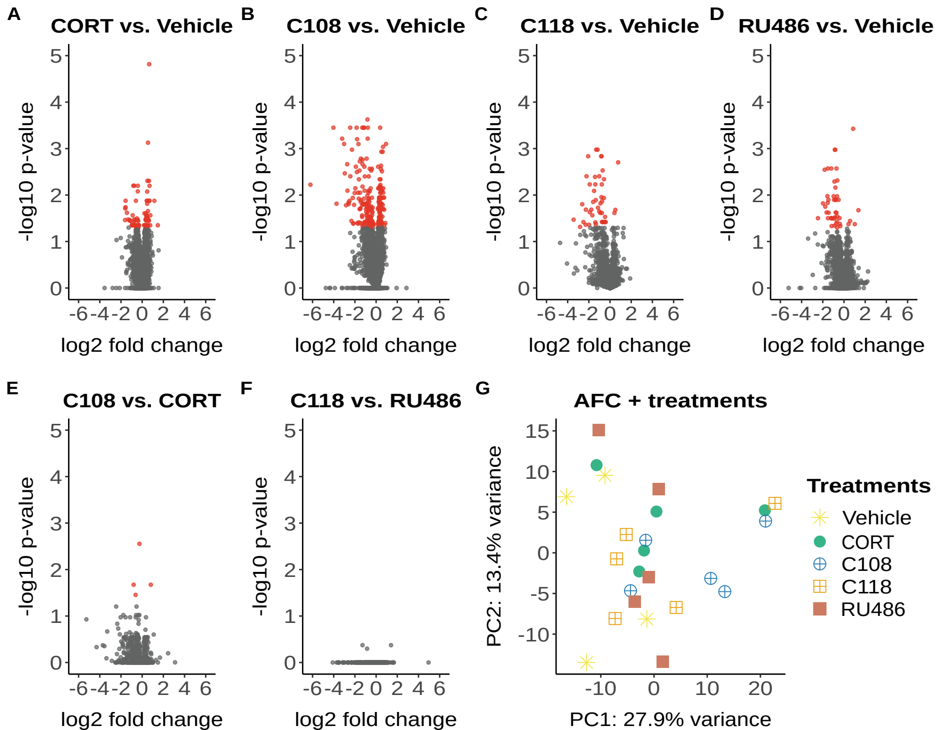

Figure 4. Pharmacological modulation of GR activity subtly affects the hippocampal transcriptome after auditory fear conditioning.

Volcano plots visualising differentially expressed genes (DEGs) of corticosterone (A), CORT108297 (B), CORT118335 (C) and RU486 (D) compared to vehicle-injected auditory fear conditioned (AFC) animals and between CORT108297 vs. corticosterone (E) and CORT118335 vs. RU486 (F). G) Principal component analysis plot visualising the effect of treatment after AFC on the hippocampal transcriptome (n = 4-5 per group). CORT: corticosterone, C108: CORT108297, C118: CORT118335.

Pharmacological transcriptome filter identifies putative GR-mediated memory associated genes

Our following step was to pinpoint which of the changes in the transcriptome ran in parallel with the regulatory effects GR exerted on memory consolidation. As corticosterone enhanced memory retrieval and both RU486 and CORT118335 impaired it, we first filtered on genes differentially expressed after additional corticosterone relative to vehicle and differentially expressed in the opposite direction by both RU486 and CORT118335. However, no transcripts met these criteria. Given the clear effects on memory retrieval, we decided to impose a bigger contrast on the transcriptome data by directly comparing the memory enhancing and impairing compounds. In order to again link this to our behavioural outcome we extended the analysis of our behavioural data to include the appropriate contrasts (**Sup. Fig. 1**). As RU486 and CORT118335 both impaired memory to similar extent, we filtered on genes differentially expressed between corticosterone in comparison to RU486 as well as CORT118335. Application of these criteria resulted in 15 genes (adjusted p-value < 0.1). To further reduce the shortlist of genes, we imposed an additional behaviour-based filter. While the freezing response after CORT108297 did not differ from vehicle-treatment, its effects on the transcriptome was substantial. Therefore, additional filtering was performed under the criterion that there was no differential expression between CORT108297 and vehicle. This further excluded two genes as potentially mediating the modulation of fear conditioning via GR (*Dbp* and *Ptgfrn*). Of this shortlist, eight were validated with a significant main effect by qPCR between the relevant groups and met the filtering criteria (**Fig. 5A**). For *Irak2* and *Nrros* we found a trend between CORT118335 and corticosterone based on the qPCR validation. These genes were kept on the shortlist as overall the data implicated their involvement. Of the other five genes, Dzip1l and Cdkn1a did show a main effect, but also a trend level / significant difference between CORT108297 and vehicle. The main effect of post-AFC treatment could not be validated for Lrig1, Tiam2 and Bcl2l1 (**Fig. 5B**). The pharmacological transcriptome filter thus identified a shortlist of eight genes that closely follow the behavioural effects of corticosterone and the two functional antagonists. Five of these genes (*Fkbp5*, *Irak2*, *Lao1*, *Mthfd2* and *Pnpla2*) are likely directly regulated by GR, based on an association to proximal GR DNA-binding in the hippocampus (**Fig. 3H**).

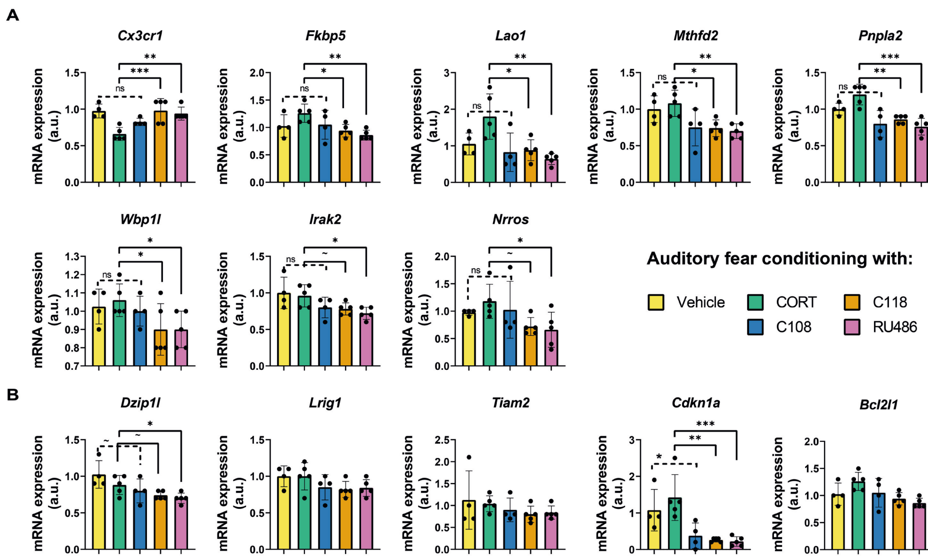

Figure 5. Application of the pharmacological transcriptome filter identifies a shortlist of genes of which the expression parallels the behavioural outcome.

qPCR analysis on vehicle, corticosterone, CORT108297, CORT118335 and RU486 groups for genes identified by applying the pharmacological transcriptome filter on the hippocampal transcriptome data (n = 4-5 per group). A) Shortlist genes validated by qPCR with a significant main effect of treatment and adhering to the pharmacological transcriptome filter. B) Genes not validated and therefore removed from the shortlist.* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001. CORT: corticosterone, C108: CORT108297 and C118: CORT118335.

Altogether, application of the pharmacological transcriptome filter resulted in eight genes for which a connection to GR-mediated fear memory was implicated based on the association of expression changes in the dorsal hippocampus in accordance with the behavioural effects.

Potential role of non-neuronal cells in GR-modulation of memory consolidation

The dorsal hippocampus is composed of various cell types and the assessed transcriptome provided an overview of all individual and potentially cell-specific effects combined. Publicly available single-cell data of the mouse hippocampal transcriptome under basal conditions (32) showed that expression of some genes on our shortlist was relatively comparable across cell types (*Lao1* and *Wbp1l*), while other genes showed a more exclusive pattern. This survey revealed that the majority of the genes identified by application of the pharmacological transcriptome filter were predominantly expressed in non-neuronal cells (*Cx3cr1*, *Irak2*, *Lao1*, *Nrros*, *Pnpla2* and *Wbp1l*), while *Fkbp5* prevailed in principal neurons and *Mthfd2* in GABAergic interneurons (**Fig. 6**). Expression of *Cx3cr1*, *Irak2* and *Nrros* was especially enriched in microglial cells, indicating a potential

role of these and other non-neuronal cells in the modulation of memory consolidation by GR.

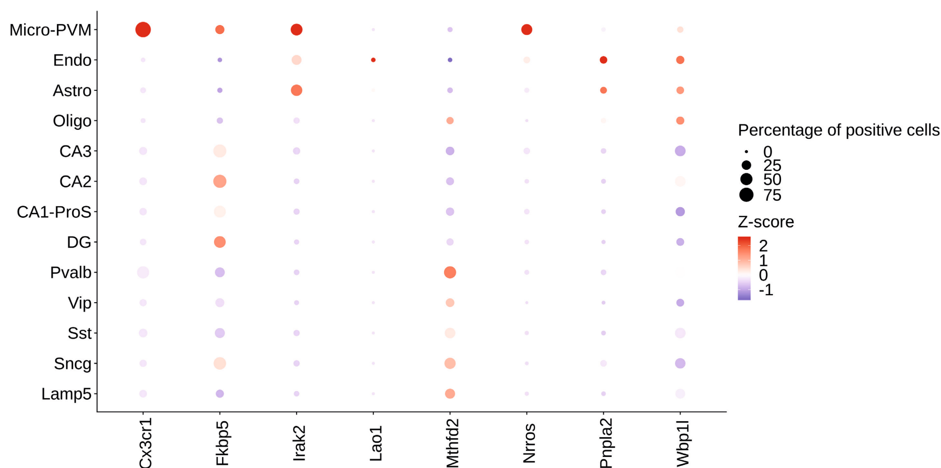

Figure 6. Single-cell expression data implicate neuronal as well as non-neuronal cells in GR-modulation of memory consolidation.

Dotplot visualizing basal single-cell expression data in the hippocampus of untrained and untreated mice for the eight short-listed genes identified by application of the pharmacological transcriptome filter. Colour intensity of the dot represents expression level per cell type based on Z-scores (the centered log-normalized average expression) and dot size depicts the percentage of the corresponding cell type expressing the gene. Astro, astrocytes; CA1-ProS, *cornu ammonis* 1/pro-subiculum pyramidal cells; CA2, *cornu ammonis* 2 pyramidal cells; CA3, *cornu ammonis* 3 pyramidal cells; DG, dentate gyrus granule cells; Endo, endothelial cells; Lamp5, lysosomal associated membrane protein family 5 positive GABA neurons; Micro-PVM, microglial cells and perivascular macrophages; Oligo, oligodendrocytes; Pvalb, parvalbumin positive GABA neurons; Sncg, synuclein gamma positive GABA neurons; Sst, somatostatin positive GABA neurons; Vip, vasoactive intestinal peptide positive GABA neurons.

DISCUSSION

To dissect the role of glucocorticoids in memory consolidation we established functional contrasts based on how GR-ligands affected the memory strength after AFC and imposed these on hippocampal transcriptome data. By utilizing the selective properties of SGRMs CORT108297 and CORT118335 alongside the endogenous agonist corticosterone (vehicle group), exogenously applied corticosterone and the classical GR antagonist RU486, we identified eight genes by which glucocorticoids may modulate memory consolidation. We argue that such a 'pharmacological transcriptome filtering' is a viable strategy to shorten 'longlists' of candidate genes from omics approaches.

In many behavioural paradigms glucocorticoids increase memory strength, and inhibiting glucocorticoid signalling impairs memory strength (5, 34). As AFC behaviour and

hippocampal memory representation is susceptible to modulation by glucocorticoids (20), we selected this paradigm to test our pharmacological transcriptome filtering approach. Of note, AFC involves multiple brain regions and fear conditioning based on cues strongly depends on the amygdala, with a smaller role for the hippocampus. Yet, both areas were shown to be equally activated after fear conditioning training when measuring ERK1/2 (19) and engrams show that fear conditioning activates hippocampal neurons as well (35-37). We therefore reasoned that the contrasts obtained by AFC memory retrieval are applicable to the hippocampal transcriptome data. Using the hippocampus allows for more tissue and less heterogenous subnuclei than in case of the amygdala.

As expected, post-AFC exogenous administration of corticosterone significantly increased average freezing levels, while RU486 and CORT118335 decreased freezing compared to the vehicle control group, presumably due to inhibiting the effects of endogenous corticosterone secreted as part of the rather stressful learning circumstances (14, 15, 20). These results confirm that glucocorticoids can modulate memory strength via GR, this being the only receptor targeted by all three compounds. The unchanged corticosterone levels after SGRMs treatment make it very unlikely that effects of the compounds occurred via alterations in HPA-axis activity. Somewhat unexpectedly, treatment with CORT108297 did not affect AFC behaviour, while previously in rats CORT108297 affected memory in the passive avoidance task to a similar extent as corticosterone (15). The difference may relate to either species or task, with different levels of endogenous glucocorticoids after AFC in comparison to the passive avoidance task. Altogether, the differential effects of pharmacological modulation of GR-activity on freezing behaviour enabled us to establish functional contrasts for subsequent filtering.

The hippocampal transcriptome was strongly affected by AFC, with functional annotation indicating synaptic changes and a role for transcription and kinase signalling. These results are in accordance with literature that emphasizes the importance of transcription in memory consolidation (38). These changes likely include stress-induced corticosterone effects, given the presence of well-characterized GR targets in the set of regulated genes and extensive overlap with GR DNA-binding. The additional effects of pharmacological modulation of GR-activity (compared to the vehicle group) were limited – not only for exogenously administrated corticosterone, but also for the classical GR antagonist RU486. These relatively small effects and the inherent variation of behavioural experiments, prevented successful application of our predetermined filtering strategy including all treatments relative to vehicle. Our group size is common for transcriptomic approaches, but in combination with the unexpected outcome of

CORT108297 on behaviour proved to be a limitation. Therefore, we defined a bigger contrast (corticosterone on the one hand versus CORT118335 and RU486 on the other hand), based on the behavioural findings and on the transcriptome data, which still proved to be powerful.

The impact inclusion of SGRMs had on our pharmacological transcriptome filtering approach is evident, as addition of CORT118335 to the transcriptome filter (compared to merely filtering based on the corticosterone vs. RU486 contrast) reduced the shortlist by 26 genes (63%, from 41 to 15 DEGs at adjusted p-value < 0.1). After qPCR validation, our approach resulted in a manageable list of eight genes for more in-depth follow-up in future studies to establish and subsequently unravel their causal role in memory consolidation. It is important to note that these follow-up studies would need to also include female mice to determine possible sex-specific effects; as we only used male mice to assess the feasibility of our approach, this is a clear limitation in the interpretation of the current dataset. Further functional analysis of potential causal target genes is hampered by the fact that this would have to involve loss of GR responsiveness of these genes, rather than knockout or knockdown *per se*. This is not trivial, since in mice such an approach has been possible only on a single occasion, based on a fortuitous naturally occurring deletion in the mouse genome (39).

Although the resulting shortlist contains the canonical GR-target gene *Fkbp5* (40, 41), it did not become a simple reiteration of established GR-target genes (42). Based on the assessed GR DNA-binding data, the seven other genes likely respond to GR in a more context-dependent manner. The role of *Cx3cr1* and *Lao1* in behaviour and memory was previously studied using knockout mouse models. *Cx3cr1* encodes for a fractalkine receptor via which microglial cells can bi-directionally communicate with neurons, a signalling pathway associated with various neurological disorders (43, 44). Behavioural assessment of *Cx3cr1* KO mice revealed an anxiolytic-like phenotype and improved contextual memory (45, 46). These effects fit with the lower expression levels we observed after corticosterone administration compared to CORT118335 and RU486. Knockout of *Lao1*, an oxidase involved in amino acid metabolism, resulted in impaired fear learning and memory, reportedly due to low acetylcholine levels (47); the present observations are in line with this earlier study. Direct involvement in behaviour or memory of any kind has to date not been studied for the remaining five genes (*Mthfd2*, *Pnpla2*, *Wbp1l*, *Nrros* and *Irak2*). Earlier, *Mthfd2* and *Nrros* affected the levels of reactive oxygen species (48, 49), of which excessive increases are associated with decreased performance in cognition-based behavioural paradigms (50, 51). The established (in)direct functions of a subset of the genes in our shortlist confirms the notion that this approach enables identification of relevant targets and guides future studies into a causal relationship.

Of note, many genes are - at a basal level - predominantly expressed in non-neuronal cells, an observation in line with growing evidence for the role of glial cells in stress and behaviour (52, 53). However, we cannot rule out that increases in gene expression occurred in cells that basally did not express said gene, indicating the need for validation. In addition, glucocorticoids were found to consistently regulate expression of non-neuronal genes and GR was shown to be important in astrocytes and microglia (42, 54, 55). Together, this also advocates for further experiments with cell-type specific knockouts of GR to examine if the modulatory effect of glucocorticoids on memory consolidation is perhaps exerted by a specific cell type. As three genes on our shortlist are predominantly expressed in microglia, we propose these cells as an interesting starting point.

To conclude, the concept of pharmacological transcriptome filtering applied in this study is based on the properties of the SGRMs, including different receptor specificity and a combination of agonistic and antagonistic effects. Binding of the SGRMs to the GR leads to a unique interactome of coregulators, which in turn results in tissue- and cell-type specific transcriptional effects (9, 10). The postulated concept relies on the assumption that treatments that have a similar functional outcome, will also similarly affect the underlying molecular mechanism. Therefore, the common denominators (in this study differentially expressed genes) are likely to be somehow involved in the functional outcome studied. While in the current study we focussed on the role of glucocorticoids in memory consolidation, the concept of pharmacological transcriptome filtering can be applied to many directions of research with an established role of glucocorticoids or other transcriptional regulators.

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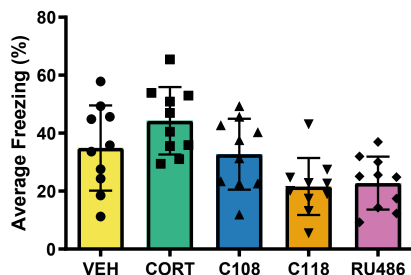
SUPPLEMENTARY FIGURE:



Contrast	P value
CORT vs. Vehicle	0.007
C108 vs. Vehicle	0.94
C118 vs. Vehicle	<0.001
RU486 vs. Vehicle	<0.001
C108 vs. CORT	<0.001
C118 vs. CORT	<0.001
RU486 vs. CORT	<0.001
C108 vs. RU486	0.003
C118 vs. RU486	0.99
C108 vs. C108	<0.001

Sup. Fig. 1. Expanded statistical analyses of behavioural data.
Average freezing of all six tones per animals at memory retrieval testing 24 hours after auditory fear conditioning ($F(29,270) = 7.025$; p-value < 0.0001) with expanded statistics in the table. VEH: vehicle, CORT: corticosterone, C108: CORT108297, C118: CORT118335.