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Diversity of glucocorticoid receptor signaling: molecular mechanisms and therapeutic implications

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Cell type specificity of glucocorticoid signaling in the adult mouse hippocampus

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ABSTRACT

Glucocorticoid stress hormones are powerful modulators of brain function and can affect mood and cognitive processes. The hippocampus is a prominent glucocorticoid target and expresses both the glucocorticoid receptor (GR – *Nr3c1*) and the mineralocorticoid receptor (MR – *Nr3c2*). These nuclear steroid receptors act as ligand-dependent transcription factors. Transcriptional effects of glucocorticoids have often been deduced from bulk mRNA measurements or spatially informed individual gene expression. However, there is only sparse data that allows insights on glucocorticoid-driven gene transcription at the cell type level. Here we used publicly available single-cell RNA sequencing data to assess the cell-type specificity of GR and MR signaling in the adult mouse hippocampus. The data confirmed that *Nr3c1* and *Nr3c2* expression differs across neuronal and non-neuronal cell populations. We analyzed co-expression with sex hormones receptors, transcriptional coregulators, and receptors for neurotransmitters and neuropeptides. Our results provide insights in the cellular basis of previous bulk mRNA results and allow the formulation of more defined hypotheses on the effects of glucocorticoids on hippocampal function.

Key words: stress hormones, corticosteroid receptors, hippocampus, single-cell RNA sequencing, transcription regulation.

INTRODUCTION

In the brain, stress responses and memory formation are essential to cope with changes in the environment [1]. The hippocampus is crucial in these processes, and highly sensitive to fluctuating levels of glucocorticoid (GC) stress hormones [2, 3]. GC levels naturally vary along the day following circadian and ultradian rhythms [4], and basal levels of endogenous GCs in the hippocampus are necessary for neuronal integrity, growth, differentiation and synaptic plasticity [5]. While acute stress induces only a temporary deviation from this balance, chronic stress or excessive GC exposure can threat the hippocampal homeostasis. All these effects are mediated by the two types of corticosteroid receptors that are expressed in the brain, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). GR and MR are nuclear steroid receptors that can act as ligand-dependent transcription factors. MR has a high GC affinity (K_d ~ 0.5nM) and accordingly is activated substantially at basal hormone levels. GR has a lower affinity (K_d ~ 5nM), and is therefore responsive to circadian GC peaks and fluctuations in the stress range [6]. Binding studies, immunohistochemistry and in situ hybridization showed that expression of *Nr3c2* gene (coding for MR) is mainly restricted to the limbic brain, specifically the hippocampus, while *Nr3c1* gene (coding for GR) is widely expressed throughout the brain [7]. So far, all genome wide studies on GR- and MR-mediated transcription in the hippocampus have been conducted with bulk tissue mRNA measurements. However, the hippocampus is a complex brain structure with a wide diversity of neuronal as well as non-neuronal cells, and with a particular spatial organization. Single-cell RNA sequencing (scRNA-seq) has allowed for a large-scale comprehensive molecular classification of cell types in the brain [8–10]. The Allen Institute for Brain Science recently sequenced ~1,2 million cells covering all regions of the adult mouse isocortex and hippocampal formation, identifying almost 380 subtypes of cells. The hippocampal data includes information on glutamatergic neurons from the dentate gyrus and cornu ammonis regions, GABAergic neurons, astrocytes, oligodendrocytes, microglia and endothelial cells [11]. Our previous in situ hybridization-based analysis on whole brain revealed spatially specific co-expression patterns of *Nr3c1* and *Nr3c2* with genes that are responsive to GCs or involved in nuclear receptor transcriptional regulation. This suggested mechanisms for regional and cellular functional specificity of GC signaling [12]. The advances in scRNA-seq carry with them new computational methods to address such co-expression at the cell type level, and allow the reconstruction of transcription factor downstream pathways [13–15]. In the current study we used existing scRNA-seq data [11] to molecularly characterize the cellular heterogeneity of GR and MR signaling in the adult mouse hippocampus. We assessed cell type expression

specificity of GR and MR downstream target genes to identify putative markers for GC responsiveness in particular cell types. Furthermore, we looked into GR and MR co-expression with sex hormone receptors, transcriptional coregulators, and receptors for neurotransmitters and neuropeptides to define for each cell type the potential pathways that may interact with hippocampal GC signaling.

MATERIALS AND METHODS

1. Single cell RNA sequencing data resources

The present study is based on the 10x scRNA-seq dataset published by the Allen Institute for Brain Science [11] and publicly available at https://portal.brain-map.org/atlases-and-data/RNA-seq/mouse-whole-cortex-and-hippocampus-10x. Briefly, the single cells were isolated from 16 different regions of the isocortex and the hippocampal formation from 54 male and female mice. The Allen Mouse Brain Common Coordinate Framework version 3 (CCFv3) ontology was used to define brain regions for profiling and boundaries for dissections. scRNA-seq data from the regions of interest were generated using 10x Genomics Chromium. For downstream processing, cells with less than 1500 detected genes as well as doublets were filtered out. The data was then clustered, and cluster names were assigned based on the Allen Institute proposal for cell type nomenclature (https://portal.brain-map.org/explore/classes/nomenclature). The topology of the taxonomy allowed to define the sex of the mouse from which the cells were isolated, the regions of interest, cell classes (glutamatergic, GABAergic or Non-Neuronal) and subclasses [11, 16]. This information was stored in the metadata table.

2. Single cell RNA sequencing data metrics and pre-processing

The metadata was used to subset cells of the hippocampus region from the gene expression matrix. We selected for 13 subclasses of hippocampal cells. The final gene count matrix consisted of 77001 cells for 26139 genes (**Fig. 1**), and was pre-processed in R v3.6.1 according to the Seurat v3.1.5 pipeline for quality control, normalization, and analysis of scRNA-seq data, using the following criteria: min.cells = 1, min.features = 100, normalized.method = LogNormalize, scale.factor = 10000, selection.method = “vst”, nfeatures = 2000. The gene counts were normalized and log-transformed across all cells which allowed for statistical comparison between cells and cell types, as previously described [17]. We performed principal component analysis (PCA), and we selected the top 50 PCs as input for the t-distributed stochastic neighbor embedding (t-SNE) dimensional reduction. Finally, the transcriptomic data was analyzed and displayed using Seurat visualization tools (**Fig. 1**).

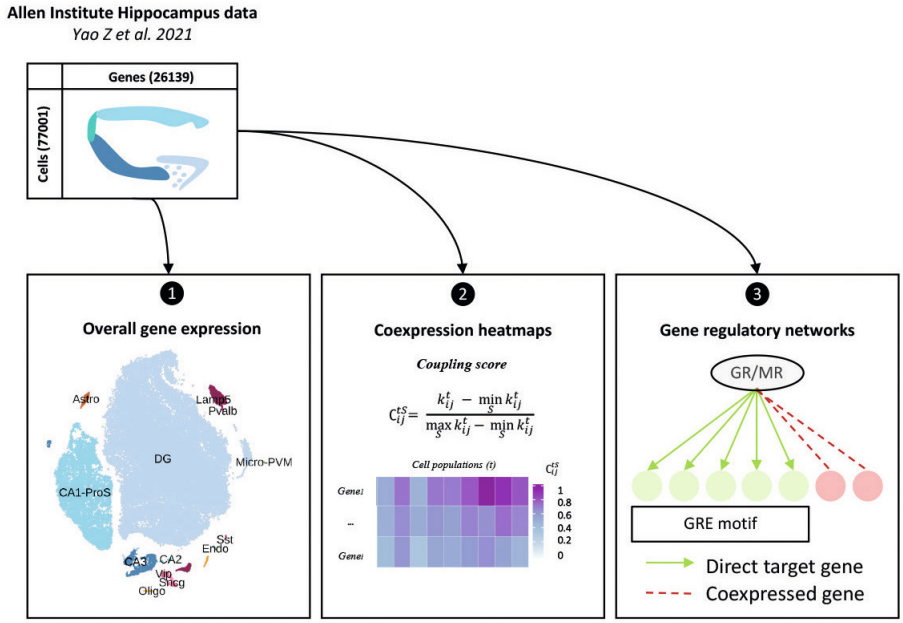


Figure 1. Schematic overview of the research strategy. Abbreviations: Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3, C_{ij}^{tS} – Coupling score – GR – Glucocorticoid receptor, MR – Mineralocorticoid receptor, GRE – Glucocorticoid response element.

3. Bulk RNA sequencing of mouse ventral hippocampus

The animal study was approved by the ethics committee of local Animal Committee of the University of Amsterdam. 8-week-old C57BL/6 J male mice were group-housed by four in conventional cages with a 12:12 h light-dark cycle and had ad libitum access to food and water. Mice received an injection of either 3mg.kg^{-1} of corticosterone (n=4) or vehicle (n=4) between 9:00 and 10:00 in the morning. Mice were killed by decapitation 3 hours after injection. The ventral hippocampus was collected for mRNA sequencing (RNA-seq). Total RNA was isolated with the NucleoSpin® RNA kit (Macherey-Nagel) and RNA quality was assessed using the RNA 6000 Nano kit on Bioanalyzer (Agilent). All samples had an RNA Integrity Number (RIN) over 6.5 with a 28/18s ratio over 1, and therefore considered suitable for sequencing. Aliquots of total RNA samples were sent for transcriptome sequencing at BGI Genomics. Stranded mRNA libraries were constructed, and 100-bp paired end sequencing was performed on the DNBseq platform resulting in over 20 million reads per sample.

RNA-seq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE184924.

The Gentrap pipeline, published as part of Bio Pipeline Execution Toolkit (Biopet, https://biopet-docs.readthedocs.io), was used for reads 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). The gene-read quantification was performed using HTSeq-count v0.6.0. HTSeq-count output files were merged into a count matrix as input for differential gene expression analysis. DEseq2 (version 1.29.4) [18] was used for normalization of the data (median of ratio's method) and identification of differentially expressed genes in R v3.4. The differential expression analysis, resulting in 16839 genes in the analysis. The contrast between vehicle and corticosterone groups was analyzed for differential expression in a pair-wise comparison. The FDR adjusted p-value of .05 was used as a cut-off to determine differentially expressed genes.

4. Selection of gene sets

Steroid receptors – This gene set contains the stress and sex hormones nuclear steroid receptors, the glucocorticoid receptor (*Nr3c1* - Nuclear receptor subfamily 3 group C member 1), the mineralocorticoid receptor (*Nr3c2* - Nuclear receptor subfamily 3 group C member 2), the androgen receptor (*Ar*), the progesterone receptor (*Pgr*), the estrogen receptors α and β (*Esr1* and *Esr2*).

GR and MR target genes – This set of genes is based on previous transcriptomic studies in rodent brain and neuronal cells after glucocorticoid treatment [19], our recent RNA-seq results in mouse ventral hippocampus after corticosterone injection, and two chromatin immunoprecipitation followed by sequencing (ChIP-seq) studies on GR and MR after injection with either 0.3 or 3mg/kg of corticosterone in rats [20, 21]. The criteria for 'target genes' were 1) regulation by GCs in previously published studies on rodent brain and 2) in our recent transcriptomic results, given that these exclusively represent mouse hippocampus, 3) the direction of regulation had to be consistent in all reporting studies; and 4) the gene had to be associated to a binding site for either GR, MR or both receptors according to the two ChIP-seq studies we used. The latter were in rat hippocampus, but it has become apparent that functional GC response elements (GREs) tend to be evolutionary conserved [22, 23].

Coregulators – The gene set of GR and MR AF-2 coregulators was based on previous profiling analysis published by Broekema et al. [24].

Neurotransmitter and neuropeptides receptor repertoire – We aimed for an exhaustive list of genes for the adrenergic, serotoninergic, cholinergic, and dopaminergic receptors according to the HUGO Gene Nomenclature Committee at the European Bioinformatics Institute (HGNC database: https://www.genenames.org/). The neuropeptides receptors list was based on the HGNC database and the previous study from Smith et al. 2020 on intracortical neuropeptide networks [25].

5. Single cell RNA sequencing coupling matrices for Nr3c1 and Nr3c2 co-expression profiles

A coupling score of *Nr3c1* and *Nr3c2* with genes of interest was calculated to rank their co-expression. First, we calculated the average expression of each gene of interest i in cell type t (x_i^t), where t is one of the 13 cell types in the adult mouse hippocampus. For each corticosteroid receptor (*Nr3c1* and *Nr3c2*), we calculated the coupling score as previously described [25], as $k_{ij}^t = \log_{10}(x_i^t \times x_j^t)$, where $i \in S$ and S is one of the gene sets described earlier, and $j \in \{Nr3c1, Nr3c2\}$. For each gene set S, we calculated the normalized coupling score C_{ij}^{tS} (**Fig.1**):

$$C_{ij}^{tS} = \frac{k_{ij}^t - \min_S k_{ij}^t}{\max_S k_{ij}^t - \min_S k_{ij}^t}$$

6. pySCENIC: assessment of GR and MR single cell gene regulatory network activity

The gene expression matrix of the clustered hippocampus scRNA-seq dataset underwent the scalable Python SCENIC (pySCENIC) workflow for single-cell gene regulatory network analysis as described by Van de Sande et al. 2020 [15]. pySCENIC reconstructs gene regulatory networks (GRNs) - *i.e.*, transcription factors together with their target genes – and assesses these *de novo* GRN activity in individual cells (**Fig. 1**). The pySCENIC workflow v0.10.3 was performed under Python v3.8.5, the output was then processed with Seurat v3.1.5 in R v3.6.1.

7. Differential expression and gene regulatory network (GRN) activity analysis of single cell RNA sequencing data

The gene count matrix for hippocampal gene expression and the GRN activity matrix underwent differential expression/activity analysis to identify genes specifically more expressed or GRNs specifically more active in certain cell types. Both differential analyses were performed using the Seurat FindAllMarkers function (Wilcoxon Rank Sum test) [17] in R v3.6.1. Besides, significant differences in gene

expression throughout cell types or within one cell type were tested with a paired two-sided Wilcoxon test (wilcox.test function) on average expression in R v3.6.1.

8. Code and data availability

The bulk RNA-seq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE184924. Open-source algorithms were used as described for single-cell analysis methods [17] and gene regulatory networks analysis [15]. The code that supports the findings of this study is openly available at the following GitHub repository: https://github.com/eviho/10XHip2021_VihoEMG. And the datasets used in the code can be downloaded from Zenodo (https://doi.org/10.5281/zenodo.5729701).

RESULTS

Nr3c1 (GR) and *Nr3c2* (MR) expression show significant cell specificity across hippocampal cell types

Our approach aimed to describe the diversity of corticosteroid receptors *Nr3c1* (GR) and *Nr3c2* (MR) signaling networks in mouse hippocampal cell types, using publicly available scRNA-seq data. We selected hippocampal cells from the Yao Z et al. mouse brain dataset [11] which resulted in 77 001 cells, divided over 13 different cell types (**Fig. 2A**). The most abundant cell types in this dataset were the dentate gyrus (DG) and cornu ammonis 1/pro-subiculum (CA1-ProS) glutamatergic neurons with respectively 58566 and 13221 cells. The two last glutamatergic neuron populations CA2 and CA3 contained respectively 143 and 1899 cells. GABAergic neurons were divided into 5 subtypes containing between 49 and 1372 cells: parvalbumin (Pvalb), somatostatin (Sst), vasoactive intestinal peptide (Vip), synuclein gamma (Sncg) and lysosomal associated membrane protein family member 5 (Lamp5) positive neurons. Finally, the data revealed 4 non-neuronal cell types: 488 astrocytes (Astro), 465 oligodendrocytes (Oligo), 73 endothelial cells (Endo) and 74 microglial cells/perivascular macrophages (Microglia-PVM) (**Fig. 2B**).

We assessed *Nr3c1* and *Nr3c2* relative expression levels throughout the hippocampal cell types. Although the t-SNE representation clearly showed a significant higher expression level of *Nr3c2* compared to *Nr3c1* in the mouse hippocampus ($log_2FC = 2.82$, p-value = .02) (**Fig. 2C**), the data was biased towards the most abundant cell types (DG and CA1-ProS). Per population, we observed a relatively higher expression of *Nr3c2* compared to *Nr3c1* in glutamatergic neurons, which was more pronounced in CA2 ($log_2FC = 3.74$, p-value < .001) (**Fig. 2D**). *Nr3c2* was actually enriched in CA2 ($log_2FC = 0.53$, p-value < .001) and the DG ($log_2FC = 0.32$, p-value <

.001) compared to other cell types (**Suppl. Table 1**). Interestingly, Nr3c2 was also more expressed than *Nr3c1* in GABAergic neurons with the biggest difference in Sncg neurons ($log_2FC = 2.75$, p-value < .001) (**Suppl. Table 1**). *Nr3c1* was more expressed in non-neuronal cell types with the biggest contrast in Micro-PVM cells where *Nr3c2* was almost absent (**Fig. 2D**). These differences in expression levels were in line with the percentage of cells expressing *Nr3c1* and *Nr3c2*. Between 50% and 100% of neurons (glutamatergic and GABAergic) were positive for *Nr3c2*, whereas only CA1-ProS, CA3 and Pvalb types passed the 50% threshold of positive cells for *Nr3c1*. As for the non-neuronal types, they contained less than 50% cells positive for either *Nr3c1* or *Nr3c2*, with a slightly higher percentage of positive cells for *Nr3c1* compared to *Nr3c2* in oligodendrocytes, microglial and endothelial cells (**Fig. 2E**).

Altogether, the results suggest a relatively higher basal expression of *Nr3c2* in mouse hippocampal neurons and astrocytes, while *Nr3c1* is relatively more expressed in oligodendrocytes, microglia, and endothelial cells.

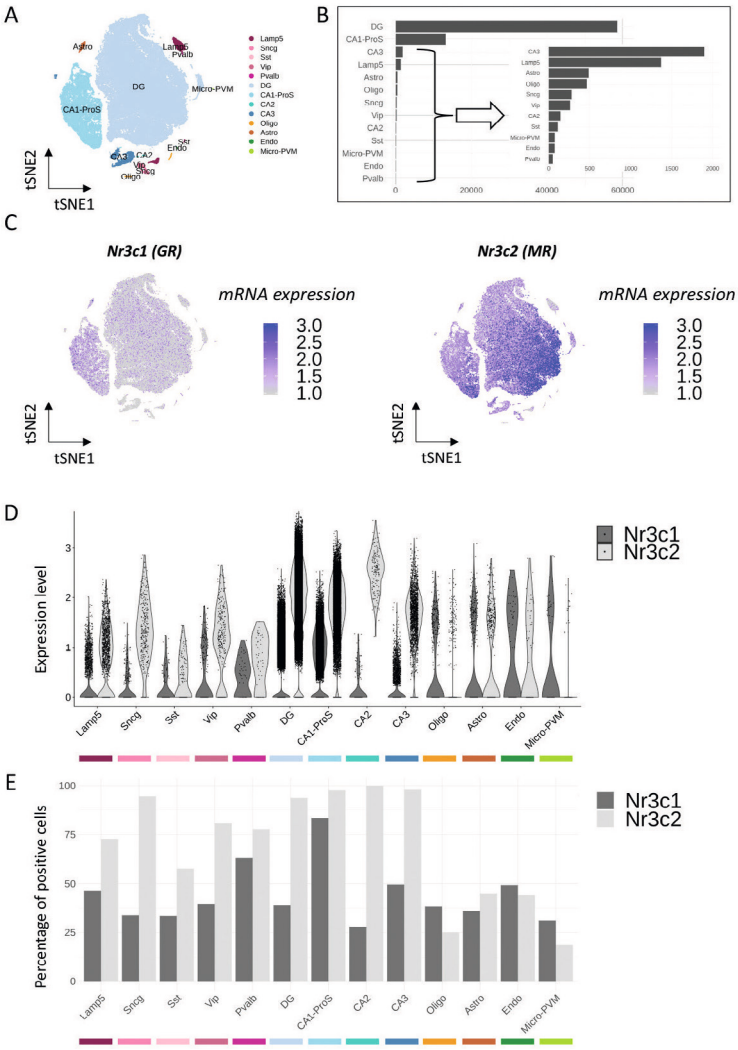


Figure 2. Cell type specificity of *Nr3c1* and *Nr3c2* expression in the adult mouse hippocampus. (A) Dimensional reduction (t-SNE) representation of mouse hippocampal cells grouped by gene expression profile similarities and assigned to known cell types. **(B)** Number of cells per cell type within the dataset. (figure legend continues on the next page).**(C)** t-SNE representation of *Nr3c1* and *Nr3c2* log-normalized mRNA expression per cell, scaled from 1 to 3 (mRNA expression). **(D)** Violin plot of *Nr3c1* and *Nr3c2* log-normalized expression (Expression Level). **(E)** Bar plot of the percentage of cells positive for *Nr3c1* and *Nr3c2*. Abbreviations: t-SNE – t-distributed stochastic neighbor embedding, Nr3c1 – Nuclear receptor subfamily 3 group C member 1, Nr3c2 – Nuclear receptor subfamily 3 group C member 2, GR – glucocorticoid receptor, MR – mineralocorticoid receptor, Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3.

Classic GR and MR target genes differentially express across hippocampal cell types

Transcription-dependent GC responsiveness of the hippocampus relies by definition on the presence of various GR and MR target genes. We studied the basal expression of GC regulated genes in different hippocampal cell types. A limited class of genes is commonly measured in bulk brain mRNA to assess GC effects [26–30]. This set includes FK506-binding protein 5 (*Fkbp5*), glucocorticoid-induced leucine zipper protein (*Tsc22d3*), period circadian regulator 1 (*Per1*) and serum/glucocorticoid regulated kinase 1 (*Sgk1*). However, the scRNA-seq data showed a clear heterogeneity for the basal expression of these genes in different hippocampal cell types (**Fig. 3A**). *Fkbp5* expression was predominant in glutamatergic neurons, particularly in the DG. In comparison, *Tsc22d3* was more expressed in GABAergic neurons and non-neuronal cells than *Fkbp5*. Furthermore, the basal expression of *Per1* suggested high cell specificity, with high expression in only 5 neuronal cell types. Finally, *Sgk1* was expressed in most hippocampal cell types but was absent in astrocytes and endothelial cells (**Fig. 3A**). The average expression was in line with the percentage of cells expressing the genes of interest. On average, 50% of glutamatergic neurons expressed *Fkbp5*, while 50% of GABAergic neurons expressed *Tsc22d3*. *Sgk1* was more present in oligodendrocytes and microglia, whereas *Tsc22d3* was more present in astrocytes and endothelial cells (**Suppl. Fig. 1A**). *Per1* was generally less expressed than any other classic target genes in whole hippocampus, which might be partially explained by circadian variation (**Fig. 3A; Suppl. Fig. 1A**). Although the analysis is done on hippocampal basal gene expression, the results suggest an heterogenous and cell type-specific response to GC signaling activation.

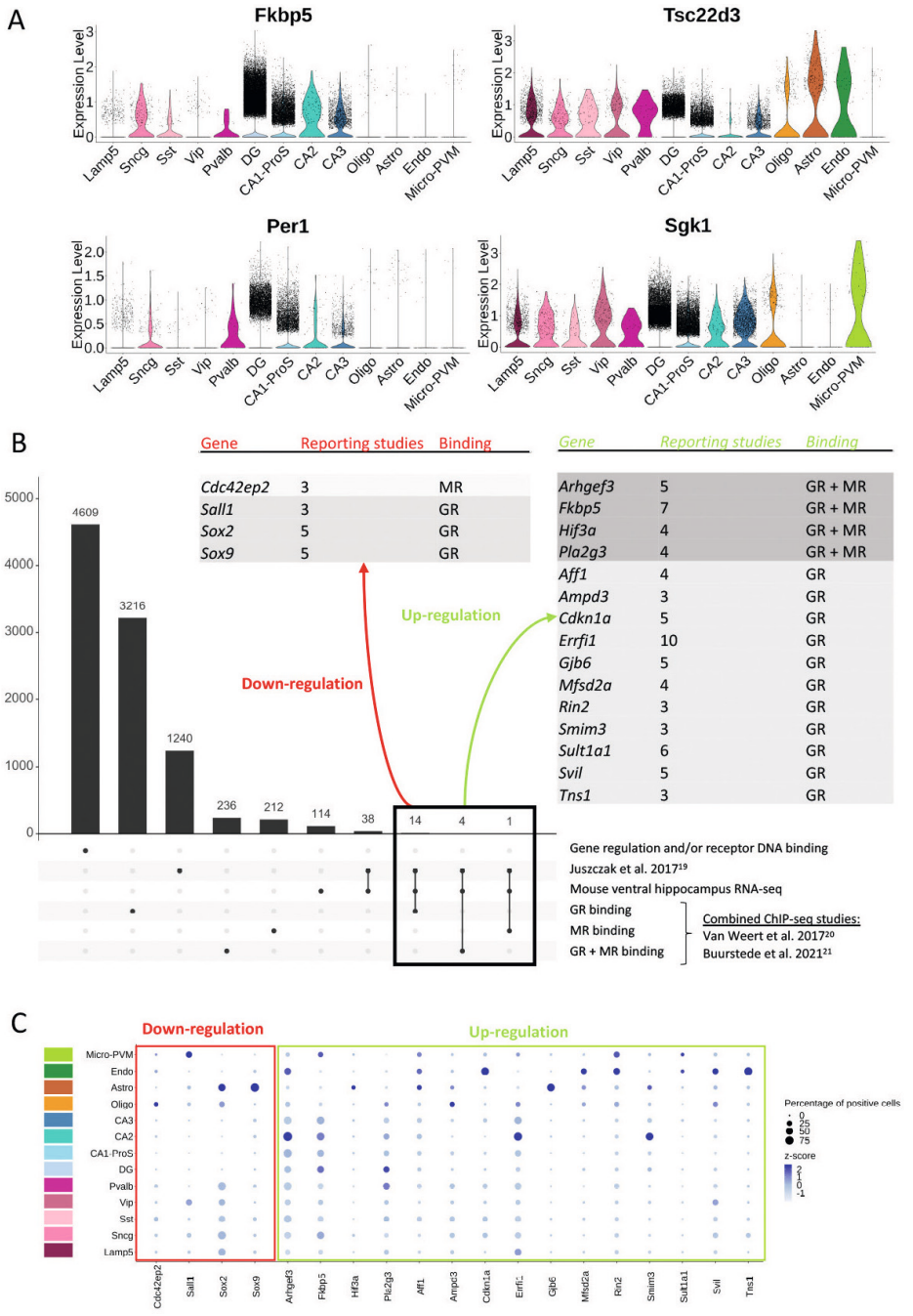


Figure 3. Cell type specificity of glucocorticoid target genes in the adult mouse hippocampus.

< Description Figure 3. (A) Violin plots representing the log-normalized expression of commonly measured glucocorticoid responsive genes *Fkbp5*, *Tsc22d3*, *Per1* and *Sgk1* (Expression Level). **(B)** List of new GR and MR target genes selection based on transcriptomic and DNA binding studies, associated with the number of transcriptomic studies reporting the gene (reporting studies), and DNA binding by GR, MR or both receptors (binding). **(C)** Dot plot representing both the centered log-normalized average expression (z-score) and the percentage of positive cells for the genes newly identified as GR and MR targets. Abbreviations: GR – glucocorticoid receptor, MR – mineralocorticoid receptor, ChIP – chromatin immunoprecipitation, RNA-seq – RNA sequencing, Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3.

Regarding MR-specific target genes, MR binding to DNA on GREs was described to be associated with NeuroD factor binding [31], and *Jdp2* was found as an MR target gene in conjunction with MR/NeuroD binding. At basal level in the scRNA-seq data, *Neurod2* was mostly expressed in glutamatergic neurons, and although relatively few cells were positive for *Jdp2*, the ones expressing it were also glutamatergic neurons (**Suppl. Fig. 1B**). *Nr3c2* expression in the DG differed throughout the cell population (**Fig. 2C**). Therefore, we assessed DG cells using a deeper level of clustering. DG cells could be further divided in six distinct sub-clusters [11, 16]. The most abundant cluster was 125_DG, where both *Nr3c2* and *Neurod2* still showed different levels of expression across the cell cluster, with a similar overall pattern (**Suppl. Fig. 1C**). This suggests that despite differentially expressing *Nr3c2* and *Neurod2*, cells in cluster 125_DG were not divergent enough in the rest of their gene expression profile to be subdivided into more cell clusters. *Jdp2* was mainly expressed in cluster 122_DG and 125_DG. However, in the absence of GC treatment, *Jdp2* expression did not strongly correlate to the contrasted expression of *Nr3c2* or *Neurod2* in the DG (**Suppl. Fig. 1C**).

A wider set of GC target genes further reveals GR and MR signaling heterogeneity across cell types

Although classic GC responsive genes already showed cellular heterogeneity of gene expression, we expanded the list of GC responsive genes to give a better recapitulation of cellular specificity of GR and MR signaling in the mouse hippocampus. We combined a published meta-analysis on GC responsive genes in rodent and human brain (17 studies) [19] with a recent RNA-seq dataset that we obtained in mouse ventral hippocampus, and ChIP-seq data assessing GR and MR DNA binding in rat hippocampus [20, 21] (**Suppl. Table 2**). This resulted in a list of 4609 genes either responsive to GC treatment or associated with a receptor binding

site. Among those genes, 3216 reported GR-specific binding to the DNA, 212 MR-specific binding, and 236 reported both GR and MR binding.

A total of 1240 genes were reported to be regulated in the previously published meta-analysis, and 114 genes were GC responsive in our recent mouse hippocampus RNA-seq dataset. We first selected for genes that were reported consistently in between the previously published meta-analysis [19] and our transcriptomic analysis. This subset of 38 genes was further filtered for genes that reported DNA binding of either GR, MR or both receptors in the ChIP-seq studies.

A total of 19 genes survived all criteria and were reported in at least three transcriptomic studies. Of these, four genes were consistently downregulated and 15 were consistently upregulated. *Cdc42ep2* was the only gene associated with MR binding, a total of 14 genes were associated with exclusive GR binding and four genes were associated to both GR and MR binding, including *Fkbp5* (**Fig. 3B**). *Tsc22d3*, *Per1* and *Sgk1* were previously reported in both transcriptomic and ChIP-seq studies but absent in the recent mouse hippocampus RNA-seq dataset (**Suppl. Table 2**). The new subset of GR and MR target genes was further analyzed in the hippocampus scRNA-seq data. Like the classic GC responsive genes, the new targets displayed a large heterogeneity in cell type basal expression (**Fig. 3C**). Genes known to be downregulated after GC treatment showed high specificity for non-neuronal cell types. *Cdc42ep2* was relatively more expressed in oligodendrocytes, *Sall1* in microglia, *Sox2* and *Sox9* in astrocytes. Among genes known to be upregulated after GC treatment, more than half were relatively more expressed in non-neuronal cells in these basal conditions. However, *Fkbp5* and *Pla2g3* were predominantly neuron specific. Moreover, *Arhgef3*, *Errfi1* and *Smim3* were preferentially expressed in CA2 (**Fig. 3C**). We also investigated the cell type specificity of genes known to be regulated by GCs but not associated with a receptor binding site. In this list of 19 genes, three were not detectable in the scRNA-seq data (*1810011O10Rik*, *Rhou*, *Lcn2*). Many genes were highly expressed in astrocytes (*e.g.*, *Dio2*), two downregulated genes (*Abi3*, *Ccr5*) were microglia specific, and three genes were widely expressed in neurons but at low levels, except for *Ccng1* which was highly expressed and abundant in CA1-ProS (**Suppl. Fig. 1D**).

The results on GR and MR downstream target genes highlighted once more the expression heterogeneity of GC target genes in mouse hippocampal cell types. Furthermore, many target genes were, under basal conditions, specifically expressed in non-neuronal cells. This indicates that transcripts from non-neuronal cells may represent a substantial part of GC target genes.

Nr3c1 and *Nr3c2* co-expression with sex hormone receptors suggests cell type-specific crosstalk

Corticosteroid receptors belong to the nuclear receptor superfamily that also includes the sex hormone receptors: the progesterone receptor (PR, coded by *Pgr*), androgen receptor (AR, coded by *Ar*), estrogen receptor α and β (ERα and ERβ, coded by *Esr1* and *Esr2*). Sex steroid receptors may interact with MR and GR, but direct interactions would by definition depend on presence and co-expression [32–34]. *Ar*, *Pgr*, *Esr1* and *Esr2* were similarly expressed in cells that came from male or female mice in the scRNA-seq with two subtle differences. Pvalb GABAergic neurons showed lower expression of *Ar* and *Pgr* in male cells, and CA3 had more positive cells and a slightly higher expression of *Pgr* in males. *Esr1* and *Esr2* were expressed in very few cells, the highest expressing cell types being the DG granule cells and CA1-ProS (**Fig. 4A**).

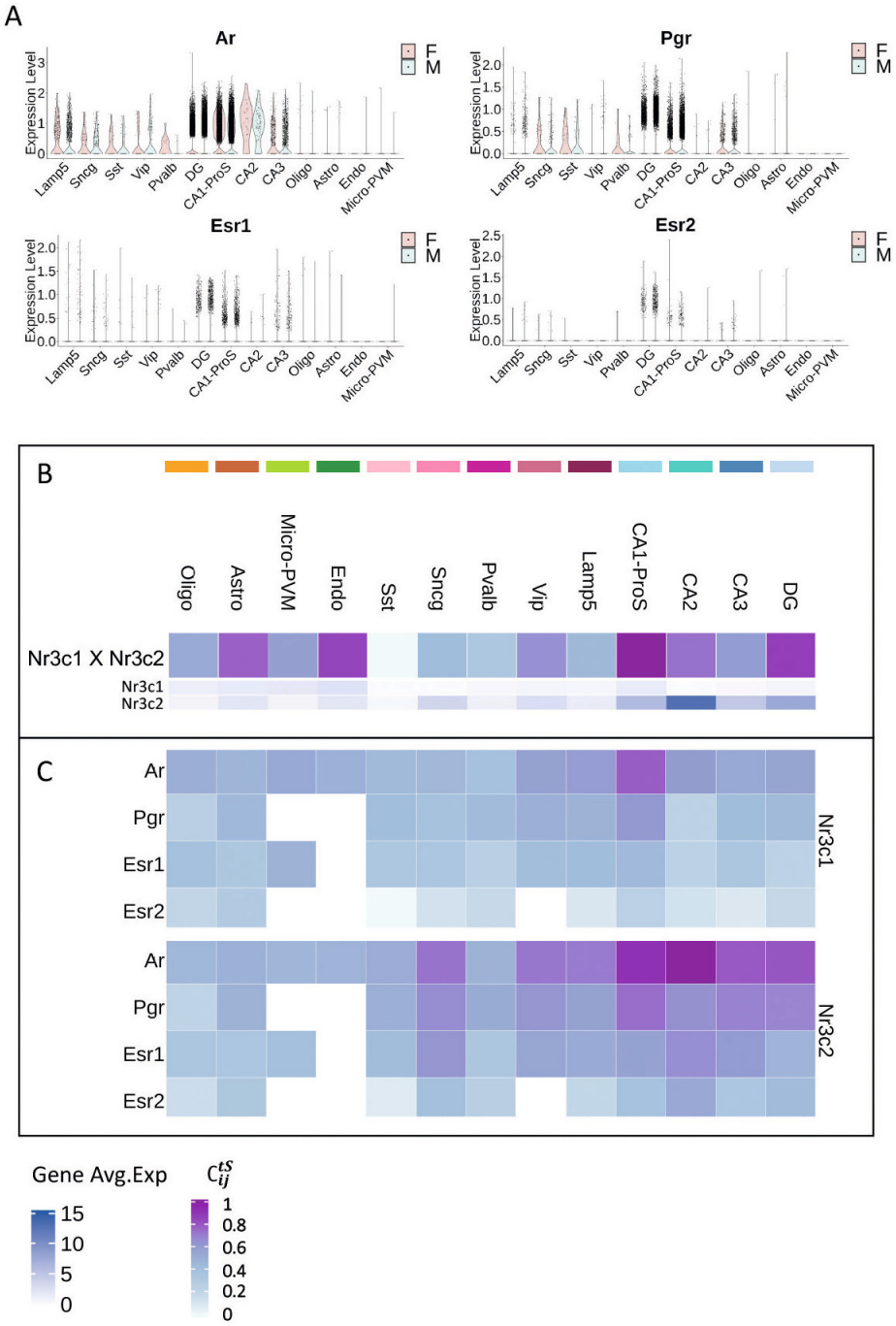


Figure 4. Cell type specificity of Nr3c1 and Nr3c2 co-expression with sex hormone receptors.

< Description Figure 4. (A) Violin plots representing the log-normalized expression (Expression Level) of sex hormone receptors *Ar*, *Pgr*, *Esr1* and *Esr2* in cells obtained from female **(F)** and male (M) mice. **(B)** Heatmap representing the coupling score C_{ij}^{tS} of *Nr3c1* with *Nr3c2*, and their respective log-normalized average expression in mouse hippocampal cell types (Gene Avg.Exp). **(C)** Heatmap representing the coupling score C_{ij}^{tS} of *Nr3c1* and *Nr3c2* with sex hormone receptors *Ar*, *Pgr*, *Esr1* and *Esr2* in mouse hippocampal cell types. Abbreviations: Nr3c1 – Nuclear receptor subfamily 3 group C member 1 (glucocorticoid receptor), Nr3c2 – Nuclear receptor subfamily 3 group C member 2 (mineralocorticoid receptor), Ar – Androgen receptor, Pgr – Progesterone receptor, Esr1 and Esr2 – Estrogen receptors α and β, F – Female, M – Male, Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3.

We next determined cell type-specific co-expression between stress and sex hormone receptors. For this, we calculated a coupling score C_{ij}^{tS} based on basal average expression of pairs of genes in the different hippocampal cell types. Corticosteroid receptors (*Nr3c1* and *Nr3c2*) showed the highest coupling score in CA1-ProS and were also highly co-expressed in the DG, CA2, endothelial cells and astrocytes (**Fig. 4B, Suppl. Table 3**). The highest coupling score between stress and sex hormone receptors was found in neuronal cells. *Nr3c1* particularly co-expressed with *Ar* and *Pgr* in CA1-ProS, while *Nr3c2* co-expressed with *Ar* mainly in glutamatergic, Lamp5, Vip and Sncg neurons, but also with Pgr in CA1-ProS (**Fig. 4C, Suppl. Table 3**). The coupling scores between *Nr3c1* and *Nr3c2* and estrogen receptors were very low due to the absence of *Esr1* or *Esr2* expression in most cells. The highest coupling score for *Esr1* and *Nr3c2* was in CA2 and Sncg, certainly driven by the high *Nr3c2* expression. We conclude that overall male and female mice have highly similar gene expression profiles for sex hormone receptors, and that co-expression of sex- and stress hormone receptors is highly cell type specific.

Nr3c1 and *Nr3c2* co-expression with AF-2 coregulators suggests cell type-specific transcriptional modulation of GC signaling

Transcriptional coactivators and corepressors are key regulators of GC-driven gene transcription. The presence of one particular coregulator can determine the outcome of GC signaling in a cell population [35–38]. In an in vitro screening assay, evidence was reported for 24 coregulators interacting with corticosteroid nuclear receptors: five with both receptor types, 17 with GR only, and two with MR only [24]. In scRNA-seq data, each of these coregulators showed a specific expression pattern throughout different hippocampal cell types. For instance – and somewhat surprising – *Ncoa2* was expressed in all cell types [39], and its highest expression level was found in microglia, while *Prox1* was mainly expressed in Vip GABAergic

neurons and in the DG, where it was significantly enriched (log_2FC=1.47, p-value < .001) (**Fig. 5A, Suppl. Table 1**).

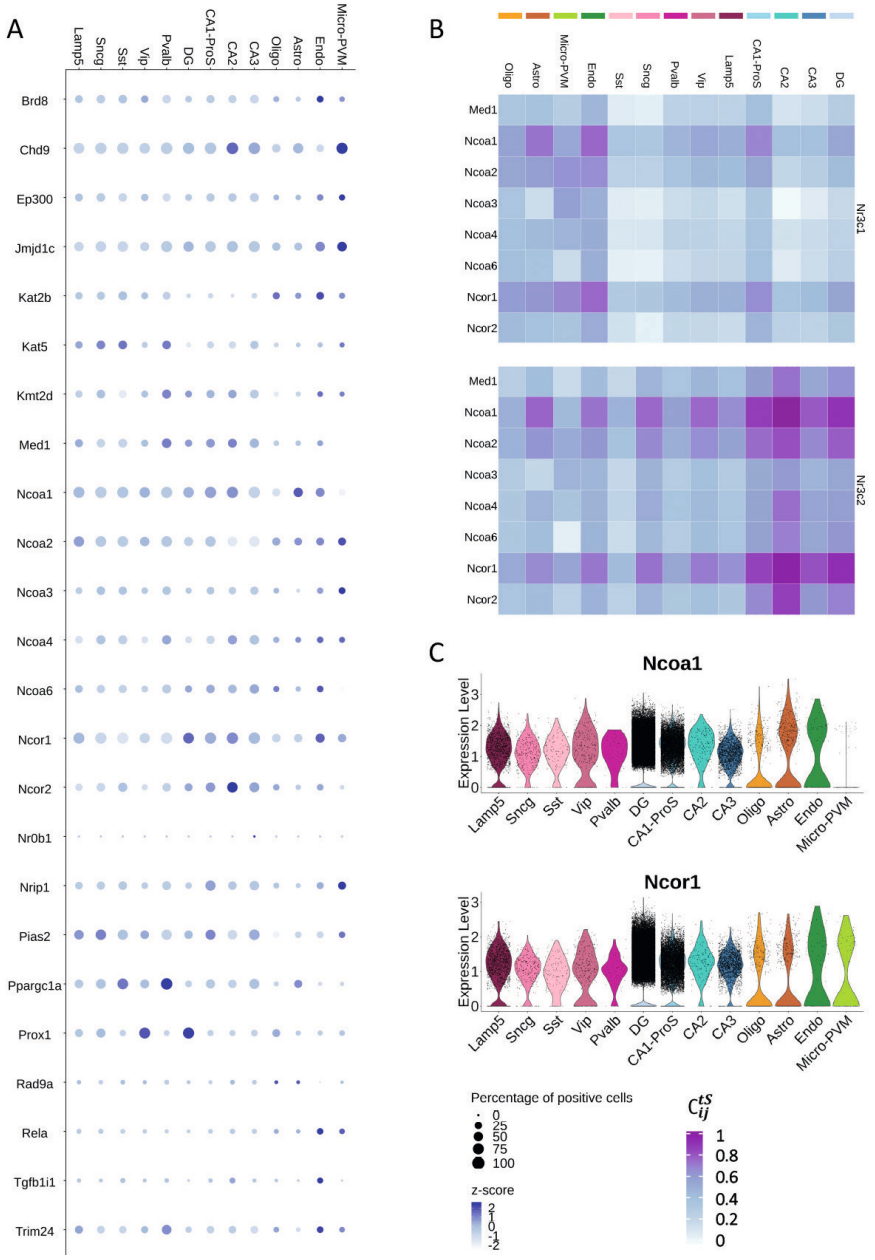


Figure 5. Cell type specificity of *Nr3c1* and *Nr3c2* co-expression with nuclear receptor coregulators.

< Description Figure 5. (A) Dot plot representing both the centered log-normalized average expression (z-score) and the percentage of positive cells for 24 nuclear receptor AF-2 coregulators known to interact with GR and/or MR according to an in vitro interaction screening assay [24]. **(B)** Heatmap representing the coupling score C_{ij}^{tS} of *Nr3c1* and *Nr3c2* with a subset of GR and MR coactivators and corepressors in mouse hippocampal cell types. **(C)** Violin plots representing the log-normalized expression (Expression Level) of the coactivator *Ncoa1* and the corepressor *Ncor1* in mouse hippocampal cell types. Abbreviations: Nr3c1 – Nuclear receptor subfamily 3 group C member 1 (glucocorticoid receptor), Nr3c2 – Nuclear receptor subfamily 3 group C member 2 (mineralocorticoid receptor), Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3, AF-2 – ligand-dependent transactivation domain 2 (helix 12), Med1 – Mediator complex subunit 1, Ncoa1 – Nuclear receptor coactivator 1, Ncoa2 – Nuclear receptor coactivator 2, Ncoa3 – Nuclear receptor coactivator 3, Ncoa4 – Nuclear receptor coactivator 4, Ncoa6 – Nuclear receptor coactivator 6, Ncor1 – Nuclear receptor corepressor 1, Ncor2 – Nuclear receptor corepressor 2.

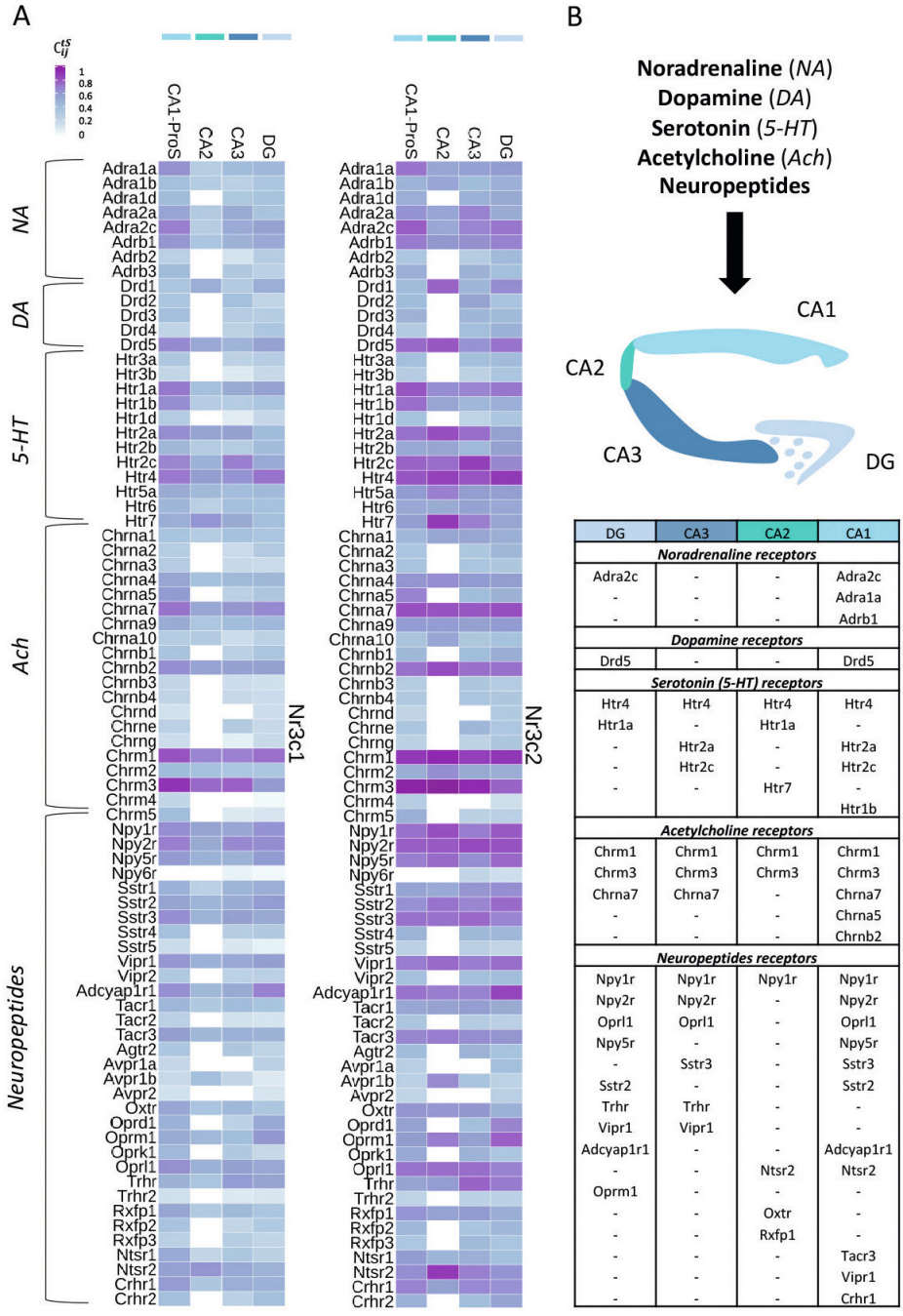
We further assessed co-expression of AF-2 coregulators with *Nr3c1* and *Nr3c2* (**Suppl. Fig. 2A, Suppl. Table 3**) for a subset of well-characterized coactivators (*Med1* and *Ncoa* family) and corepressors (*Ncor1* and *Ncor2*) (**Fig. 5B**). There was a clear co-expression with the coregulators in non-neuronal cells for *Nr3c1* and in glutamatergic neurons for *Nr3c2*. Interestingly, both *Nr3c1* and *Nr3c2* strongly co-expressed with *Ncoa1* and *Ncor1*, which showed the exact same pattern of co-expression throughout cell types. Ncoa1 and *Ncor1* showed the highest coupling scores with *Nr3c1* and *Nr3c2* in CA1-ProS, astrocytes, and endothelial cells, and with *Nr3c2* in other glutamatergic neurons, as well as Vip and Sncg GABAergic neurons (**Fig. 5B**). *Ncoa1* and *Ncor1* were expressed almost at the same level in all hippocampal cell types; except for microglia which did not express *Ncoa1* (**Fig. 5C**). Therefore, the co-expression of these co-regulators with stress hormone receptors is mainly driven by the cell specificity of *Nr3c1* and *Nr3c2* expression, with the notable exception of microglia.

Neurotransmitter and neuropeptide receptors differential co-expression with *Nr3c1* and *Nr3c2* suggests synapse-specific inputs

We next focused on neurotransmitter and neuropeptide pathways in the hippocampal glutamatergic tri-synaptic path, which is the best characterized synaptic transmission route in the hippocampus. In this glutamatergic circuit, excitatory projections from the entorhinal cortex reach the DG granule cells through the perforant path, and the DG mossy fibers project to CA3 pyramidal neurons which in turn stimulate CA1 neurons through the Schaffer collateral pathway [40].

In addition to the tri-synaptic path, CA1 also receive direct and strong excitatory projections from CA2 [41].

Although the sensory information mostly arrives in the DG, the CA-regions also receive inputs from other brain regions. Afferent synapses to the tri-synaptic path are not only glutamatergic, but they also include neurotransmitters such as noradrenaline (NA), dopamine (DA), or serotonin (5-hydroxytryptamine, 5-HT), acetylcholine (Ach) and neuropeptides. We addressed the co-expression of genes coding for NA, DA, 5-HT, Ach and 33 neuropeptide receptors with *Nr3c1* and *Nr3c2* (**Suppl. Table 3**), to determine how these pathways could interact with GC signaling in the hippocampal tri-synaptic circuit. NA receptors were mainly of the alpha-1a, alpha-2a/c and beta-1 types. They co-expressed with *Nr3c1* in CA1-ProS, and with *Nr3c2* also in CA2, CA3 and the DG (**Fig. 6A - NA**).



DG	CA3	CA2	CA1
Noradrenaline receptors			
Adra2c	-	-	Adra2c
-	-	-	Adra1a
-	-	-	Adrb1
Dopamine receptors			
Drd5	-	-	Drd5
Serotonin (5-HT) receptors			
Htr4	Htr4	Htr4	Htr4
Htr1a	-	Htr1a	-
-	Htr2a	-	Htr2a
-	Htr2c	-	Htr2c
-	-	Htr7	-
			Htr1b
Acetylcholine receptors			
Chrm1	Chrm1	Chrm1	Chrm1
Chrm3	Chrm3	Chrm3	Chrm3
Chrna7	Chrna7	-	Chrna7
-	-	-	Chrna5
-	-	-	Chrnb2
Neuropeptides receptors			
Npy1r	Npy1r	Npy1r	Npy1r
Npy2r	Npy2r	-	Npy2r
Oprl1	Oprl1	-	Oprl1
Npy5r	-	-	Npy5r
-	Sstr3	-	Sstr3
Sstr2	-	-	Sstr2
Trhr	Trhr	-	-
Vipr1	Vipr1	-	-
Adcyap1r1	-	-	Adcyap1r1
-	-	Ntsr2	Ntsr2
Oprm1	-	-	-
-	-	Oxtr	-
-	-	Rxfp1	-
-	-	-	Tacr3
-	-	-	Vipr1
-	-	-	Crhr1

Figure 6. Cell type specificity of *Nr3c1* and *Nr3c2* co-expression with neurotransmitter and neuropeptide receptors in the hippocampal tri-synaptic pathway.

< Description Figure 6. (A) Heatmap representing the coupling score C_{ij}^{ts} of *Nr3c1* and *Nr3c2* with adrenergic, dopaminergic, serotoninergic, cholinergic and neuropeptides receptors in excitatory neurons of the hippocampal tri-synaptic pathway. **(B)** Table of the neurotransmitter and neuropeptide receptors above threshold in terms of coupling with *Nr3c1* and *Nr3c2* expression (coupling score C_{ij}^{ts} > 0.6). Abbreviations: Nr3c1 – Nuclear receptor subfamily 3 group C member 1 (glucocorticoid receptor), Nr3c2 – Nuclear receptor subfamily 3 group C member 2 (mineralocorticoid receptor), DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3, NA – Noradrenaline, DA – Dopamine, 5-HT – 5-hydroxytryptamine, Ach - Acetylcholine.

For DA receptors, *Drd5* co-expressed strongly with *Nr3c1* in CA1-ProS and with *Nr3c2* co-expressed in all glutamatergic neurons. *Drd1* co-expressed with *Nr3c2* in CA2 and the DG (**Fig. 6A - DA**). Many 5-HT receptors were strongly co-expressed with *Nr3c1* or *Nr3c2* in all regions of the tri-synaptic circuit, particularly *Htr1a*, *Htr2a*, *Htr2c* and *Htr4* (**Fig. 6A – 5HT**). The most consistent co-expressed Ach receptors throughout the tri-synaptic circuit were *Chrm1* and *Chrm3* (**Fig. 6A - Ach**). Neuropeptide Y (NPY) receptors 1, 2 and 5 were strongly co-expressed with *Nr3c2* in all cell types, whereas they were more specific to CA1-ProS and the DG for *Nr3c1*, which reflects specificity of steroid receptors more than of these three types of NPY receptors. *Sstr2* and *Sstr3* were the most co-expressed somatostatin receptors, while *Vipr1* was the most strongly co-expressed vasoactive intestinal peptide receptor. *Adcyap1r1* (pituitary adenylate cyclase-activating polypeptide type I receptor) was highly co-expressed with *Nr3c1* in CA1-ProS and with *Nr3c2* in all glutamatergic neurons. Tachykinin receptor *Tacr3*, opioid receptor *Oprl1* and corticotropin-releasing hormone (CRH) receptor *Crhr2* were co-expressed the strongest with *Nr3c1* in CA1-ProS. *Nr3c2* co-expressed with tachykinin, arginine-vasopressin, oxytocin, opioid, thyrotropin-releasing hormone (TRH), relaxin, neurotensin and CRH receptors in several glutamatergic synapses (**Fig. 6A - Neuropeptides**).We selected for the genes that had a coupling score above 0.6 both with *Nr3c1* or *Nr3c2* to get an overview of the strongest correlated neurotransmitter and neuropeptide receptors with GC signaling in the tri-synaptic circuit (**Fig. 6B**). For instance, NA receptors are most robustly co-expressed with *Nr3c1* and *Nr3c2* in the DG and CA1-ProS. Neurotransmitter and neuropeptide receptors co-expression with corticosteroid receptors was more selective in GABAergic neurons and non-neuronal cells. For instance, in microglia *Nr3c1* (and *Nr3c2*) showed high co-expression with *Adrb1* and *Adrb2*. The coupling score with *Ntsr2* was particularly high in astrocytes (**Suppl. Fig. 2B**).

Nr3c1 and *Nr3c2* escape *de novo* gene regulatory network analysis

It is known that cell-specific gene regulation relies essentially on coordination of the activity of transcription factors [42]. Recent progress in high-throughput sequencing allows the reconstruction of transcription factor downstream networks.

We applied the pySCENIC pipeline to see whether we could identify putative MR and GR dependent regulatory networks in particular cell types [15]. The pySCENIC workflow is divided in three steps: first, it computes co-expression modules of a transcription factor (TF) with all correlated genes based on the scRNA-seq count matrix. Then, these co-expression modules are further refined by selecting genes with the TF-specific DNA motif in their promoter region, generating the gene regulatory network (GRN) modules. Finally, the refined GRN activity is measured in each individual cell, by scoring the component gene expression per GRN, and is used for new clustering (**Fig. 7A**).

In this analysis, we based the t-SNE dimensional reduction on GRN activity, rather than gene expression. The t-SNE included the same 13 cell types, but the clustering grouped the cells differently. The most notable change was the disappearance of GABAergic neurons specificities. These neurons grouped together as one cluster which means that all GABAergic neuronal types have very similar GRN activity profile (**Fig. 7B**), as previously described using pySCENIC in scRNA-seq brain data [15, 43]. During the refinement of co-expression modules into GRNs, the co-expression modules with less than 80% of genes containing a binding site for the TF in their promoter region were excluded. Nr3c1 and Nr3c2 GRN activity could not be calculated due to not passing this threshold of motif discovery. Nevertheless, the GRN analysis allowed the identification of some cell type-specific gene networks in the mouse hippocampus (**Suppl. Fig. 3A, Suppl. Table 4**). For instance, the neuronal GRN Hsf3(+), the GABAergic GRN Maf(+) and the glutamatergic GRN Neurod2(+) showed cell type specific activity (**Suppl. Fig. 3B**). To further characterize the mouse hippocampus cell diversity, we performed a differential activity analysis on GRNs to find the most active GRN for each cell type (**Fig. 7C, Suppl. Table 5**). GRNs were more specific in non-neuronal cells. For instance, Otx1(+) is the most active GRN in astrocytes being expressed in 94% of astrocytes and only 1% of all-other cells, with an activity enrichment log-fold change of 4.24 (**Suppl. Table 5**).

Although we could not determine genes involved in Nr3c1(+) and Nr3c2(+) regulatory networks and their differential activity in hippocampal cell types, the pySCENIC allowed for a better characterization of other transcription factor downstream networks in mouse hippocampus. This can in turn be important in determining the cellular context of stress hormone receptor activity.

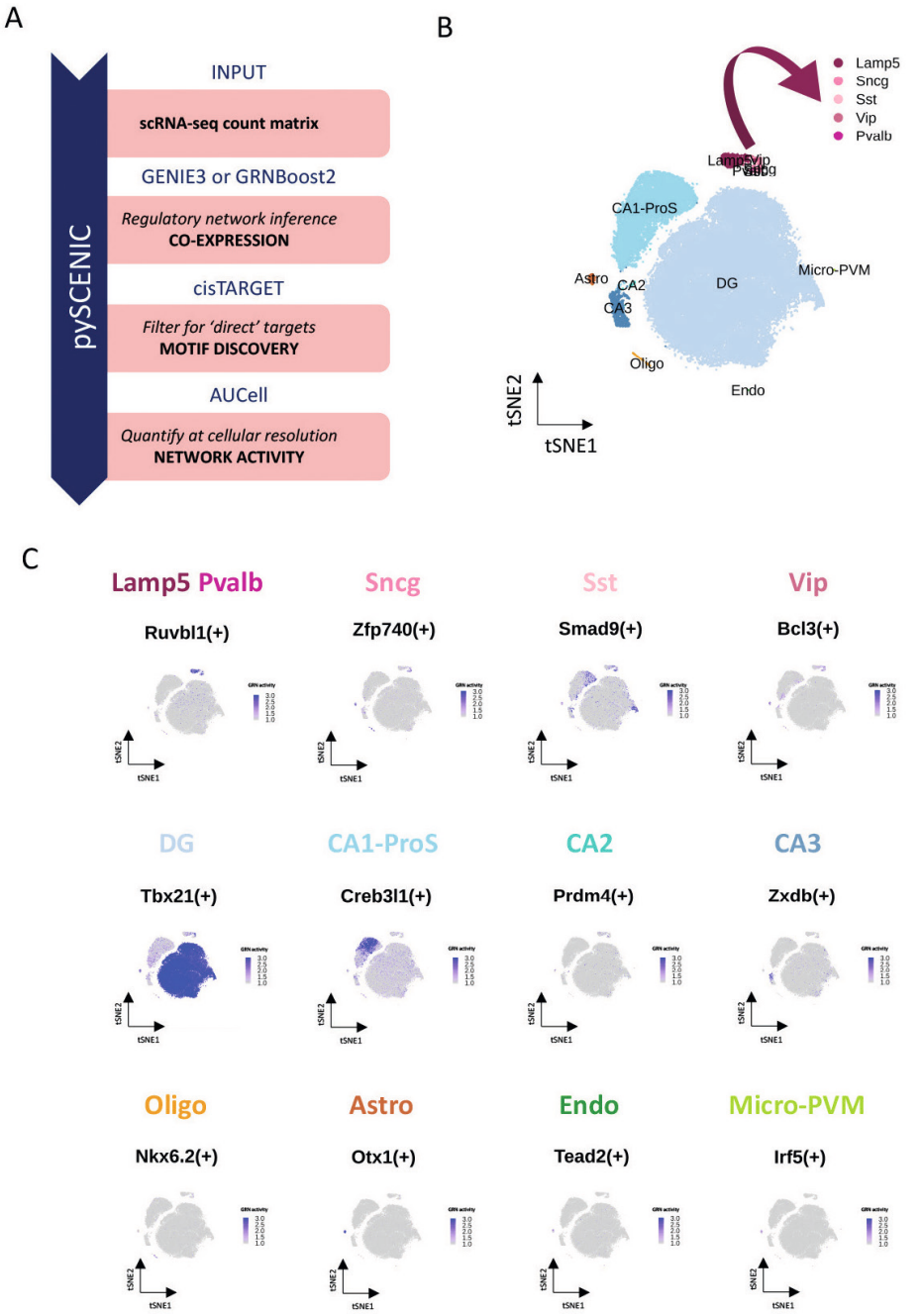


Figure 7. Mouse adult hippocampus gene regulatory networks (GRNs). (A) Description of the pySCENIC pipeline. **(B)** Dimensional reduction (t-SNE) representation of mouse hippocampal cells grouped based on GRN activity similarities and assigned to known cell types. (legend continues on the next page)

(C) t-SNE representation of each hippocampal cell population most active GRN activity level per cell, scaled from 1 to 3. The sign (+) allows the distinction between a transcription factor gene (e.g *Neurod2*) and this same transcription factor network (e.g Neurod2(+)). Abbreviations: scRNA-seq – Single-cell RNA sequencing, GRN – Gene regulatory network, Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/ Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3.

DISCUSSION

We set out to describe the cell-specific gene expression in the hippocampus in order to better understand MR and GR-mediated signaling. In a non-treated context, corticosteroid receptor genes *Nr3c1* (GR) and *Nr3c2* (MR), classic GC responsive genes and newly categorized target genes showed a very heterogenous basal expression throughout hippocampal cell types, and likely predicted cell type-specific responsiveness to GC signaling activation. Furthermore, the results on co-expression suggested cell type-specific crosstalk between sex and stress hormones, as well as a possible cell type-specific transcriptional coregulation. Our results also summarize the heterogeneity in stress hormone receptor co-expression with neurotransmitter and neuropeptide receptors in the tri-synaptic hippocampal circuit. Finally, despite providing no further insight on GR and MR downstream gene regulatory network cell specificity, the pySCENIC pipeline revealed the cell-specific activity of 376 transcription factor gene regulatory networks in the mouse hippocampus. These later results further emphasize the hippocampal cell heterogeneity in terms of gene transcription activity.

Our results confirm high MR mRNA expression in glutamatergic neurons (**Fig. 2D**), in line with its previously reported presence, and its role in mediating effects in hippocampal pyramidal and granule cell excitability [44–48]. MR expression in CA2 glutamatergic cells stands out, and recent study showed that neuronal MR deletion resulted in the disappearance of CA2 molecular identity [49]. It is interesting to note that GABAergic neurons have appreciable levels of MR mRNA. So far, based on predominant presence in the granular and pyramidal cell layers, the glutamatergic cells have received most attention. However, the widespread presence of MR challenges the notion of purely cell-autonomous effects in glutamatergic neurons. This expands the focus of future work looking into the basis of the MR-mediated effects on cognitive and emotional processing [50, 51]. On the other hand, MR binding to DNA earlier was linked to NeuroD factors, and this seems to reflect mainly glutamatergic neurons (**Suppl. Fig. 1B-C**). Immunohistochemical co-expression

studies will therefore be a valuable addition to this, and other findings at the mRNA level.

Our data for GR also validate some known notions, like the relatively low expression of GR mRNA in CA3 pyramidal cells (**Fig. 2D**) [52, 53].Presence of both receptor types in the glutamatergic CA1 neurons fits well with GR and MR cell-autonomous opposite effects in CA1 [54]. GR is certainly expressed in DG granule cells, but the percentage of positive cells is – perhaps surprisingly – modest. This may explain why corticosterone-sensitivity of DG excitability and gene expression is markedly different from CA1 pyramidal neurons [55–57]. The DG is arguably the most complex structure in the hippocampus in terms of cellular diversity and organization [58]. A possible reason for the DG heterogeneity is hippocampal neurogenesis, leading to cells in different stages of neuronal maturation. Recent results suggest that neuronal progenitor cells and their progeny have intrinsic GC sensitivity and display a dorsoventral differential response to long-term GC exposure [59].

These results could explain the contrast we observed in MR expression. The data supported differential GC sensitivity in the DG but did not allow to further subdivide DG cells because of their overall very similar pattern of gene expression. The level of clustering we used in the deeper analysis of the DG divided the region in only six subclusters. It is likely that more depth in the scRNA-seq associated with clustering based on neurogenesis markers would provide further insights on MR expression in neurons at different maturation stages.

GR mRNA expression was also high in oligodendrocytes, astrocytes, microglia, and endothelial cells (**Fig. 2D**). Functionality of GR in glial cell types has previously been established, for example with cell type-specific knockout mouse models [60–62]. Indeed, in a mouse model for Cushing's disease (AdKO) we observed clear changes for astrocytes, microglia and oligodendrocytes [63]. For all these cell types, effects of GCs, stress, and/or GR antagonists (direct and indirect) have been reported in rodents and human studies [64–67]. Specifically, microglial cells are clearly responsive to stress and GCs, and have recently been reported to play a role in synaptic plasticity [68, 69]. Interestingly, the signaling repertoire of GR in microglia is unique for the brain, in that *Ncoa1* (coding for the Steroid Receptor Coactivator-1 or SRC-1) is hardly expressed, and *Ncoa2* (coding for the SRC-2/GRIP1) may be a predominant GR coregulator (**Fig. 5A**), analogous to immune-modulatory GR effects in the periphery [70, 71]. A cell type-specific coregulator repertoire may allow more selective targeting of GR using selective receptor modulators that distinguish between downstream signaling pathways [35–38]. For instance, in an

epilepsy model, treatment with the selective GR modulator CORT108297 limited reactive microgliosis in the mouse dentate gyrus without affecting an increase in astrogliosis [72].

The set of MR/GR target gene that we used relied on studies that all addressed brain or neuronal tissue. Yet, there were many differences in species, genetic background and age, exact tissue, the type of intervention, the dosage and type of GC used, and the latency between treatment and sample collection (**Suppl. Table 2**). We could not provide a complete description of the conditions across the studies, as they sometimes failed to mention housing and light cycle conditions, the animal sex, or the timing of their intervention. Therefore, while we trust our criteria selected robustly responding GC target genes, the list is by no means exhaustive. Expression of MR/GR target genes clearly differed between cell types, but basal expression does not necessarily reflect the cell type-specific GC responsiveness. For instance, *Sgk1* is known to be strongly and apparently quite selectively induced in white matter [73, 74]. However, our results showed that *Sgk1* basal mRNA levels are high in all neuronal cell types, oligodendrocytes, and microglia (**Fig. 3A**). This is an example of a gene where basal expression does not fully correlate with MR and/or GR mediated effects. However, only very few target genes show such almost binary on-off responses after GC elevations. Therefore, we expect that increased levels of Fkbp5 mRNA reflect responses in glutamatergic neurons, and those of Tsc22d3 mRNA mainly responses in other cell types.

An additional argument in favor of basal expression predicting “target-ness” is that an increased mRNA level in a relatively small cell population will be diluted by steadily high expression levels in other more abundant cell types. However, this all remains to be proven based on experimental data addressing responses in specific cell types. The uncertainty of cell-specific target genes applies to a lesser extent for genes that are downregulated, as this can only occur in cell types that initially expressed the gene of interest. Specific expression of downregulated genes seems to concern mainly non-neuronal cell types (**Fig. 3C, Suppl. Fig. 1D**); for microglia clearly pointing to GR rather than MR-mediated responses.

Susceptibility and prevalence of stress-related neuropsychiatric and neurodegenerative pathologies differ between men and women [75], and prevalence of these stress-related disorders increases in females upon drastic hormonal changes [76]. Many of these disorders have been associated to altered structure, function, and neurogenic processes within the hippocampus [77–81], suggesting a possible sex dimorphism in GC effects on hippocampal function. Our

results showed that cell-specific GR and MR mRNA levels correlated substantially with AR and PR mRNA (**Fig. 4C**). This could suggest a direct crosstalk between those receptors, as AR and PR can bind to GREs [82]. On the other hand, interactions with ER likely do not have a great impact in the hippocampus, given the low expression of *Esr1* and *Esr2* (**Fig. 4A, Fig. 4C**). Thus, the quite large literature on estrogen effects on hippocampal function [83–85] points to involvement of membrane estrogen receptors [86, 87] and/or interactions in afferent brain areas.

The hippocampal tri-synaptic path receives various inputs from other brain regions and harbors a large diversity of synapses with receptors for NA, DA, 5-HT, Ach and neuropeptides. In our results, CA1 showed the highest number of NA, DA, 5-HT, and Ach receptors that were strongly co-expressed with GR and MR (**Fig. 6B**). Previous studies showed that NA, DA and 5-HT can suppress the perforant path input to CA1 by reducing postsynaptic potentials [88]. This suggests a possible interaction between GR/MR and neurotransmitter receptor signaling that could influence CA1 synaptic activity, conform with the early work by Joëls et al. [89]. Basal forebrain cholinergic neurons which project to the hippocampus are involved in stress adaptation and cognition [90]. The cholinergic system interacts with GC signaling in processes such as hippocampal-dependent memory reconsolidation [91]. Our results suggest that the Ach receptors likely to be involved in this crosstalk are *Chrm1*, *Chrm3* and *Chrna7* (**Fig. 6B**). In humans, higher NPY levels in serum and plasma were correlated to adaptive coping following stress as well as PTSD resilience [92–94]. A study in rats suggested that NPY interneuron activation in the DG contributed to trauma resilience in a model for PTSD [95]. Our results suggest that *Npy1r*, *Npy2r* and *Npy5r* expression is highly coupled with GR and MR mRNA levels in the DG (Fig. 6B). Conceivably, NPY and GC signaling communicate via interaction of those receptors in the rodent DG (inter)neurons.

Hippocampal oxytocin was found to be important for social discrimination [96], and oxytocin can prevent stress-induced hippocampal synaptic dysfunction and impairment of long-term potentiation and memory [97]. Our results suggest that oxytocin signaling interference with GC signaling is mainly restrained to the hippocampal cornu ammonis region (**Fig. 6B**). Our data also confirm the predominant role of CA2 specific AVPR1B receptors in stress-related signaling, in conjunction with MR (**Fig. 6A**) [49, 98].

GR and MR activation may affect neuronal development [99], as exemplified in CA2 pyramidal cells for MR [49] and the DG granule cells for GR [100]. This may be linked to corresponding downstream regulatory pathways. However, when looking

for transcriptional networks, GR and MR did not meet the selective criteria for the pipeline motif discovery because their co-expression modules had less than 80% of genes with a detected binding site in their promoter region. The pySCENIC motif discovery is limited to 10kb down- and up-stream of gene transcription start sites, while GR (and supposedly MR) binding sites are often further from their target gene start sites [21]. For hippocampal target genes, an in silico GRE interspecies screening of GC-responsive genes showed that GREs were between 30kb downstream and 175kb upstream of transcription start sites of GR target gene start site – a typical example being *Adra1b* which is co-expressed with GR in pyramidal cells (**Fig. 6A**) [23]. In addition, the inability for the pySCENIC pipeline to detect MR network may have been related to an overestimation of potential MR target genes. MR mRNA levels were high in most cells in the hippocampus and significantly correlated to a total of 7319 genes. Consequently, its direct genomic targets may have been diluted by other correlated genes, leading to loss of statistical power. Nevertheless, the dominant co-expression modules provided the cellular context in which MR and GR can bind to chromatin, and this may well be relevant, as exemplified by the Neurod2(+) GRN that may be linked to MR target genes (**Suppl. Fig. 3B**).

Although our data in part recapitulate previous published transcriptomic studies, the cell type-specific expression of genes that potentially interact with MR and GR allows for a reinterpretation of GC signaling in the adult mouse hippocampus. With the lack of an actual single cell transcriptomic dataset after GC treatment, the cell type-specific expression of MR/GR downstream targets suggests gene markers to study responsiveness of particular cell types. Moreover, the co-expression of potentially interacting factors, such as other steroid receptors and transcriptional coregulators, defines where direct interactions can take place, and may help to more specifically target the receptors with selective modulators [38]. We hope the results allow the formulation of more defined future hypotheses on stress hormone effects on hippocampal function.

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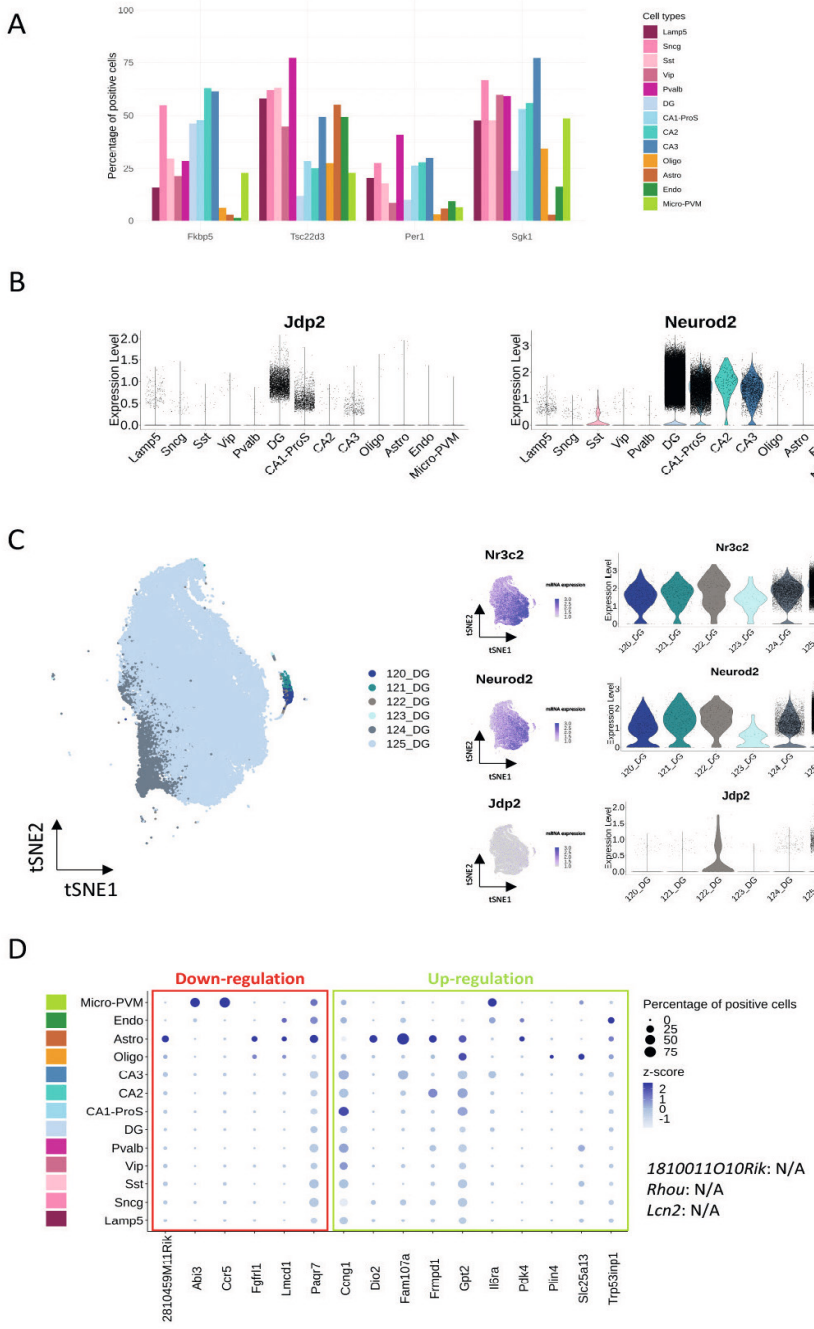
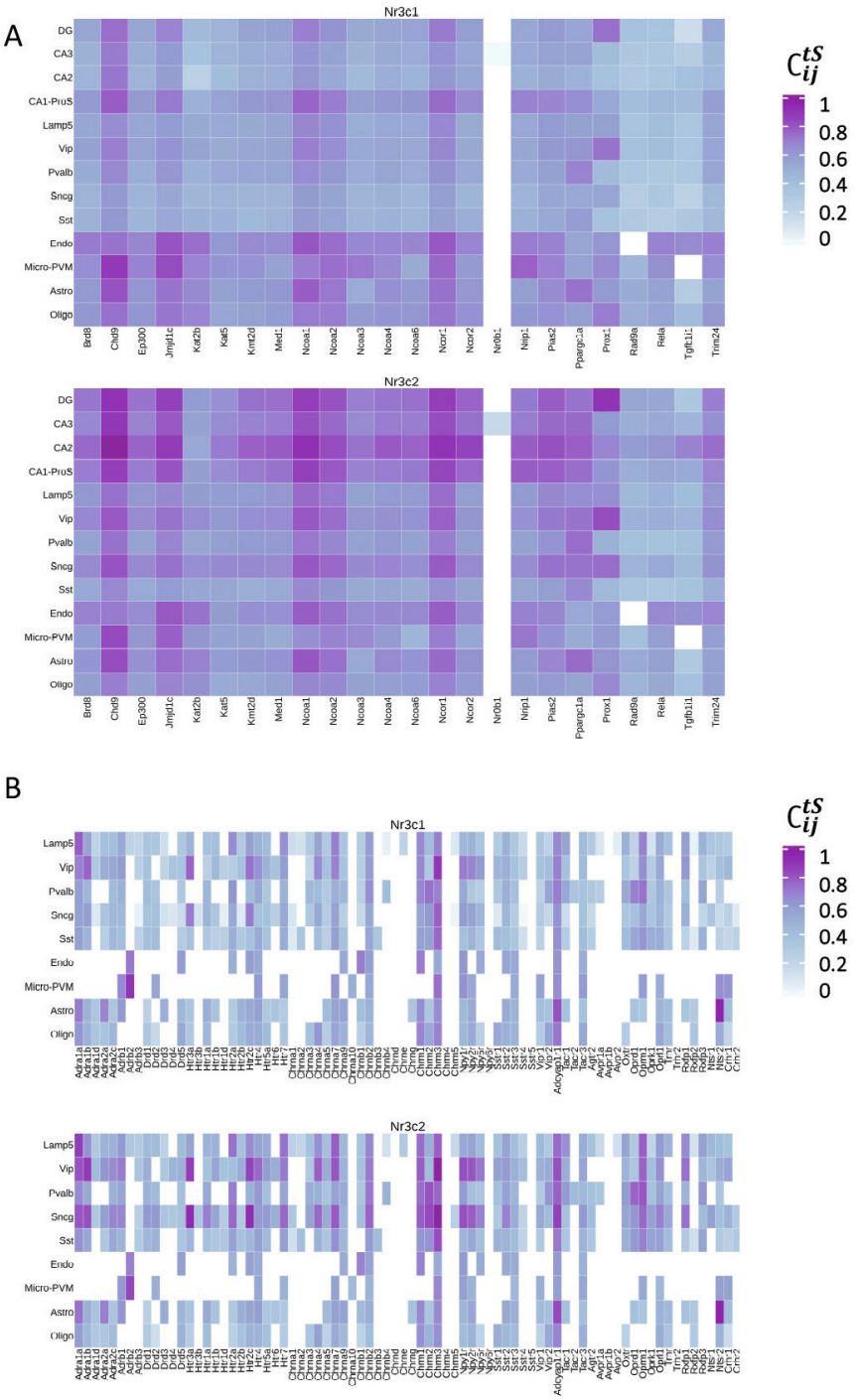
> Description Supplementary figure 1. (A) Bar plot of the percentage of cells positive for classic glucocorticoid target genes *Fkbp5*, *Tsc22d3*, *Per1* and *Sgk1*. **(B)** Violin plots representing the log-normalized expression of MR-specific target gene *Jdp2* and binding partner Neurod2. **(C)** Dimensional reduction (t-SNE) representation (scaled log-normalized expression) and violin plots (log-normalized expression) of *Nr3c2*, *Neurod2* and *Jdp2* in different dentate gyrus subclusters of cells. **(D)** Dot plot representing both the centered log-normalized average expression (z-score) and the percentage of positive cells for the genes identified as glucocorticoid responsive but with no evidence of GR nor MR binding. *1810011O10Rik*, *Rhou* and *Lcn2* were not detected in the dataset. Abbreviations: Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3.

APPENDIX

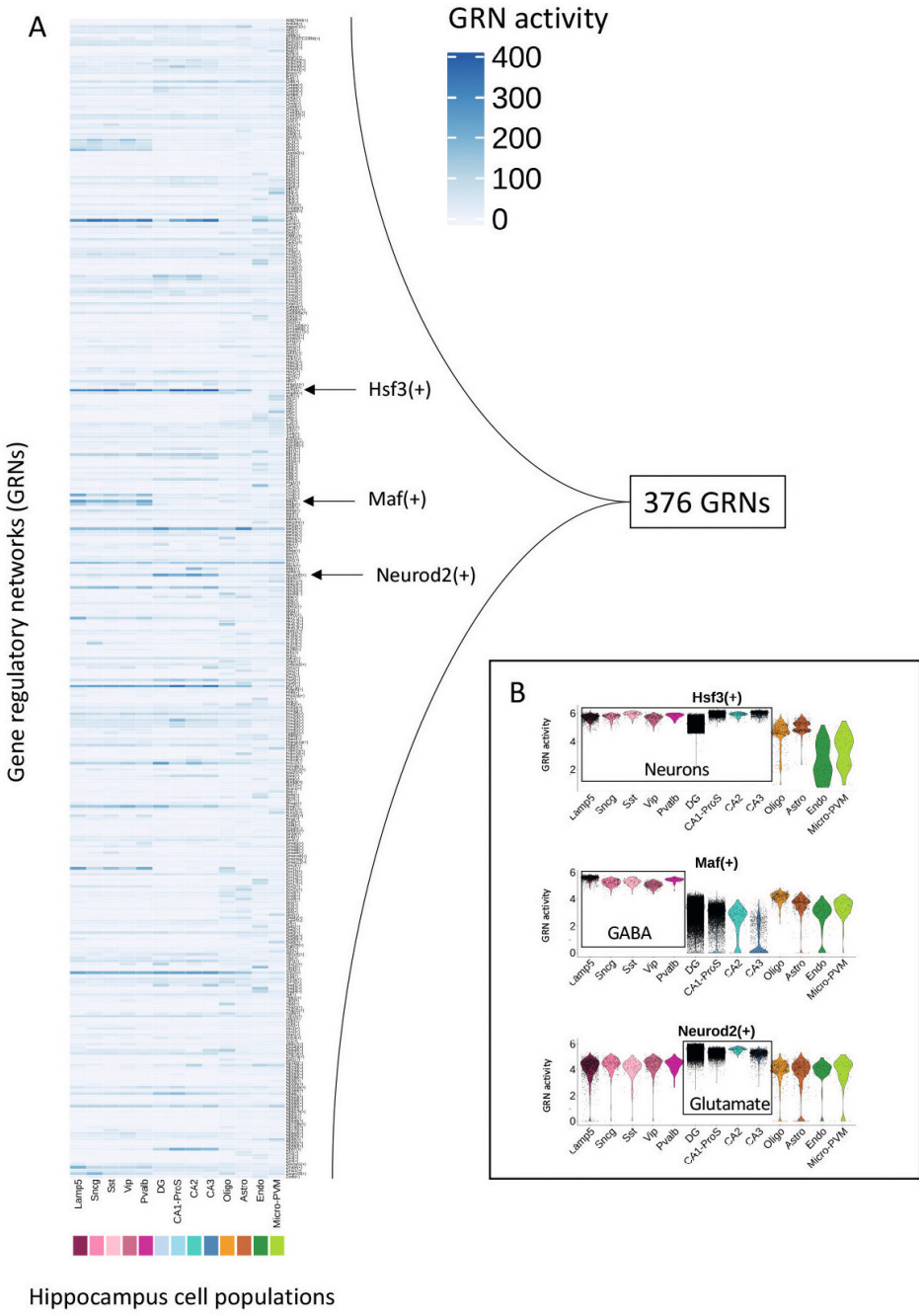


Supplementary Figure 1.



Supplementary Figure 2.

< Description Supplementary Figure 2. (A) Heatmap representing the coupling score C_{ij}^{ts} of AF-2 co-activators and co-repressors with *Nr3c1* and *Nr3c2* in mouse hippocampal cell types. **(B)** Heatmap representing the coupling score C_{ij}^{ts} of *Nr3c1* and *Nr3c2* with adrenergic, dopaminergic, serotoninergic, cholinergic and neuropeptides receptors in GABAergic neurons and non-neuronal cells. Abbreviations: Nr3c1 – Nuclear receptor subfamily 3 group C member 1 (glucocorticoid receptor), Nr3c2 – Nuclear receptor subfamily 3 group C member 2 (mineralocorticoid receptor), Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3, AF-2 – ligand-dependent transactivation domain 2 (helix 12).



Supplementary Figure 3.

< Supplementary Figure 3. (A) Heatmap representing the cell type-specific activity of 376 gene regulatory networks (GRNs) in the adult mouse hippocampus. **(B)** Violin plots representing the log-normalized activity of Hsf3(+), Maf(+) and Neurod2(+) gene regulatory networks, which are respectively relatively higher in neuronal cells, GABAergic cells and glutamatergic cells. The sign (+) allows the distinction between a transcription factor gene name (e.g *Neurod2*) and this same transcription factor network (e.g Neurod2(+)). Abbreviations: GRN – Gene regulatory network, Nr3c1 – Nuclear receptor subfamily 3 group C member 1 (glucocorticoid receptor), Nr3c2 – Nuclear receptor subfamily 3 group C member 2 (mineralocorticoid receptor), Astro – Astrocytes, Oligo – Oligodendrocytes, Endo – Endothelial cells, Micro-PVM – Microglia/ Perivascular macrophages, Lamp5 – Lysosomal associated membrane protein family member 5, Vip – Vasoactive intestinal peptide, Pvalb – Parvalbumin, Sncg – Synuclein gamma, Sst – Somatostatin, DG – Dentate Gyrus, CA1-ProS – Cornus ammonis 1-Prosubiculum, CA2 – Cornus ammonis 2, CA3 – Cornus ammonis 3, AF-2 – ligand-dependent transactivation domain 2 (helix 12).

Supplementary tables (cf. online publication)

Supplementary Table 1. Differential expression analysis of adult mouse hippocampus gene expression matrix. gene: gene name; cluster: cell type; avg_ logFC: $\log_2$(FC) of the average expression within the cluster vs. outside the cluster; pct.1: % of positive cells for the gene within the cluster; pct.2: % positive cells for the gene outside the cluster; p_val_adj: adjusted p-value (p_val_adj = 0 means that the adjusted p-value is inferior to 2.2e-308 and is therefore too low to be displayed in R).

Supplementary Table 2. Output of the meta-analysis on glucocorticoid responsive genes based on previous transcriptomic and ChIP-sequencing studies. included. studies: detailed description of transcriptomic and ChIP-sequencing studies included in the meta-analysis (1); RNA.seq_current.study: outcome of differential gene expression analysis of mouse ventral hippocampus bulk RNA-seq data with corticosterone vs. vehicle treatment (2); GC_responsive.genes: list of all genes reported in the included transcriptomic and ChIP-sequencing studies (3); gene. search: search function for individual gene outcome based on meta-analysis results and description of the meta-analysis information (4); List_main.targets: list of the genes described within the manuscript in Fig. 3B, Fig.3C and Supp. Fig. 1D (5).

Supplementary Table 3. Coupling score C_{ij}^{ts} matrices raw data. Coupling scores for Nr3c1 and Nr3c2 with each other (1), with sex hormone receptors (2), with AF-2 coregulators (3), and with neurotransmitter and neuropeptide receptors (4).

Supplementary Table 4. Mouse hippocampus gene regulatory network (GRN) normalized activity matrix. Gene regulatory networks (GRNs) X Cell types.

Supplementary Table 5. Differential activity analysis of adult mouse hippocampus gene regulatory networks (GRNs). cluster: cell type; network: name of the transcription factor gene regulatory network; avg_logFC: $\log_2$(FC) of the average GRN activity within the cluster vs. outside the cluster; pct.1: % of positive cells for the GRN within the cluster; pct.2: % positive cells for the GRN outside the cluster; p_val_adj: adjusted p-value (p_val_adj = 0 means that the adjusted p-value is inferior to 2.2e-308 and is therefore too low to be displayed in R).