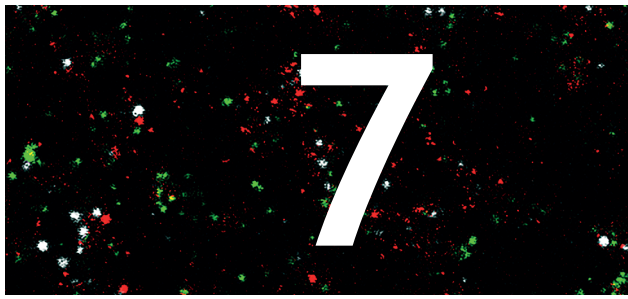
Stress, obesity and mood disorders: towards breaking a vicious cycle

Koorneef, L.L.



Do corticosteroid receptor mRNA levels predict the expression of their target genes?

Lisa L. Koorneef
Eva M. G. Viho
Lucas F. Wahl
Onno C. Meijer

In preparation

ABSTRACT

The corticosteroid stress hormones affect brain function via high-affinity mineralocorticoid receptors (MRs) and lower affinity glucocorticoid receptors (GRs). MR and GR not only differ in affinity for ligand, but also have distinct, sometimes opposite, actions on neuronal excitability and on other cellular and higher order parameters. MR and GR mRNA expression is often used as a proxy for the responsiveness to stress hormones. In this study we explored in male mice how GR and MR mRNA levels are associated with the expression of a shared target gene, glucocorticoid-induced leucine zipper (GILZ, coded by *Tsc22d3*), using *in situ* hybridization. In the hippocampus and hypothalamus, mRNA levels of MR rather than GR mostly correlated with GILZ mRNA under basal conditions and after a corticosterone injection at the bulk tissue level. Analyzed per individual cell, these correlations were much weaker. Using publicly available single cell RNA sequencing data, we again observed that MR and GR mRNA levels were only weakly correlated with target gene expression in both glutamatergic and GABAergic neurons. MR levels can be limiting for receptor action, but many other cell-specific and region-specific factors ultimately determine corticosteroid receptor action. Altogether, our results argue for caution whilst interpreting the consequences of changed receptor expression for the response to glucocorticoids.

INTRODUCTION

Glucocorticoids have profound effects on adaptive and cognitive processes in the brain, which allows for adaptation to stressors. Glucocorticoids bind to the high-affinity mineralocorticoid receptor (MR, coded by *Nr3c2*) and lower affinity glucocorticoid receptor (GR, coded by *Nr3c1*), which each coordinate distinct processes [1]. MR and GR act in large measure as ligand-activated transcription factors. They have a very similar central DNA binding domain (DBD), via which they bind to accessible glucocorticoid response elements (GREs) at the genome. Although MR and GR share downstream target genes, they also each regulate specific target genes based on additional interactions at the DNA [2, 3]. GR can additionally repress gene transcription via 'negative GREs' or by interfering with the activity of other transcription factors such as AP-1 and NF-kB via tethering [4-6].

In the hippocampus, where MR has its highest expression, GR and MR are mainly known to functionally oppose each other, but synergistic actions have also been described [1, 7-9]. At the cellular level (e.g. neuronal excitability), these opposite actions are observed as a u-shaped dose-dependent response to corticosterone [7, 10, 11]. In those cases, the hormone effects depend on the balance between GR and MR mediated actions. At the organism level, this has led to the theory that a proper GR:MR balance is essential for behavioral adaptation and neuroendocrine functioning [12, 13]. In theory, a combination of transactivation at low steroid levels via MR, followed by transrepression via GR at higher steroid levels would allow for such a u-shaped transcriptional response. However, regardless of the directionality of the MR and GR mediated effects, receptor expression levels are thought to be important for hormone responsiveness.

Because glucocorticoid effects on the brain are crucial for stress adaptation and mental health, many studies assess steroid responsiveness by measurement of MR and GR mRNA levels. Particularly for MR this may be relevant, as its high affinity leads to substantial ligand occupancy even at basal (trough) levels of hormone. Regulation of receptor abundance would then be a way to change the strength of what is a tonic signal via MR [14]. The lower affinity of GRs makes corticosteroid hormone concentration a first limiting step. However, once GRs get activated, receptor number may be limiting for particular transcriptional and cellular responses.

In this study we explored in the mouse brain how GR and MR mRNA levels, and the GR:MR balance, are correlated with the expression of shared target gene glucocorticoid-induced leucine zipper (GILZ, coded by *Tsc22d3*). This association was investigated at both the tissue and cellular level in the hippocampus and paraventricular nucleus of the hypothalamus (PVN) of mice given a vehicle or corticosterone injection prior to sacrifice. We chose the hippocampus and the PVN as our regions of interest, because GR and MR are both expressed and functionally important in these brain structures.

GR, MR and GILZ mRNA levels were measured with *in situ* hybridization (ISH), as ISH is one of the few quantitative methods with spatial resolution. To further distinguish between neuronal cell types, the relation between corticosteroid receptors and target gene expression was also analyzed using the recent Allen Brain Atlas single-cell RNA sequencing dataset of the mouse hippocampus.

MATERIALS AND METHODS

Animals

This study has been approved by the institutional ethics committee on animal care and experimentation at the Leiden University Medical Center. The 8-week old male C57Bl/6J mice (Charles River, Sulzfeld, Germany) were housed in ventilated cages with a 12 h light : 12 h darkness regime and *ad libitum* access to food and water. Mice were injected subcutaneously with 200 µL solvent (EtOH:PBS 1:20) or 1.5 mg/kg corticosterone (Sigma- Aldrich, Zwijndrecht, Netherlands) 1 h prior to killing by cervical dislocation. Brains were collected and stored at -80° for ISH.

In situ hybridization

Frozen, unfixed brain slices (12 µm) were collected on glass slides at bregma -0.772 mm (PVN) and -1.532 mm (hippocampus). 4-plex ISH was performed using the RNA-Scope fluorescent multiplex assay and RNAscope 4-plex ancillary kit (Advanced Cell Diagnostics, Newark, CA) and opal fluorophores 520, 570, 620 and 690 (PerkinElmer, Hoogvliet Rotterdam, the Netherlands). Nuclei were counterstained with DAPI staining (Advanced Cell Diagnostics). Results of the FKBP5 hnRNA probe that we included were later excluded from the analysis, as the signal was too low. Pictures were captured with a confocal microscope (SP8 WLL, Leica Microsystems).

Image analysis

All pictures were analysed in 8-bit with ImageJ Software (NIH, USA). Cells were selected based on nuclear DAPI staining. First, Gaussian blur was used to autofill nuclei and the contrast of all DAPI images was enhanced. Nuclei were then selected by a fixed threshold and the outer edge was enlarged by a fixed size, i.e., 5 pixels for CA1, CA3 and PVN and 0.5 for the nucleus-dense DG. Touching nuclei were separated with the watershed function, after which nuclei were selected using the 'analyse particles' function. Quality of selections were manually verified and overlapping cells were deleted from the analysis. The signal of each probe was then measured within these cell selections with automatic thresholding. The 'otsu' method was used for the quantification of the MR signal in all regions, and of the GR and GILZ signal in CA1, CA3 and PVN [15]. The 'moments' method

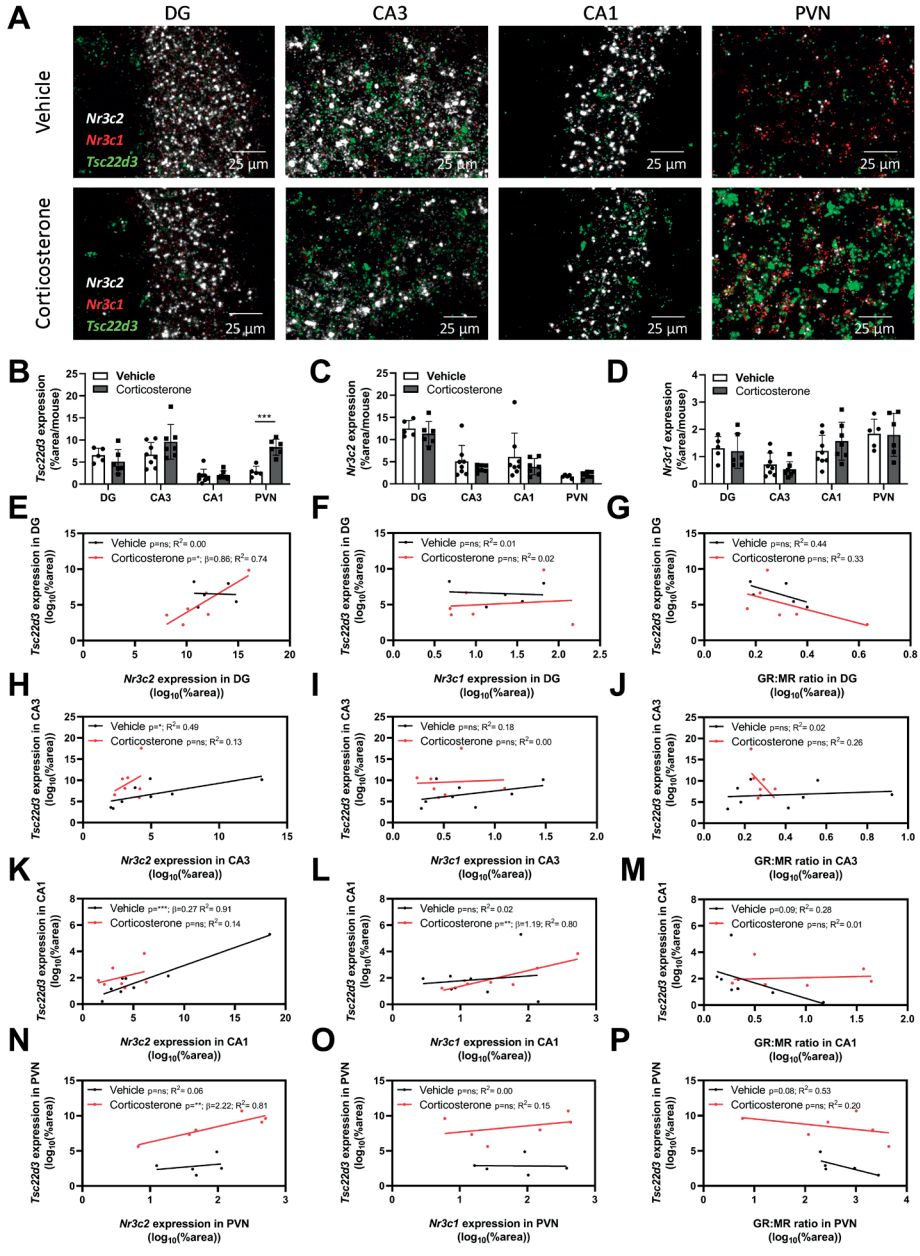
was used to measure GR and GILZ signal in the DG [16]. Expression was defined as the percentage area covered by the signal compared to the total cell area. Cells were classified as GILZ-positive if the percentage area was >0 in CA3, CA1 and PVN, and >3 in the DG. To display the cell distribution per brain region and treatment, cells were analysed with the single-cell clustering package Seurat v.3.1.5 in R v4.0.0 [17]. The same package was used to determine the number of genes expressed per cell, as well as the distribution of MR, GR and GILZ mRNA expression per cell and per mouse [17].

Single-cell RNA sequencing data resources, metrics and processing

Single cell data were obtained from the SMART-seq single-cell RNA sequencing dataset published by the Allen Institute for Brain Science [18]. The gene expression matrix and the table of cell metadata were downloaded from https://portal.brain-map.org/atlases-and-data/rnaseq/mouse-whole-cortex-and-hippocampus-smart-seq. The metadata were used to subset cells of the hippocampal formation from the gene expression matrix. We selected 9 subclasses of hippocampal cells according to the Common Cell Type Nomenclature guidelines, including 5 GABAergic neuronal cell types and 4 glutamatergic neuronal cell types [19]. The final hippocampus gene expression matrix resulted in 6474 cells for 39236 genes. The newly obtained matrix was pre-processed according to the Seurat v.3.1.5 pipeline in R v4.0.0, using the following criteria: *min.cells = 1, min.features = 100, 2500 < nFeature_RNA < 13000, nCount_RNA < 1.00e+06, normalized.method = "LogNormalize", scale.factor = 10000, selection.method = "vst", nfeatures = 2000* [17]. We performed principal component analysis and selected the top 50 principal components. These components were used as input for the t-distributed stochastic neighbor embedding reduction (t-SNE), using the following settings: *dims = 1:50, perplexity = 50, max_iter = 1000*. The transcriptomic data were analyzed and displayed as scatterplots with linear regression using ggplot2 tools in R v4.0.0.

Statistical analysis

Simple linear regression and t-tests were performed with GraphPad Prism (version 8.1.1.330, GraphPad Software, La Jolla, CA, USA). Stepwise multiple linear regression was performed in SPSS Statistics (version 25.0, IBM Corporation, Armonk, NY, USA) and single-cell RNA sequencing data were analyzed in R v4.0.0. All data are expressed as mean ± S.E.M. All *P*-values are two-tailed and $P < 0.05$ was considered as statistically significant. Two groups having equal S.D. were analyzed with an independent sample t-test. Two groups having different S.D. were analyzed with a Welch's t-test. To calculate significance of correlates at the tissue level, a stepwise multiple linear regression was performed with GILZ mRNA expression as dependent variable, and GR mRNA expression, MR mRNA expression and GR:MR ratio as independent variables. Significance of correlates at the single cell level were calculated with simple linear regression. In all


A
DG
CA3
CA1
PVN
Vehicle
Corticosterone
Nr3c2
Nr3c1
Tsc22d3
25 µm
B
C
D
Vehicle
Corticosterone
Tsc22d3 expression (%area/mouse)
Nr3c2 expression (%area/mouse)
Nr3c1 expression (%area/mouse)
***
DG
CA3
CA1
PVN
E
F
G
Tsc22d3 expression in DG (log10(%area))
Vehicle p=ns; R2= 0.00
Corticosterone p=*; β=0.86; R2= 0.74
Vehicle p=ns; R2= 0.01
Corticosterone p=ns; R2= 0.02
Vehicle p=ns; R2= 0.44
Corticosterone p=ns; R2= 0.33
Nr3c2 expression in DG (log10(%area))
Nr3c1 expression in DG (log10(%area))
GR:MR ratio in DG (log10(%area))
H
I
J
Tsc22d3 expression in CA3 (log10(%area))
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Corticosterone p=ns; R2= 0.13
Vehicle p=ns; R2= 0.18
Corticosterone p=ns; R2= 0.00
Vehicle p=ns; R2= 0.02
Corticosterone p=ns; R2= 0.26
Nr3c2 expression in CA3 (log10(%area))
Nr3c1 expression in CA3 (log10(%area))
GR:MR ratio in CA3 (log10(%area))
K
L
M
Tsc22d3 expression in CA1 (log10(%area))
Vehicle p=***; β=0.27 R2= 0.91
Corticosterone p=ns; R2= 0.14
Vehicle p=ns; R2= 0.02
Corticosterone p=**; β=1.19; R2= 0.80
Vehicle p=0.09; R2= 0.28
Corticosterone p=ns; R2= 0.01
Nr3c2 expression in CA1 (log10(%area))
Nr3c1 expression in CA1 (log10(%area))
GR:MR ratio in CA1 (log10(%area))
N
O
P
Tsc22d3 expression in PVN (log10(%area))
Vehicle p=ns; R2= 0.06
Corticosterone p=**; β=2.22; R2= 0.81
Vehicle p=ns; R2= 0.00
Corticosterone p=ns; R2= 0.15
Vehicle p=0.08; R2= 0.53
Corticosterone p=ns; R2= 0.20
Nr3c2 expression in PVN (log10(%area))
Nr3c1 expression in PVN (log10(%area))
GR:MR ratio in PVN (log10(%area))

← **Figure 1: MR mRNA levels predict GILZ mRNA expression in vehicle and corticosterone-treated mice in the hippocampus and PVN.** Mice received a vehicle or corticosterone injection 1 h prior to killing. A) *In situ* hybridization was performed to visualize MR (*Nrc3c2, white*), GR (*Nrc3c1, red*) and GILZ (*Tsc22d3, green*) mRNA in the hippocampal DG, CA3 and CA1 and in the paraventricular nucleus (PVN). Mean expression of *Tsc22d3, Nr3c2* and *Nr3c1* in all cells were calculated per mouse. B) Corticosterone induced *Tsc22d3* expression in PVN, but not in DG, CA3 and CA1. C-D) Corticosterone did not influence *Nrc3c1* and *Nrc3c2* expression in hippocampus and PVN. E-G) In the DG, *Nr3c2* expression, but not *Nrc3c1* or GR:MR ratio predicted *Tsc22d3* expression in corticosterone-treated mice. H-M) *Tsc22d3* expression correlated with *Nr3c2* in CA3 and CA1 of vehicle-treated mice, and with *Nr3c1* in CA1 of corticosterone-treated mice. N-P) In the PVN, *Nr3c2* expression, but not *Nr3c1* or GR:MR ratio, predicted *Tsc22d3* expression in corticosterone-treated mice. Values are means ± S.E.M. of n = 5-8 animals. Significance was calculated with an independent sample t-test in B-D) and with stepwise multiple linear regression in E-P). The graphs show the significance (p), slope (ß) and strength (R^2) of the correlations found.*P < 0.05, **P < 0.01, ***P < 0.001.

regression analyses the pattern of residuals was random, supporting the linear fit of the model. Pearson's correlation coefficient was calculated to determine the strength of relationships.

RESULTS

Corticosterone induced GILZ mRNA expression in the PVN, but not in hippocampal regions.

Cellular GR (*Nr3c1*), MR (*Nr3c2*), and GILZ (*Tsc22d3*) mRNA levels were quantified with ISH on brains of mice that were pretreated with vehicle or corticosterone. MR and GILZ mRNAs were highly expressed in all regions of the hippocampus and PVN (**Fig. 1A**). GR mRNA levels were high in PVN, CA1 and DG, but lower in the CA3 (**Fig. 1A**). To quantify the expression of each gene per cell, cells were identified by enlarging the outer edge of nuclei which had been selected by DAPI staining. The effect of corticosterone treatment at the bulk tissue level was determined by calculating the mean mRNA expression of GILZ, MR and GR in all cells per mouse. Corticosterone induced GILZ mRNA expression significantly in the PVN, but not in the DG, CA3 and CA1 (**Fig. 1B**). Corticosterone did not significantly affect MR and GR mRNA levels in any of the regions examined (**Fig. 1C-D**).

MR mRNA levels predict GILZ mRNA expression in vehicle and corticosterone-treated mice in the hippocampus and PVN.

To investigate if GR and MR mRNA levels and GR:MR ratio predict GILZ mRNA expression, a stepwise multiple linear regression was performed on the mean values per mouse. MR mRNA levels strongly correlated with GILZ mRNA in the DG of corticosterone-treated mice (R^2=0.74, **Fig.1E**). No significant correlation was found between GR and GILZ mRNA in this region (**Fig. 1F**). A non-significant, inverse correlation was found between GR:MR ratio and GILZ mRNA expression, likely simply reflecting the positive correlation of GILZ

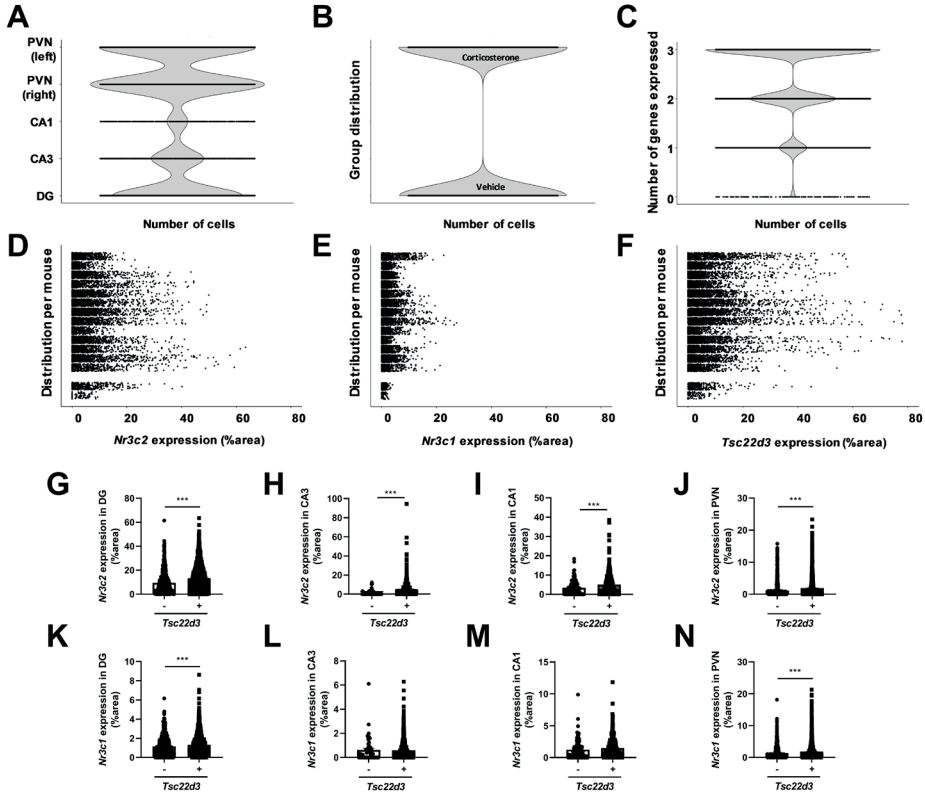


Figure 2: MR mRNA levels are higher in GILZ-positive cells in the hippocampus and PVN. Mice received a vehicle or corticosterone injection 1 h prior to killing. *In situ* hybridization was performed to quantify MR (*Nrc3c2*), GR (*Nrc3c1*) and GILZ (*Tsc22d3*) mRNA in the hippocampal DG, CA3 and CA1 and in the paraventricular nucleus (PVN). A) PVN and DG were the most nucleus dense brain regions. B) When cells from all regions were pooled, an equal number of cells came from vehicle- and corticosterone-treated mice. C-F) Most cells expressed both *Nr3c2, Nr3c1,* and *Tsc22d3*, but expression highly varied within and between animals. G-J) *Nrc3c2* expression was higher in GILZ-positive than in GILZ- negative cells in all investigated regions. K-N) *Nrc3c1* expression was higher in GILZ-positive cells in DG and PVN, but not in CA1 and CA3. Values are means ± S.E.M. of n = 5-8 animals. Significance was calculated with a Welch's t-test. *** $P < 0.001$.

and MR mRNA. (**Fig. 1G**). In the CA3 and CA1, MR mRNA levels predicted GILZ mRNA expression in vehicle-treated animals (R^2 = 0.49 and 0.91 respectively, **Fig. 1H-K**). In the CA1, also GR mRNA levels were strongly correlated with GILZ mRNA in corticosterone-treated animals (R^2= 0.80, **Fig. 1L**). GR:MR ratio was not strongly associated with GILZ mRNA in this region (**Fig. 1M**). In the PVN, MR mRNA levels significantly correlated with GILZ mRNA expression in corticosterone-treated animals (R^2= 0.81, **Fig. 1N-P**), but not under basal conditions. In conclusion, MR mRNA levels predicted GILZ mRNA expression both under basal and corticosterone conditions in the hippocampus, and after corticosterone treatment in the PVN, while GR mRNA levels predicted GILZ mRNA

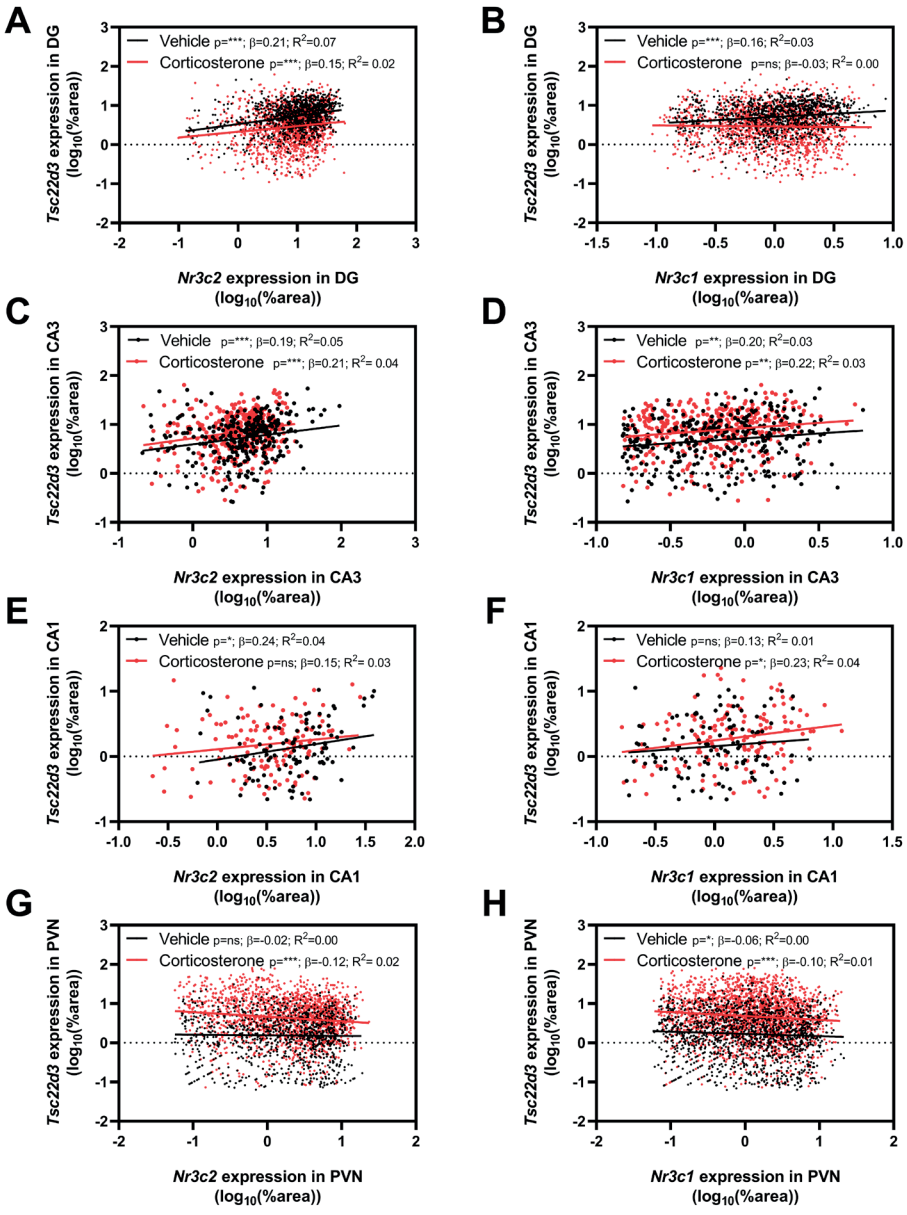
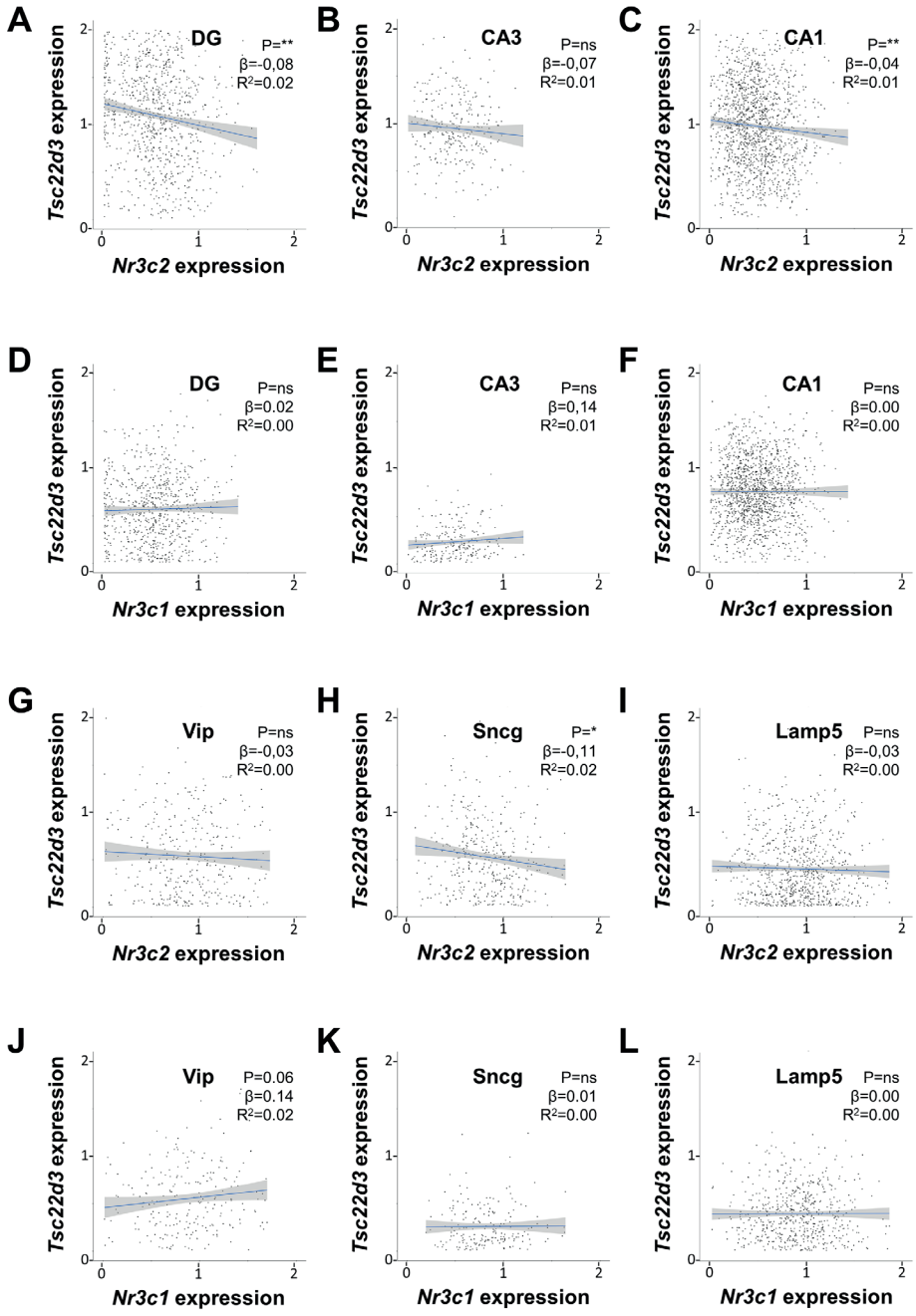


Figure 3: MR and GR mRNA levels poorly predict *Tsc22d3* expression in hippocampal glutamatergic and GABAergic neurons. Mice received a vehicle or corticosterone injection 1 h prior to killing. *In situ* hybridization was performed to quantify MR (*Nrc3c2*), GR (*Nrc3c1*) and (*Tsc22d3*) mRNA in the hippocampal DG, CA3 and CA1 and in the paraventricular nucleus (PVN). Cells from all mice were pooled per treatment. Significant, but very weak correlations between *Nr3c2*, *Nr3c1* and *Tsc22d3* were found in both vehicle- and corticosterone-treated animals in A-F) DG, CA3, CA1 and in G-H) PVN. All expression data were log10-transformed. Significance was calculated with simple linear regression. The graphs show the significance (p), slope (ß) and strength (R^2) of the correlations found * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.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P=**
β=-0,08
R²=0.02
B
CA3
P=ns
β=-0,07
R²=0.01
C
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P=**
β=-0,04
R²=0.01
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DG
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R²=0.00
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β=0.00
R²=0.00
Tsc22d3 expression
Nr3c2 expression
Nr3c1 expression

← **Figure 4: MR and GR mRNA levels do not predict GILZ mRNA expression in hippocampal glutamatergic and GABAergic neurons.** Cellular MR (*Nr3c2*), GR (*Nr3c1*) and GILZ (*Tsc22d3*) mRNA levels were obtained from a previously published single-cell sequencing data set of naïve mouse hippocampi. All expression values are log-normalized and scaled according to the Seurat pipeline. MR and GR mRNA levels poorly predicted *Tsc22d3* expression in A-F) glutamatergic neurons of the DG, CA3 and CA1-regions or in G-L) various subclasses of GABAergic neurons, i.e. Vip, Sncg and Lamp5 neurons. Data were analyzed with simple linear regression. The graphs show the 95% confidence interval, as well as the significance (p), slope (β) and strength (R^2) of the correlations found. * $P < 0.05$, ** $P < 0.01$.

only in the CA1 of corticosterone-treated animals. GR:MR ratio was not an independent determinant for GILZ gene regulation and therefore excluded from further analyses.

Cellular MR, GR, or GILZ mRNA levels highly vary within and between animals.

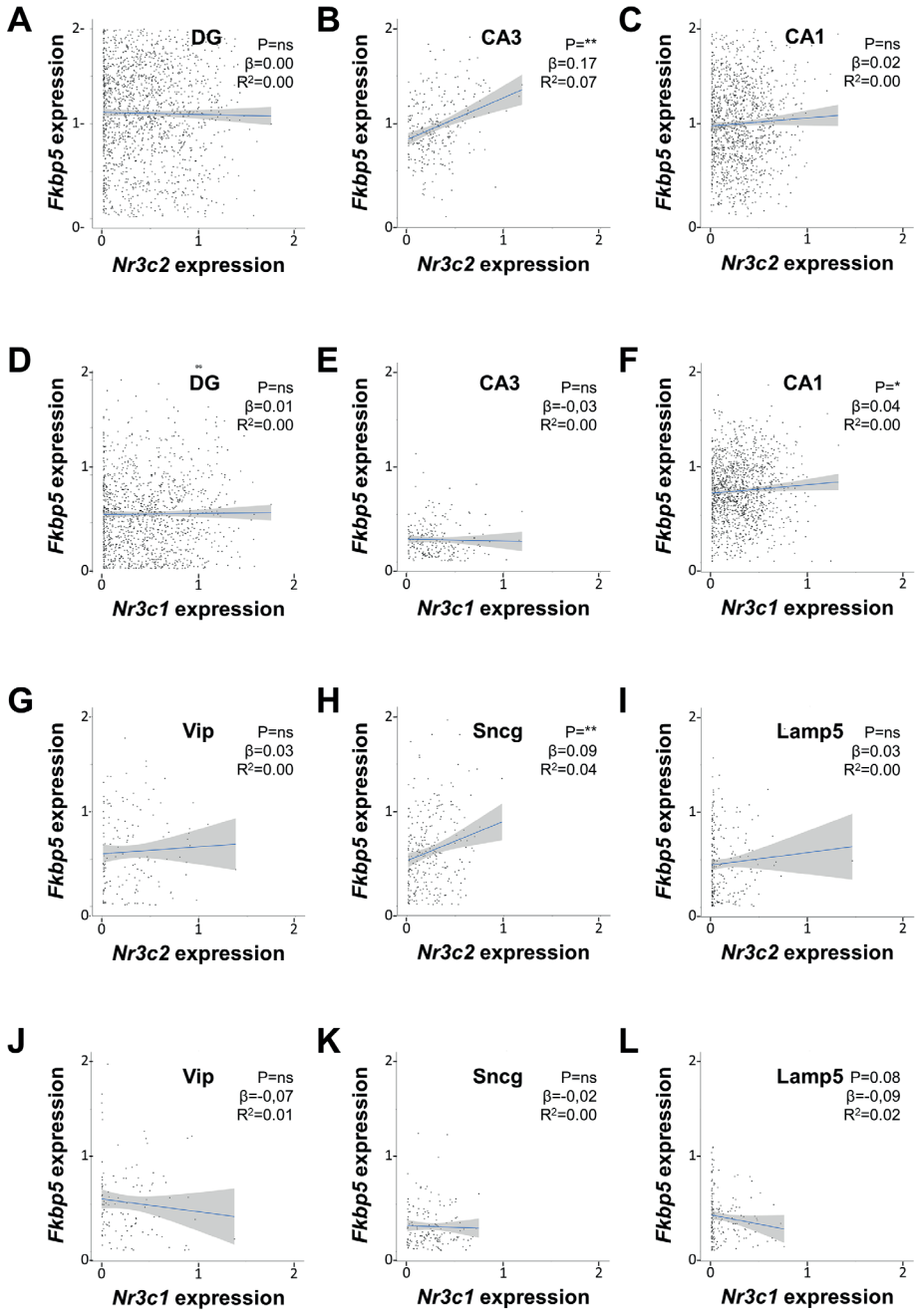
To explore the relationship between receptor levels and GILZ mRNA expression at the cellular level, we first assessed the distribution and variability of the dataset. Most of the cells were detected in the PVN and DG, while the CA1 was the least nucleus-dense (**Fig. 2A**). The number of cells in each treatment group was comparable when cells from all regions were pooled (**Fig. 2B**). The majority of cells expressed both corticosteroid receptors and GILZ and very few cells expressed none of the three genes of interest (**Fig. 2C**). When clustered per mouse, it was apparent that the cellular expression of MR, GR, or GILZ mRNA highly varied within and between animals (**Fig. 2D-F**). Of the two mice with the lowest total cell number, DG cells were excluded from analysis due to technical issues (**Fig. 2D-F**).

MR mRNA levels are higher in GILZ-positive cells in the hippocampus and PVN

To evaluate the enrichment of corticosteroid receptors in GILZ-responsive cells in hippocampus and PVN, we measured MR and GR mRNA expression in GILZ-negative and positive cells per region. MR mRNA levels were higher in GILZ-positive cells than in GILZ-negative cells in all investigated regions (**Fig. 2G-J**). However, GR mRNA levels were higher in GILZ-positive cells only in the DG and PVN (**Fig. 2K-N**). The higher expression of the GILZ target gene in the presence of corticosteroid receptors may in itself indicate functionality of the receptors.

MR and GR mRNA levels weakly predict GILZ mRNA expression at the cellular level in the hippocampus and PVN

To explore whether GR and MR mRNA levels could predict GILZ mRNA at the cellular level, single linear regression was performed on all cells of mice within the same treatment group. Significant, positive correlations between MR, GR and GILZ mRNA expression were found in vehicle- and corticosterone-treated animals in all hippocampal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R²=0.01
K
Sncg
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R²=0.00
L
Lamp5
P=0.08
β=-0,09
R²=0.02
Fkbp5 expression
Nr3c2 expression
Nr3c1 expression

← **Figure 5: MR and GR mRNA levels do not predict *Fkbp5* expression in hippocampal glutamatergic and GABAergic neurons.** Cellular MR (*Nr3c2*), GR (*Nr3c1*) and GILZ (*Tsc22d3*) mRNA levels were obtained from a previously published single-cell sequencing data set of naïve mouse hippocampi. All expression values are log-normalized and scaled according to the Seurat pipeline. MR and GR mRNA levels poorly predicted *Fkbp5* expression in A-F) glutamatergic neurons of the DG, CA3 and CA1-regions, or in G-L) various subclasses of GABAergic neurons, i.e. Vip, Sncg and Lamp5 neurons. Data were analyzed with simple linear regression. The graphs show the 95% confidence interval, as well as the significance (p), slope (β) and strength (R^2) of the correlations found. * $P < 0.05$, ** $P < 0.01$.

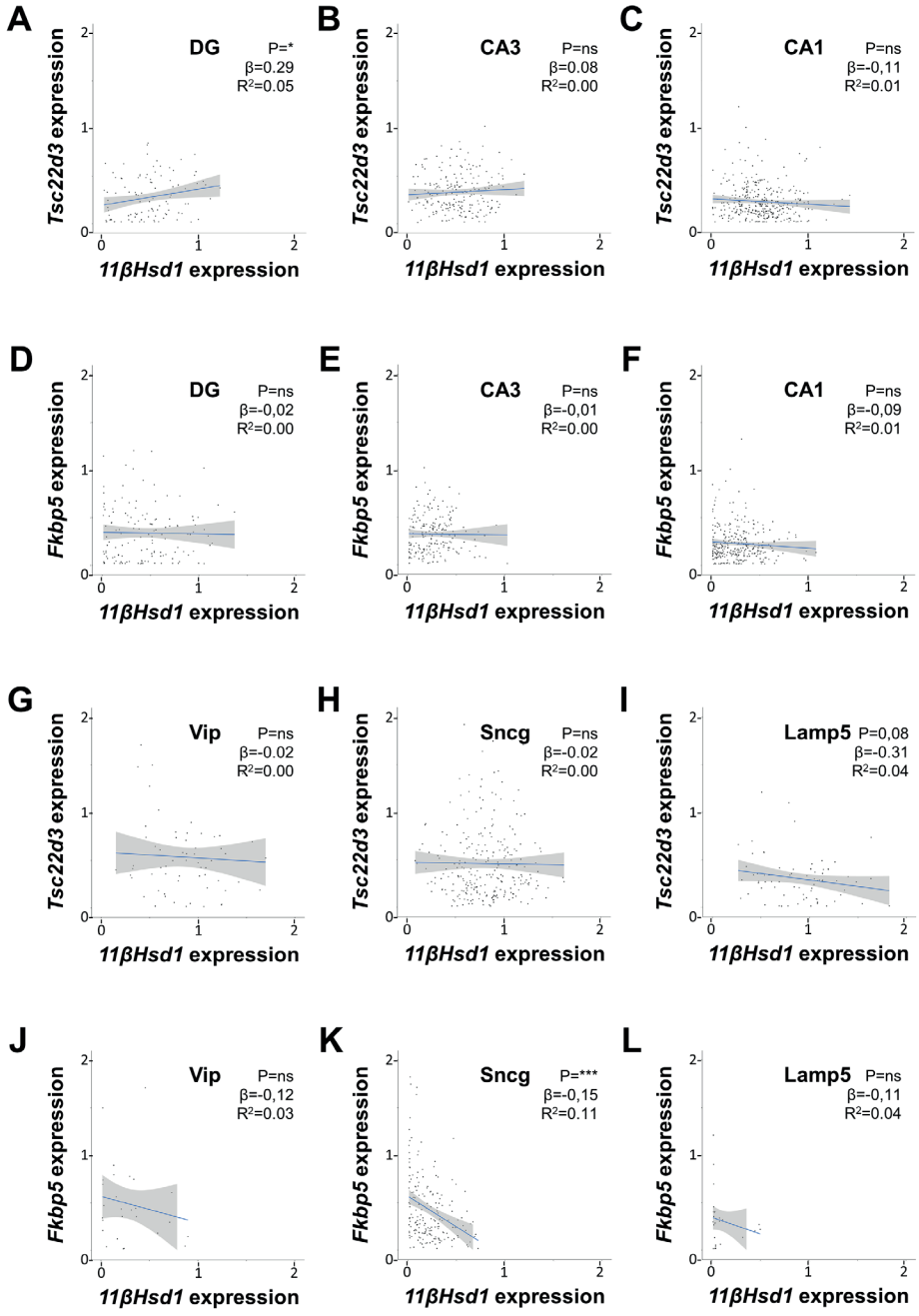
regions (**Fig. 3A-F**). However, the correlations were generally very weak (R^2<0.08), also when data were evaluated at the cellular level per mouse individually (data not shown). Similar results were obtained in the PVN (R^2 < 0.03, **Fig. 3G-H**). Therefore we conclude that, despite some significant correlations, GR and MR mRNA levels poorly predict GILZ mRNA expression at the cellular level, as measured with ISH.

MR and GR mRNA levels poorly predict GILZ mRNA expression in hippocampal glutamatergic and GABAergic neurons under basal conditions

The ISH data do not differentiate between neuronal cell types and the weak correlations may additionally be due to the low sensitivity of the assay or undersampling. We therefore explored the association between corticosteroid receptors and GILZ mRNA expression using a previously published single-cell RNA sequencing dataset of naïve mouse hippocampi. In this dataset, 4 glutamatergic and 5 GABAergic neuronal types could be identified. Glutamatergic neurons were distinguished based on the subclassification of hippocampal regions (CA-regions and dentate gyrus). The CA2-region was excluded from regression analyses due to low cell counts. In glutamatergic neurons, MR mRNA levels were significantly and negatively correlated with GILZ mRNA in the CA1 and the DG, but not in CA3 (**Fig. 4A-C**). However, strength of these correlations was again very weak (R^2 < 0.03). No significant correlations were found between GR and GILZ mRNA levels in glutamatergic neurons (**Fig. 4D-F**). From the 5 GABAergic neurons identified, the parvalbumin and somatostatin neurons were excluded from analyses because of low cell counts, leaving the lysosomal associated membrane protein (Lamp5), synuclein gamma (Sncg) and vasoactive intestinal peptide (Vip) subclasses. A significant, but very weak negative correlation was found between MR and GILZ mRNA in Sncg neurons, but not in Lamp5 and Vip neurons (R^2 = 0.02, **Fig. 4G-I**). GR mRNA levels did not correlate with GILZ mRNA in GABAergic neurons (**Fig. 4J-L**). In conclusion, MR and GR mRNA levels poorly predicted GILZ mRNA expression in both glutamatergic and GABAergic neurons.

MR and GR mRNA levels do not predict *Fkbp5* expression in hippocampal glutamatergic and GABAergic neurons

To assess the generality of our findings, we extended our analysis in the single cell sequencing dataset to another shared GR and MR target gene, i.e. FK506 binding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DG
P=*
β=0.29
R²=0.05
Tsc22d3 expression
11βHsd1 expression
B
CA3
P=ns
β=0.08
R²=0.00
Tsc22d3 expression
11βHsd1 expression
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CA1
P=ns
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R²=0.01
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11βHsd1 expression
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DG
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β=-0,02
R²=0.00
Fkbp5 expression
11βHsd1 expression
E
CA3
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β=-0,01
R²=0.00
Fkbp5 expression
11βHsd1 expression
F
CA1
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Fkbp5 expression
11βHsd1 expression
G
Vip
P=ns
β=-0.02
R²=0.00
Tsc22d3 expression
11βHsd1 expression
H
Sncg
P=ns
β=-0.02
R²=0.00
Tsc22d3 expression
11βHsd1 expression
I
Lamp5
P=0,08
β=-0.31
R²=0.04
Tsc22d3 expression
11βHsd1 expression
J
Vip
P=ns
β=-0,12
R²=0.03
Fkbp5 expression
11βHsd1 expression
K
Sncg
P=***
β=-0,15
R²=0.11
Fkbp5 expression
11βHsd1 expression
L
Lamp5
P=ns
β=-0,11
R²=0.04
Fkbp5 expression
11βHsd1 expression

← **Figure 6: *11ßHsd1* expression weakly correlates with GILZ or FKBP5 mRNA expression in hippocampal glutamatergic and GABAergic neurons.** Cellular 11ßHSD1, GILZ and FKBP5 mRNA levels were obtained from a previously published single-cell sequencing data set of naïve mouse hippocampi. All expression values are log-normalized and scaled according to the Seurat pipeline. A-F) *11ßHsd1* expression poorly predicted *Tsc22d3* or *Fkbp5* expression in A-F) glutamatergic neurons of the DG, CA3 and CA1-regions, or in G-L) various subclasses of GABAergic neurons, i.e. Vip, Sncg and Lamp5 neurons. Data were analyzed with simple linear regression. The graphs show the 95% confidence interval, as well as the significance (p), slope (ß) and strength (R^2) of the correlations found. * $P < 0.05$, *** $P < 0.001$.

protein 5 (*Fkbp5*). Using the same single-cell RNA sequencing dataset, GR and MR mRNA levels were correlated with *Fkbp5* expression in hippocampal glutamatergic and GABAergic neurons. A significant, but very weak correlation was found between MR and FKBP5 mRNA levels in glutamatergic neurons of the CA3-region, but not in DG and CA1 (R^2=0.07, **Fig. 5A-C**). GR mRNA levels did not significantly predict *Fkbp5* expression in glutamatergic neurons (**Fig. 5D-F**). Only in GABAergic Sncg neurons MR mRNA levels significantly, but weakly predicted *Fkbp5* expression (R^2=0.04, **Fig. 5G-I**). GR mRNA levels were not associated with *Fkbp5* in hippocampal GABAergic neurons (**Fig. 5J-L**). To conclude, *Fkbp5* expression could not robustly be predicted by corticosteroid receptor expression in hippocampal glutamatergic and GABAergic neurons.

11ßHsd1 expression weakly correlates with GILZ and FKBP5 mRNA expression in hippocampal glutamatergic and GABAergic neurons

Next to receptor levels, ligand levels importantly determine the transcriptional activity of corticosteroid receptors. The HPA-axis controls circulating glucocorticoid hormone levels, while local variations in hormone levels are caused by activity of the 11ß-hydroxysteroid dehydrogenase type 1 (*11ßHsd1*) enzyme, which converts glucocorticoids from their inactive to their active form. Therefore, we investigated how *11ßHsd1* expression relates to GILZ and FKBP5 mRNA levels at the single cell level. In glutamatergic neurons, *11ßHsd1* poorly predicted GILZ and FKBP5 mRNA expression (R^2<0.06, **Fig. 6A-F**). No significant correlations were found between 11ßHSD1 and GILZ mRNA in GABAergic neurons (**Fig. 6G-I**). A significant, but weak correlation was found between *11ßHsd1* and *Fkbp5* expression in Sncg neurons (R^2=0.11, **Fig. 6J-L**). Taken together, *11ßHsd1* expression did not explain target gene expression in either glutamatergic or GABAergic neurons.

DISCUSSION

This study explored how GR and MR mRNA levels and GR:MR balance predict receptor activity, as measured by mRNA expression of target genes GILZ and FKBP5. At the tissue level, MR mRNA levels were strongly positively correlated with GILZ mRNA under vehicle

conditions in CA1 and CA3, and under corticosterone conditions in DG and PVN. In addition, MR mRNA levels were consistently higher in GILZ-positive cells. GR mRNA levels did not correlate with GILZ mRNA expression, except in the CA1 of corticosterone-treated animals. The effect of GR:MR ratio on GILZ mRNA expression was a net sum of the individual effects of GR and MR. At the cellular level, MR and GR mRNA levels generally poorly predicted GILZ mRNA levels, both in data generated by ISH and single-cell RNA sequencing. Similarly, MR and GR mRNA levels were not associated with *Fkbp5* expression at the single cell level. Taken together, it can be revealed at the tissue level that MR, but not GR mRNA levels likely limits the mRNA expression of GILZ in the hippocampus and PVN, but not at the cellular level.

In line with the notion that MR activity is controlled by the relative abundance of receptors due to the high affinity for corticosterone and its occupancy under basal conditions, we observed that MR mRNA levels are limiting for GILZ mRNA expression at the tissue level. It differed per hippocampal subfield whether MR was limiting under low or high ligand conditions. This may be due to region-specific translocation dynamics, which was previously shown to differ between CA1 and DG, although this does not directly explain our findings [20]. There may also be regional differences in post-translational modifications or expressed splice variants of MR, which both influence the sensitivity and transcriptional activity of the receptor [21-23]. Glucocorticoid levels, and thus receptor occupancy, can additionally differ per subregion due to the activity of glucocorticoid-amplifying enzyme 11βHSD1. In our study *11βHsd1* expression did not predict GILZ mRNA expression at basal level, but mRNA levels do not necessarily reflect enzymatic activity. Therefore, it is possible that local 11βHSD1 activity was higher in regions where MR mRNA levels limited target gene expression in vehicle treated animals [24-26]. Supporting that ligand levels limit GR action, GR did not limit GILZ mRNA expression in most investigated regions including CA3, where overall GR expression is low. Since GR was limiting in a region where GR is highly expressed, this again underlines the importance of other cell- or region-specific factors which, next to absolute receptor levels, eventually determine GR-mediated transcription.

We did not find a transcriptional basis for the u-shaped response to glucocorticoids that is functionally observed. The transcriptional changes underlying such a relationship perhaps reflect regulation of MR and GR specific target genes, which would code for proteins with opposite actions [2]. This is also suggested by chromatin immunoprecipitation-sequencing studies which show that GRE binding is the dominant mode of signaling in the rodent hippocampus, arguing against GR-mediated transrepression through tethering to other transcription factors [3, 27, 28]. While a U-shaped response to hormone has been described in the CA1, a bell-shaped and linear relationship has been described for DG and PVN, respectively [8, 9]. Since we did not find differences in

transcriptional regulation of GILZ between these regions, this further supports that the opposite actions of MR and GR involve separate target genes.

Despite the strong correlations at the tissue level, GR and MR mRNA levels poorly predicted GILZ or FKBP5 mRNA expression at the cellular level. It is possible that tissue-correlations reflected an association between receptors and target genes expressed in different cells. In that case, these correlations may not reflect causality between receptor levels and target gene expression within the same cell. This is, however, not very likely, as most cells expressed both receptors and GILZ mRNA. In addition, the enrichment of MR in cells expressing GILZ suggests that MR could even be a driver of basal GILZ mRNA expression. The lack of effect at the cellular level may rather reflect a sensitivity issue of the used techniques or undersampling. For the ISH technique, different cell sizes may have introduced considerable variation in the %area readout parameter. Furthermore, the directionality of the correlations between receptors and target genes may differ per cell type (positive/negative/no effect), which could have reduced the overall effect. This effect dilution is especially observed in the highly heterogenous cell population of the PVN, where significance at the mouse level (R^2=0.81) became negligible (R^2=0.02) at the cellular level. However, when we differentiated between glutamatergic and GABAergic neurons in a single-cell RNA sequencing dataset, we did not find stronger correlations or major differences in directionally between these two cell types.

In this study we chose to measure mRNA levels of corticosteroid receptors as these are often used to assess receptor status (e.g. [29-31]). However, it is likely that parameters other than mRNA levels can better approximate actual receptor activity. Since mRNA levels do not always reflect protein status, protein levels may better reflect the 'capacity' of the receptor [32, 33]. Besides, the transcriptional activation of genes is determined by many other factors, including the bio-availability of the ligand, on/off binding kinetics at the genome, posttranslational modifications of the receptors and the exact protein composition of the receptor-ligand transcription complex [34-36]. Therefore, it is likely that multiple factors need to be included in the regression model to robustly predict target gene expression at the cellular level.

Eventually, we showed that MR mRNA levels, and GR mRNA levels to a smaller extent, could predict GILZ mRNA expression at the tissue level, but not at the cellular level. To assess response to hormone, mRNA levels of corticosteroid receptors and the relative GR:MR balance should be interpreted cautiously in future studies, as many other cell- and region-specific factors determine the glucocorticoid-driven transcriptional outcome.

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