Genomic glucocorticoid signaling in the hippocampus: understanding receptor specificity and context dependency
Weert, L.T.C.M. van

Citation
Weert, L. T. C. M. van. (2021, November 16). *Genomic glucocorticoid signaling in the hippocampus: understanding receptor specificity and context dependency*. Retrieved from https://hdl.handle.net/1887/3240129

CHAPTER 3

Identification of mineralocorticoid receptor target genes in the mouse hippocampus

Lisa T.C.M. van Weert[1,2,3]*, Jacobus C. Buurstede[1]*, Hetty C.M. Sips[1], Sabine Vettorazzi[4], Isabel M. Mol[1], Jakob Hartmann[5], Stefan Prekovic[6], Wilbert Zwart[6], Mathias V. Schmidt[7], Benno Roozendaal[2,3], Jan P. Tuckermann[4], R. Angela Sarabdjitsingh[8], Onno C. Meijer[1]

[1] Department of Medicine, Division of Endocrinology,
Leiden University Medical Center, Leiden, The Netherlands
[2] Department of Cognitive Neuroscience,
Radboud University Medical Center, Nijmegen, The Netherlands
[3] Donders Institute for Brain, Cognition and Behaviour,
Radboud University, Nijmegen, The Netherlands
[4] Institute of Comparative Molecular Endocrinology,
University of Ulm, Germany
[5] Department of Psychiatry, McLean Hospital,
Harvard Medical School, Belmont, MA, USA
[6] Division of Oncogenomics, Oncode Institute,
The Netherlands Cancer Institute, Amsterdam, The Netherlands
[7] Department of Stress Neurobiology and Neurogenetics,
Max Planck Institute of Psychiatry, Munich, Germany
[8] Department of Translational Neuroscience, UMC Utrecht Brain Center,
University Medical Center Utrecht, The Netherlands
*These authors contributed equally

Abstract

Brain mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) respond to the same glucocorticoid hormones, but can have differential effects on cellular function. Several lines of evidence suggest that MR-specific target genes must exist, and might underlie distinct effects of the receptors. Our goal was to identify MR-specific target genes in the hippocampus, a brain region where MR and GR are co-localized and play a role in the stress response. Using genome-wide binding of both receptor types, we previously identified MR-specific, MR-GR overlapping and GR-specific putative target genes. We now report altered gene expression levels of such genes in the hippocampus of forebrain MR knockout (fbMRKO) mice, sacrificed at the time of their endogenous corticosterone peak. Of those genes associated with MR-specific binding, the most robust effect was a 50% reduction in *Jun dimerization protein 2* (*Jdp2*) mRNA levels in fbMRKO mice. Downregulation was also observed for the MR-specific *Nitric oxide synthase 1 adaptor protein* (*Nos1ap*) and *Suv3 like RNA helicase* (*Supv3l1*). Interestingly, the classical glucocorticoid target gene *FK506 binding protein 5* (*Fkbp5*), that is associated with MR and GR chromatin binding, was expressed at substantially lower levels in fbMRKO mice. Subsequently hippocampal *Jdp2* was confirmed to be upregulated in a restraint stress model, posing *Jdp2* as a bona fide MR target that is also responsive in an acute stress condition. Thus, we show that MR-selective DNA binding can reveal functional regulation of genes, and further elucidates distinct MR-specific effector pathways.

Introduction

Endogenous glucocorticoid hormones affect brain function via two closely related nuclear receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The ligand concentration in part determines the specific MR/GR responses. High affinity MRs are occupied by endogenous corticosteroids at basal conditions, and have been found to be more relevant in the initial phase of a stress response (1, 2). In contrast, the lower affinity GRs get activated only at higher glucocorticoid levels, around the peak of the circadian rhythm and during a stress response. While GRs are expressed widely throughout the central nervous system, brain glucocorticoid binding MRs are mainly restricted to limbic areas (3).

In the hippocampus MR and GR are crucial for spatial memory and the modulation of cognition, mood and behavior (3). Within the CA1 hippocampal subregion, MR and GR mediate opposite glucocorticoid effects on pyramidal neuron excitability (4), via transcriptional mechanisms (5). Also spatial learning in rodents is differentially affected by MR and GR signaling, with MR modulating response selection and GR being essential for memory consolidation (6, 7). Because of intrinsic MR-mediated effects that oppose those of GR, it has long been argued that MR-specific target genes must exist (8). The existence of MR-specific transcriptional coregulators (9, 10) also argues this point. However, many effects that can be attributed specifically to MR function so far are rapid non-genomic effects, mediated by the membrane variant of the receptor (11, 12).

Several classical genomic MR-targets have been described in various tissues over the past two decades, such as *FK506 binding protein 5* (*Fkbp5*) (13), *glucocorticoid-induced leucine zipper* (*Gilz*) (14), *period circadian clock 1* (*Per1*) (15) and *serum/glucocorticoid regulated kinase 1* (*Sgk1*) (16). However, these genes are all known to be also GR responsive (17-20). Of note, the two receptors can bind their target DNA as homodimers, but also heterodimerization of MR/GR has been described (15). While MR-selective transrepression and transactivation may occur (21, 22), to date, no hippocampal genomic targets have been reported that are strictly MR-dependent. Transcriptional changes have been attributed to MR function (23), but were not formally proven to be direct targets of the receptor and might thus be affected by MR activity in an indirect manner. However, while Glucocorticoid Response Element (GRE) presence seems crucial for both MR and GR DNA binding in the hippocampus, binding sites for NeuroD transcription factors were found selectively at MR-bound loci (24). NeuroD factors could coactivate glucocorticoid-induced

3

transactivation and were indeed present near MR-specific binding sites, suggesting that specific GRE-dependent MR target genes do exist.

The current study assessed if direct MR binding to the hippocampal DNA led to expression regulation of the nearby gene. Based on our recent work that defined MR-specific, MR-GR overlapping and GR-specific chromatin binding sites and corresponding putative target genes within the rat hippocampus (24), we examined mRNA levels of several genes in each of these categories. Forebrain MR knockout (fbMRKO) mice showed altered expression for a subset of genes, including downregulation of the mixed MR/GR target *Fkbp5*, and the MR-specific *Jun dimerization protein 2* (*Jdp2*), *Nitric oxide synthase 1 adaptor protein* (*Nos1ap*) and *Suv3 like RNA helicase* (*Supv3l1*) mRNA levels. Subsequently, corticosterone responsiveness of *Jdp2*, one of the genes having an MR-bound promoter, was validated in mice that were exposed to different durations of restraint stress.

Material and methods

Animals

Male homozygous forebrain-specific MR knockout (fbMRKO) and control c57bl/6 mice (n=7) aged 8-9 weeks, were housed on a 12-hour light/12-hour dark reversed cycle (lights off at 9:00AM). The fbMRKO mice were generated using MR^{flox} mice, having MR exon 3 flanked by loxP sites, and mice expressing Cre recombinase controlled by the CAMKIIα gene (25). Male MR$^{flox/flox}$CamKCre$^{Cre/wt}$ mice were crossed with female MR$^{flox/flox}$ mice to generate fbMRKO (MR$^{flox/flox\_Cre}$) and control (MR$^{flox/flox\_wt}$) offspring. As the breeding unexpectedly generated more fbMRKO than control mice, only part of the control animals were littermates. No differences were found in expression levels between littermate and non-littermate controls in any of the genes measured. Mice were transferred to a novel cage 20 min before harvesting the tissue, and sacrificed around the time of their endogenous corticosterone peak, between 9:30AM-12:00PM. We assessed the expression of MR, overlapping and GR putative target genes in this condition, as both receptor types are activated at peak of the diurnal corticosterone rhythm. The novel cage was included in the protocol to ensure MR and GR binding for ChIP analysis in the same animals, under the assumption that mRNA levels will not be affected in this short time span. From all mice trunk blood was collected, and hippocampal hemispheres were freshly dissected and snap-frozen in liquid nitrogen for later analysis.

For validation of *Jdp2* downregulation, male fbMRKO (n=14) and littermate controls (n=10) aged 8-12 weeks, were housed on a 12-hour light/12-hour dark cycle (lights on at 7:00AM). Mice were bred as described above. Sacrifice took place under baseline conditions, between 9:30-10:30AM. Brains were collected and snap-frozen in liquid nitrogen for later analysis.

For MR binding site validation in the mouse brain, male c57bl/6 mice (n=5) aged 16-19 weeks, were housed on a 12-hour light/12-hour dark cycle (lights on at 8:00AM), and were sacrificed in the afternoon 60 min after an IP injection of 3.0 mg/kg corticosterone (Sigma) dissolved in 5% ethanol in saline, ensuring MR binding. Hippocampal hemispheres were freshly dissected and snap-frozen in liquid nitrogen for later analysis.

Male Balb/c mice (n=3-6) aged 8-15 weeks, were housed on a 12-hour light/12-hour dark cycle (lights on at 6:00AM), and were exposed to various periods of restraint stress (0-30-60-120-240 min) and sacrificed directly afterwards, between 9:30AM-2:00PM. At this time of the diurnal corticosterone trough, both MR and GR DNA binding can be enhanced in response to stress (15) and consequential gene expression changes compared to non-stressed control mice could be revealed. From all mice trunk blood was collected, and hippocampal hemispheres were freshly dissected and snap-frozen in liquid nitrogen for later analysis.

All experiments were performed according to the European Commission Council Directive 2010/63/EU and the Dutch law on animal experiments and approved by the animal ethical committee from Utrecht University, University of Amsterdam, or the German Regierungspräsidium Tübingen.

Plasma measurements

Trunk blood was centrifuged for 10 min at 7000x*g*, after which plasma was transferred to new tubes. Corticosterone levels of the fbMRKO experiment were determined using an Enzyme ImmunoAssay (EIA, Immunodiagnostic Systems), and ACTH and corticosterone levels of the restraint stress mice were determined using an Enzyme-Linked ImmunoSorbent Assay (ELISA, IBL International), according to the manufacturers' instructions.

Target gene selection

MR-specific, MR-GR overlapping and GR-specific binding sites were annotated to the nearest gene (24). In order to increase the chances of correct annotation and identifying functional target genes, we focused on binding sites located intragenic or in the proximal promoter (up to -5 kb). Furthermore, hippocampal expression (26) of the putative target genes, the degree of coexpression with NeuroD factors (Neurod1/2/6) and face validity of chromatin immunoprecipitation-sequencing (ChIP-seq) peaks were assessed. The total numbers of putative target genes measured for MR-specific, overlapping (including classical targets), and GR-specific subset were 12, 10 and 9 respectively.

Table 1. Primer sequences used for qPCR on mouse hippocampal ChIP samples. See Table 3 for binding site details.

Binding site	Nearest gene	Forward & reverse (5'>3')	Product length (bp)
GR3000_1726	*Acsl6*	CCTGCCAGGAGAGCAGATG TGTGCAGGAAGGCAAGTTCT	178
MR3000_740 GR3000_34	*Fkbp5*	TGCCAGCCACATTCAGAACA TCAAGTGAGTCTGGTCACTGC	122
MR3000_1054	*Jdp2*	AAGTAAGACCGCGACCTACA AAATACCCAGTGCAGAGACGAA	192
MR300_473 GR3000_599	*Kif1c*	GCTGGGGTGTACACAGATGG TGACTAGCCAGAGCAGTATGTC	156
GR3000_106	*Mrpl48*	AGCTGTGCTTTGGAAGCCTA CATAAGGTGGGCCACACTCC	170
MR300_196	*Nos1ap*	CCTCCGATGCTGCTTGGATA CAGACCGAGCCAGCGATAAG	197
MR3000_738 GR3000_12	*Per1*	GGAGGCGCCAAGGCTGAGTG CGGCCAGCGCACTAGGGAAC	73
MR300_503	*Rilpl1*	CAGGCAGATGCCAGGCT CCCATGCCTGTTCCTCTAGT	106
MR3000_359	*Supv3l1*	TGCAGGGATTCGATGGACAG CTCTGAGCCACCTCTCAAGC	165
MR3000_641 GR3000_1603	*Zfp219*	AGTCCATCACATTCTGTTGCTTTC TAGTCAGCTATGACCATGCAGT	131

ChIP-qPCR

For MR binding validation in the mouse, we performed ChIP-qPCR on hippocampal tissue of wild type mice (n=5) as described previously (27). Hippocampal hemispheres were cryosectioned at 30 µm before crosslinking with 2 mM disuccinimidyl glutarate, followed by 1% formaldehyde. Fixated tissue was suspended, nuclei were isolated and sonicated

for 10 rounds (30 seconds ON/30 seconds OFF) using a Biorupter Pico (Diagenode). Chromatin of two hemispheres of the same animal were pooled and used for a single ChIP sample (500 µL) to measure MR binding with 5 µg of anti-MR antibody (21854-1-AP, ProteinTech). Immunoprecipitation was performed with 50 µL magnetic Protein A beads (Dynabeads™, Invitrogen). Background signal was detected for each sample with a sequential ChIP using 5 µg of control IgG antibody (ab37415, Abcam). Pellets were dissolved in 50µL 10mM Tris-HCl pH 8. Subsequently, qPCR was performed on 5x diluted ChIP samples, with primers that were designed to span the GRE of the MR binding sites and are listed in **Table 1**.

Real-time quantitative PCR

Mouse hippocampal hemispheres were homogenized in TriPure (Roche) by shaking the tissue with 1.0-mm-diameter glass beads for 20 seconds at 6.5 m/s in a FastPrep-24 5G instrument (MP Biomedicals). Total RNA was isolated, cDNA was generated and RT-qPCR was performed as described previously (24). As *Actb* (beta-actin) expression was regulated between fbMRKO and control mice, genes of interest were normalized against the in both experiments stably expressed housekeeping gene *Rplp0*, encoding a ribosomal protein. Primer sequences are listed in **Table 2**.

In situ hybridization

Frozen brains were sectioned at 18 µm in a cryostat microtome, collected on Super Frost Plus slides, and stored at -80°C until further use. *In situ* hybridization using ^{35}S UTP-labeled ribonucleotide probes for *Jdp2* was performed as described previously (28).

Table 2. Primer sequences used for RT-qPCR on mouse hippocampus.

Gene	Full name	Forward & reverse (5'>3')	Product length (bp)
Acsl6	Acyl-CoA synthetase long-chain family member 6	TCTCAGGGAATGGACCCTGT CCTCTTGGTAGGACAGCCAC	135
Bhlhb9	Basic helix-loop-helix domain containing, class B9	AACTCACCTGGCCAGCAATC CTCTGGCTGCCTTGGGATTT	187
C4ST1 (Chst11)	Chondroitin 4-sulfotransferase 1	GAATTTGCCGGATGGTGCTG AGCAGATGTCCACACCGAAG	117
Camk1d	Calcium/calmodulin-dependent protein kinase ID	GCATCGAGAACGAGATTGCC CCAGACACAAGTTGCATGACC	114
Camkk2	Calcium/calmodulin-dependent protein kinase kinase 2	AGAACTGCACACTGGTCGAG ACCAGGATCACAGTTGCCAG	85

Gene	Full name	Forward & reverse (5'>3')	Product length (bp)
Fkbp5	FK506 binding protein 5	TCCTGGGAGATGGACACCAA TTCCCGTACTGAATCACGGC	113
Gilz (Tsc22d3)	Glucocorticoid-induced leucine zipper	TGGCCCTAGACAACAAGATTGAGC CCACCTCCTCTCTCACAGCAT	78
Hsd17b11	Hydroxysteroid (17-beta) dehydrogenase 11	CGCAGGACCCTCAGATTGAA GGAGCAGTAAGCCAGCAAGA	167
Jdp2	Jun dimerization protein 2	TACGCTGACATCCGCAACAT CGTCTAGCTCACTCTTCACGG	100
Kif1c	Kinesin family member 1C	TTAATGCCCGTGAGACCAGC AAGCTTTTGGGGGCATCCTT	106
Mrpl48	Mitochondrial ribosomal protein L48	CAGTATGTCCACCGCCTCTG CTCGCTCATGGGTGGTAAGG	145
Nos1ap	Nitric oxide synthase 1 adaptor protein	TGGAATTCAGCCGAGGTGTG GGAAGGGAGCAGCATTCGAG	131
Nr3c1 (GR)	Nuclear receptor subfamily 3, group C, member 1	CCCTCCCATCTAACCATCCT ACATAAGCGCCACCTTTCTG	89
Nr3c2 (MR)	Nuclear receptor subfamily 3, group C, member 2	TCCAAGATCTGCTTGGTGTG CCCAGCTTCTTTGACTTTCG	239
Per1	Period circadian clock 1	ACGGCCAGGTGTCGTGATTA CCCTTCTAGGGGACCACTCA	162
Rilpl1	Rab interacting lysosomal protein-like 1	ACGAGCTCAAGTCCAAGGTG AGTCGCTTGATCCCCGATTC	148
Rplp0	Ribosomal protein, large, P0	GGACCCGAGAAGACCTCCTT GCACATCACTCAGAATTTCAATGG	85
Sgk1	Serum/glucocorticoid regulated kinase 1	AGAGGCTGGGTGCCAAGGAT CACTGGGCCCGCTCACATTT	129
Supv3l1	Suv3 like RNA helicase	CTCACTCGGCCTCTAGACAAG TCCACGTCCAGAGAATGGGA	170
Zfp219	Zinc finger protein 219	GATCTGCAGCGCTACTCCAA TGCACGAGTCTCAGACCAAC	96

Statistics

In the fbMRKO experiment, independent *t*-tests were used, taking *P*<0.01 as significance cut-off to correct for multiple gene testing. For the ChIP-qPCR validation we performed one-tailed paired *t*-tests. The predictable directionality, i.e. MR signal is higher than background IgG signal, justifies the use of a one-tailed test. As one may argue that a decrease in signal would also be relevant, we note that significant *P*-values were all <0.025, and therefore would also be significant using a two-tailed test. We considered a paired test appropriate as MR and IgG are measured on the same chromatin sample, and this allows correction for the corresponding background levels. Again, one-tailed unpaired *t*-tests gave essentially the same results. For one of the genes, *Nos1ap*, one of the samples

was excluded from analysis because of a missing value due to non-detectable IgG levels. For the time course of restraint stress, a one-way ANOVA was performed with Holm-Sidak's multiple comparison post-hoc tests. In the *in situ* measurements of the fbMRKO animals, unpaired *t*-tests were performed. Results were considered significantly different when $P<0.05$ unless stated otherwise. GraphPad Prism 7 was used to analyze the data. All graphs show individual values and data are further depicted with means ± SEM.

Table 3. Selected putative target genes to validate.

Binding site	GRE sequence (rat/mouse)	Annotation	Distance from TSS (bp)	Associated gene	ABA hippocampal expression
MR300_225	AGAACATTATGTTCC AAAACATCAGGATCC	Intron	116761	*Camkk2*	10.34
MR3000_360	GGAACACTCTCTTCC GGAACTCTCTCTTCC	Intergenic	-1071	*Hsd17b11*	3.98
MR3000_1054	AGAGCTCTTTGTGTT AGAATTCTTTGTGTT	Intergenic	-3983	*Jdp2*	13.58
MR300_196	CTCACACTTTCTCCC CTAGCACTCTCTCCC	Intron	233500	*Nos1ap*	11.50
MR300_503	CAACCTCTTTCTTCC CAACCCTCTTTCTCC	Intron	12715	*Rilpl1*	15.52
MR3000_359	TGTGCTTTCTGTTCC GGTGCTTTTTGTTAC	Intron	1661	*Supv3l1*	0.83
MR300_713 GR3000_248	AGAGCAGGCTGTTCT AAAACAGCCTGGTCT	Intron	95108	*Camk1d*	2.64 (mainly CA)
MR3000_740 GR3000_34	AGAACAGGGTGTTCT AGAACAGGGTGTTCT	Intron	62931	*Fkbp5*	8.86
MR300_473 GR3000_599	GGGACTGGAAGTTCC GGAACTTCCAGTCCC	Intron	9921	*Kif1c*	2.94
MR300_738 GR3000_12	GGAACATCGTGTTCT GGAACATCGTGTTCT	Intergenic	-3357	*Per1*	3.06
MR3000_641 GR3000_1603	ACACCAGGATGTTCC ACACCAGGATGTTCC	Intergenic	-2125	*Zfp219*	2.62
GR3000_1726	TGAACTTGCAGCGTT TGAGCTTGCAGCATT	Intergenic	-1931	*Acsl6*	15.52
GR3000_647	AGGACTGTTAGTACT AGGGCTTTTAGTACT	Intergenic	-3526	*Bhlhb9*	9.05
GR3000_193	AGAACTGTCTGCACC AGAACTCTCCATCAG	Intron	121265	*C4ST1*	7.28
GR3000_106	GGCTCTCCTTGTGCT GGCTCTCCTTGTGCC	Intron	24445	*Mrpl48*	4.71

Additional binding site information can be found in supplemental data of (24). Sequences represent the rat GRE (upper) and mouse GRE (lower) with mismatches to the rat sequence in red. GRE = glucocorticoid response element, TSS = transcription start site, ABA = Allen Brain Atlas

Results

In order to explore the functional effects of previously detected MR/GR DNA binding, i.e. transcription regulation, binding sites were associated to their nearest gene. This resulted in lists of MR-specific, MR-GR overlapping and GR-specific putative target genes (24). Gene expression levels, for a subset of each category (**Table 3**), were measured in forebrain-specific MR knockout (fbMRKO) mice at the time of their diurnal corticosterone peak. MR mRNA expression was indeed abolished, and GR mRNA was slightly upregulated in the hippocampus of fbMRKO mice (**Figure 1A**), confirming earlier reports (25). MR protein levels also showed efficient knockdown (29). Furthermore, no differences were found in plasma corticosterone levels of these animals at the time of sacrifice (**Figure 1B**). As the studied target loci were originally detected in the rat brain (24), we validated MR binding in mice. ChIP-qPCR confirmed hippocampal MR binding at the *Jdp2* (P = 0.0124), *Kif1c* (P = 0.0087), *Nos1ap* (P = 0.0172), *Rilpl1* (P = 0.0098), and *Zfp219* (P = 0.0049) loci in wild type (WT) mice, while this signal did not exceed background IgG levels at the GR-specific sites near *Acsl6* (P = 0.4410) and *Mrpl48* (P = 0.2142) (**Figure 1C**). Only for *Supv3l1* (P = 0.1784) we were unable to detect the expected MR binding. Also for classical target genes *Fkbp5* (P = 0.0246) and *Per1* (P = 0.0066) an MR enrichment was demonstrated.

Several MR-specific putative targets showed lower expression levels in the fbMRKO compared to WT mice (**Figure 2A**). The most robust effect was found in the *Jdp2* mRNA levels, which were reduced by 50% (P < 0.0001). Other differentially expressed genes were MR-specific *Nos1ap* (P = 0.0005) and *Supv3l1* (P = 0.0061), and MR-GR overlapping *Camk1d* (P = 0.0016) and *Kif1c* (P = 0.0022), which were also all downregulated in the fbMRKO compared to WT mice (**Figure 2A, 2B**). Moreover, two of the GR-specific genes, *Acsl6* (P = 0.0002) and *Mrpl48* (P = 0.0065) were expressed at lower levels, and *C4ST1* showed a trend of lowered expression (P = 0.0138) (**Figure 2C**).

Besides the brain-related putative MR/GR target genes, we measured the expression of the classical target genes *Fkbp5*, *Gilz*, *Per1* and *Sgk1* (**Figure 2D**). These genes are all known to be bound and/or regulated by both MR and GR; our identified MR-GR overlapping target subset contained *Fkbp5* and *Per1* (**Table 3**). Of the four classical targets only *Fkbp5* was downregulated in fbMRKO mice, to 44% of the levels observed in WT animals (P < 0.0001).
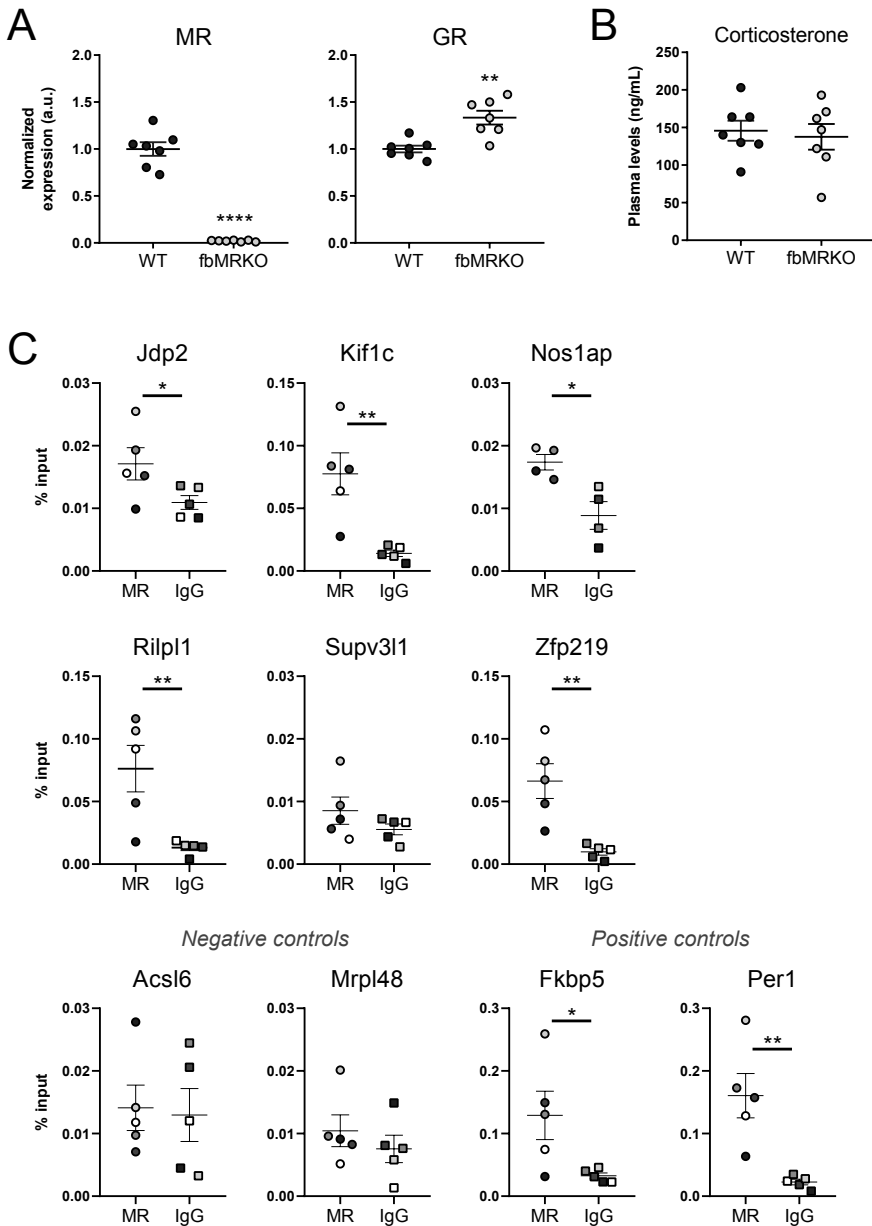
Figure 1. Validation of MR detection in wild type (WT) mice and absence of MR in forebrain MR knockout (fbMRKO) mice. **A)** Hippocampal mRNA levels showing MR downregulation and slight GR upregulation, and **B)** unaltered plasma corticosterone levels in fbMRKO versus wild type (WT) mice; assessed by independent *t*-tests. **C)** MR binding assessed by ChIP-qPCR in the hippocampus of WT mice, along with an IgG background signal per sample; assessed by one-tailed paired *t*-tests. Corresponding measurements are depicted in the same color. GR-specific targets *Acsl6* and *Mrpl48* served as negative controls; classical glucocorticoid targets *Fkbp5* and *Per1* served as positive controls. a.u. = arbitrary unit, * $P<0.05$, ** $P<0.01$, **** $P<0.0001$
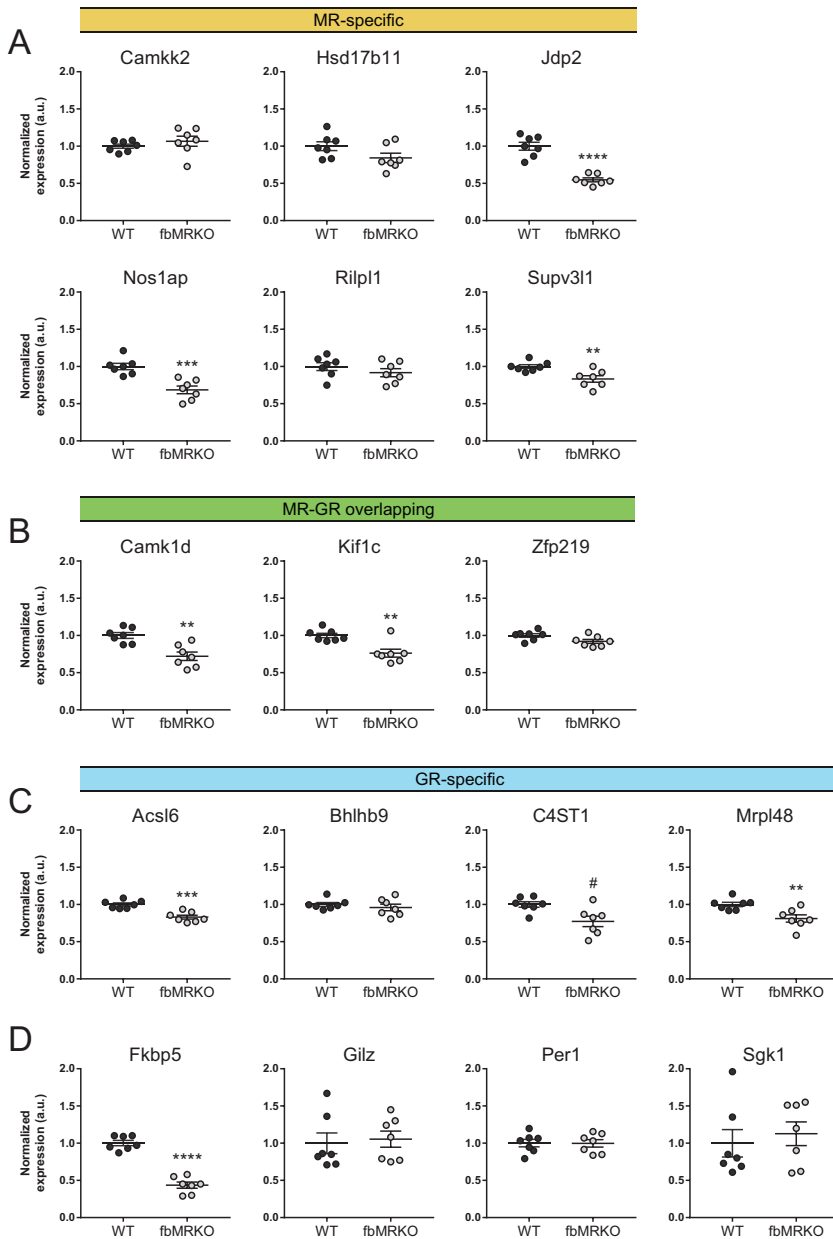
Figure 2. Hippocampal mRNA levels of glucocorticoid target genes assessed in wild type (WT) and forebrain MR knockout (fbMRKO) mice. Gene expression of **A)** MR-specific, **B)** overlapping and **C)** GR-specific targets and **D)** classical glucocorticoid targets in fbMRKO versus WT mice; assessed by independent *t*-tests with $P<0.01$ as significance cut-off. Other genes measured, but not differentially expressed between WT and fbMRKO mice: *Adam23*, *Arl8b*, *Dgkb*, *Els1*, *Myo16* and *Nob1* as MR-specific targets; *Grb2*, *Luzp1* and *Map1lc3b* as overlapping targets; *Arntl*, *B3galt1*, *Map2k5*, *Pglyrp1* and *Slc3a2* as GR-specific targets. a.u. = arbitrary unit, # $P<0.05$ (considered a trend), ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$
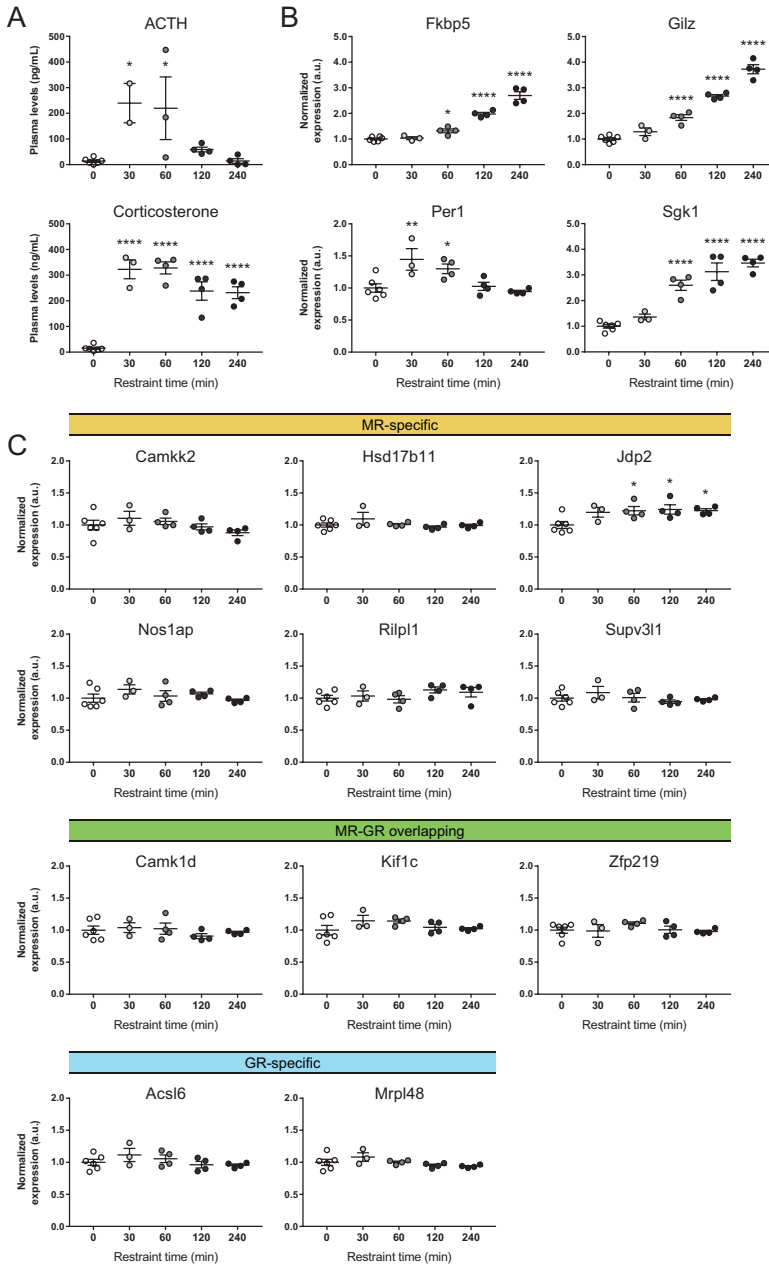
Figure 3. Hippocampal mRNA levels of glucocorticoid target genes assessed in a restraint stress model. **A)** Plasma ACTH and corticosterone levels after different durations of restraint stress. **B)** Validation of time-dependent classical glucocorticoid target gene activation upon restraint stress. **C)** Gene expression of MR-specific, overlapping and GR-specific targets after different durations of restraint stress. All assessed by one-way ANOVA with Holm-Sidak's post-hoc tests. ACTH = adrenocorticotropic hormone, a.u. = arbitrary unit, * $P<0.05$, ** $P<0.01$, **** $P<0.0001$

Next, we aimed to show regulation of the target genes in an acute stress context. Even though MR is substantially occupied by ligand under basal glucocorticoid levels, MR (and GR) DNA binding and subsequent transcriptional effects can be enhanced by a rise of corticosterone (15). Hippocampal gene expression was assessed in mice that were exposed to restraint stress of different durations (0-30-60-120-240 min). Plasma corticosterone levels were increased after all durations of restraint stress, but tend to return to baseline at 120 min and 240 min, in line with the fact that ACTH levels were normalized at these time points (**Figure 3A**).

Of the classical glucocorticoid target genes, *Fkbp5*, *Gilz* and *Sgk1* were upregulated after 60, 120 and 240 min of restraint (**Figure 3B**). *Per1* showed a transient increase, with elevated levels at 30 min and 60 min, which had declined again from 120 min restraint stress. Interestingly, the MR-exclusive target gene *Jdp2* that was mostly affected in the fbMRKO mice showed an increase in response to stress (**Figure 3C**), in animals that were exposed to restraint for 60 to 240 min. Other genes associated with MR and/or GR binding loci that we had selected for validation did not show transcriptional effects upon restraint stress (**Figure 3C**).

Finally, we confirmed *Jdp2* downregulation measured by *in situ* hybridization in an independent experiment in fbMRKO (**Figure 4**). In absence of MR, *Jdp2* mRNA levels were decreased in the principal neurons of the dorsal hippocampus, as apparent from significant lower expression in the CA2 (P = 0.0001), CA3 (P = 0.0357) and dentate gyrus (P = 0.0005) subregions. For the CA1 this occurred at the trend level (P = 0.0901).
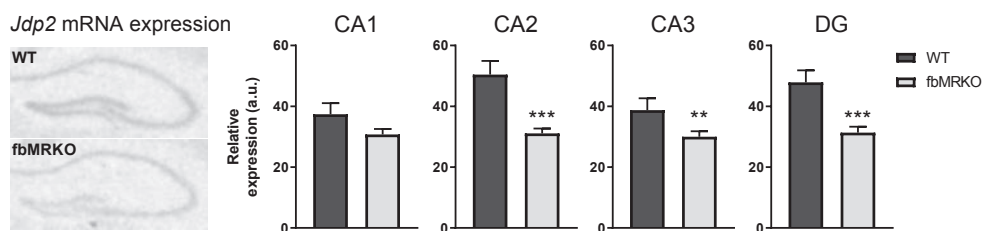

Figure 4. Validation of hippocampal *Jdp2* downregulation in forebrain MR knockout (fbMRKO) mice compared to wild type (WT) mice, detected by *in situ* hybridization; assessed by unpaired *t*-tests. On the left is depicted a representative scanned autoradiograph film per genotype. Gene expression is quantified per subregion of the hippocampus: cornu ammonis (CA)1, CA2, CA3 and the dentate gyrus (DG). a.u. = arbitrary unit, ** P<0.01, *** P<0.001

Discussion

Based on non-overlapping MR-GR binding sites, we defined putative MR-specific and GR-specific hippocampal target genes. We identified *Jdp2* as a likely MR-specific transcriptional target, that is both downregulated in fbMRKO mice and upregulated in response to restraint stress. Also *Nos1ap* and *Supv3l1*, two other genes linked to MR-specific binding sites, were expressed at a lower levels in fbMRKO mice, but did not change upon restraint stress. Classical glucocorticoid target genes *Fkbp5*, *Gilz*, *Per1* and *Sgk1* all responded to restraint stress by increased transcription. Of these targets, only *Fkbp5* showed a substantially lower hippocampal expression in the absence of MR.

Both technical and biological factors could explain the limited success in validating MR-specific genomic targets. The annotation of binding sites to the nearest gene is not without error, as it is possible that another neighboring gene is affected by the binding locus assessed. We do not have data on spatial chromatin organization or RNA polymerase activity in the same experimental setup, which could enable the proper linking of binding loci to the actual site of transcriptional activity (30). To lower the chance of false positive annotations, we did focus on binding sites that were located within genes or (proximal) promoter regions. However, even in the case that the putative target is legitimate, we might still have false negative results on gene expression changes. Because the hippocampus consists of several subregions and various cell types, we could be unable to detect MR-dependent regulation that is constrained to a subset of hippocampal cells. While the ChIP-seq signal can be strong enough to withstand dilution, gene regulation might be diluted when the average gene expression over the whole hippocampus is assessed, as fold change in hippocampal mRNA expression tends to be modest in response to steroids (31). Despite possible false negative results, we were able to find robust changes in several MR-specific and classical glucocorticoid target genes.

It is of note that gene regulation by MR knockout and restraint stress was validated in a mouse model, while the MR/GR binding loci were obtained from experiments in rats. We were able to show MR binding in the mouse hippocampus at five out of six MR targets originally detected in the rat brain. Evolutionary conservation can increase the predictive value of functional GREs (32, 33). Moreover, as brain MR/GR-mediated regulation is considered part of a general adaptive response, one would expect genes regulated in rat to also be affected in mice. However, the species difference is an additional potential cause for absence of mRNA regulation.

3

The fbMRKO animals become MR deficient during embryonic development, and loss of MR protein is completed after birth (25). In our experiment downregulated MR expression was validated, and slight upregulation of GR expression in the hippocampus was observed as described before (25). It is possible that MR-dependent gene expression is normalized due to compensation by GR or other factors. We cannot exclude that such compensatory mechanisms might as well affect expression of *Jdp2*, *Nos1ap* and *Supv3l1* in the fbMRKO mice. Also, redundancy in gene regulation is not uncommon, and while complete dependence of target genes to a single transcription factor can happen (34), it is rare in case of MR and GR signaling. In addition, binding of nuclear receptors such as MR can have permissive effects on chromatin, and could be necessary but not sufficient for transcription. In fact, as little as 13% of GR binding sites can be linked to transcriptional activity (35). Thus, the lack of transcriptional effects might reflect a context dependency.

To start looking at MR regulation in a relevant context, we chose a restraint stress paradigm in wild type mice as a more physiological setting. Mice were stressed in the morning, to make sure that basal corticosterone levels were low, and MR activation not necessarily fully maximal (36). The classical glucocorticoid target genes all responded in this acute stress situation, and of the MR-specific targets identified in the fbMRKO mice only *Jdp2* expression was affected. Non-regulated genes in the restraint stress experiment might still be MR-dependent, but at a lower EC50 (37), or in different contexts, like in behavioral paradigms in which fbMRKO animals show changed phenotypes, such as working memory in a radial maze (25).

For the genes associated with GR-specific chromatin binding, *Acsl6* and *Mrpl48* showed lower expression levels in the fbMRKO mice. In general, the effect size on specifically GR-associated target gene expression was less pronounced. The fact that these GR targets are downregulated, while expression of GR itself is slightly upregulated in fbMRKO mice seems contradictory. However, this could be a result of indirect effects of MR deficiency. Another explanation is that GR binding takes place at a negative GRE, where GR leads to repression (instead of activation) of the nearby gene (38, 39).

More interestingly, several overlapping targets were downregulated in fbMRKO mice: the newly identified *Camk1d* and *Kif1c*, and the classical target *Fkbp5*. This suggests that MR is needed for expression of these genes in the hippocampus. The GR compensatory upregulation does not seem to prevent dysregulation of these combined target genes in the absence of MR. It is likely that heterodimerization of MR and GR is involved in the regulation of overlapping binding sites. *Fkbp5* expression was recently shown to

be modulated by MR-GR heterodimers (15). The observation that *Fkbp5* expression is lowered in fbMRKO mice, can represent functional consequences of the absence of one of the heterodimerization partners. Fkbp5 is part of an ultra-short feedback loop, where it is induced by glucocorticoids, while in turn Fkbp5 prevents GR activation (40). Besides the observed upregulation of GR expression itself, the lowered *Fkbp5* levels could contribute to a compensatory mechanism by relieving repression of GR function in order to overcome the lack of MR signaling.

Overall, the *Jdp2* gene was the most robust MR target identified in this study. Initially Jdp2 was discovered as a negative regulator of activator protein-1 (AP-1) function, by dimerizing to c-Jun and preventing transcriptional effects (41). Later it was found that Jdp2 can also act in a stimulating fashion, that is as coactivator for the progesterone receptor (42). In this latter study Jdp2 was also shown to have a coactivating effect on transactivation by GR, as was confirmed by Garza et al. (43). We found *Jdp2* to be a bona fide MR target. A feedforward mechanism could be speculated, in which MR can increase Jdp2 levels, which in turn could enhance GR activity. A recent ChIP-seq study in mouse neuroblastoma cells found the Jdp2 binding motif near both MR- and GR-bound sites (44). Besides the differential affinity of MR and GR for their hormone, temporal responses to glucocorticoids could be accounted for by such a feedforward loop. Feedforward models have been described before for GR (45) and other nuclear receptors (46, 47). It is worth noting that Jdp2 has been implicated in AP-1 modulation during fear extinction (48), and polymorphisms in the *Nos1ap* gene have been linked to posttraumatic stress disorder and depression (49), demonstrating also a functional role of these genes in the stress system.

In conclusion, we found three novel hippocampal MR-specific target genes, that are *Jdp2*, *Nos1ap* and *Supv3l1*, of which *Jdp2* is also responsive in an acute stress situation. Dissecting the glucocorticoid response in MR-specific, common and GR-specific pathways will enable us to better understand the stress physiology and pathophysiology of stress-related disorders.

Acknowledgements

References

1. Reul JM, de Kloet ER. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology. 1985;117(6):2505-11.

2. Joels M, Karst H, DeRijk R, de Kloet ER. The coming out of the brain mineralocorticoid receptor. Trends in neurosciences. 2008;31(1):1-7.

3. de Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. Nature reviews Neuroscience. 2005;6(6):463-75.

4. Joels M, de Kloet ER. Mineralocorticoid receptor-mediated changes in membrane properties of rat CA1 pyramidal neurons in vitro. Proceedings of the National Academy of Sciences of the United States of America. 1990;87(12):4495-8.

5. Karst H, Karten YJ, Reichardt HM, de Kloet ER, Schutz G, Joels M. Corticosteroid actions in hippocampus require DNA binding of glucocorticoid receptor homodimers. Nature neuroscience. 2000;3(10):977-8.

6. Oitzl MS, de Kloet ER. Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. Behavioral neuroscience. 1992;106(1):62-71.

7. Oitzl MS, Reichardt HM, Joels M, de Kloet ER. Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(22):12790-5.

8. Datson NA, van der Perk J, de Kloet ER, Vreugdenhil E. Identification of corticosteroid-responsive genes in rat hippocampus using serial analysis of gene expression. The European journal of neuroscience. 2001;14(4):675-89.

9. Pascual-Le Tallec L, Lombes M. The mineralocorticoid receptor: a journey exploring its diversity and specificity of action. Molecular endocrinology. 2005;19(9):2211-21.

10. Yang J, Fuller PJ, Morgan J, Shibata H, McDonnell DP, Clyne CD, et al. Use of phage display to identify novel mineralocorticoid receptor-interacting proteins. Molecular endocrinology. 2014;28(9):1571-84.

11. Joels M, Sarabdjitsingh RA, Karst H. Unraveling the time domains of corticosteroid hormone influences on brain activity: rapid, slow, and chronic modes. Pharmacological reviews. 2012;64(4):901-38.

12. Karst H, Berger S, Turiault M, Tronche F, Schutz G, Joels M. Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(52):19204-7.

13. Latouche C, Sainte-Marie Y, Steenman M, Castro Chaves P, Naray-Fejes-Toth A, Fejes-Toth G, et al. Molecular signature of mineralocorticoid receptor signaling in cardiomyocytes: from cultured cells to mouse heart. Endocrinology. 2010;151(9):4467-76.

14. Soundararajan R, Zhang TT, Wang J, Vandewalle A, Pearce D. A novel role for glucocorticoid-induced leucine zipper protein in epithelial sodium channel-mediated sodium transport. The Journal of biological chemistry. 2005;280(48):39970-81.

15. Mifsud KR, Reul JM. Acute stress enhances heterodimerization and binding of corticosteroid receptors at glucocorticoid target genes in the hippocampus. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(40):11336-41.

16. Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, et al. Epithelial sodium channel regulated by aldosterone-induced protein sgk. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(5):2514-9.

17. Baughman G, Wiederrecht GJ, Chang F, Martin MM, Bourgeois S. Tissue distribution and abundance of human FKBP51, and FK506-binding protein that can mediate calcineurin inhibition. Biochemical and biophysical research communications. 1997;232(2):437-43.

18. D'Adamio F, Zollo O, Moraca R, Ayroldi E, Bruscoli S, Bartoli A, et al. A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. Immunity. 1997;7(6):803-12.

19. Conway-Campbell BL, Sarabdjitsingh RA, McKenna MA, Pooley JR, Kershaw YM, Meijer OC, et al. Glucocorticoid ultradian rhythmicity directs cyclical gene pulsing of the clock gene period 1 in rat hippocampus. Journal of neuroendocrinology. 2010;22(10):1093-100.

20. Webster MK, Goya L, Ge Y, Maiyar AC, Firestone GL. Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. Molecular and cellular biology. 1993;13(4):2031-40.

21. Meijer OC, de Kloet ER. Corticosterone and serotonergic neurotransmission in the hippocampus: functional implications of central corticosteroid receptor diversity. Critical reviews in neurobiology. 1998;12(1-2):1-20.

22. Meinel S, Ruhs S, Schumann K, Stratz N, Trenkmann K, Schreier B, et al. Mineralocorticoid receptor interaction with SP1 generates a new response element for pathophysiologically relevant gene expression. Nucleic acids research. 2013;41(17):8045-60.

23. Meijer OC, de Kloet ER. A role for the mineralocorticoid receptor in a rapid and transient suppression of hippocampal 5-HT1A receptor mRNA by corticosterone. Journal of neuroendocrinology. 1995;7(8):653-7.

24. van Weert LTCM, Buurstede JC, Mahfouz A, Braakhuis PSM, Polman JAE, Sips HCM, et al. NeuroD Factors Discriminate Mineralocorticoid From Glucocorticoid Receptor DNA Binding in the Male Rat Brain. Endocrinology. 2017;158(5):1511-22.

25. Berger S, Wolfer DP, Selbach O, Alter H, Erdmann G, Reichardt HM, et al. Loss of the limbic mineralocorticoid receptor impairs behavioral plasticity. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(1):195-200.

26. Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, et al. Genome-wide atlas of gene expression in the adult mouse brain. Nature. 2007;445(7124):168-76.

27. Singh AA, Schuurman K, Nevedomskaya E, Stelloo S, Linder S, Droog M, et al. Optimized ChIP-seq method facilitates transcription factor profiling in human tumors. Life Sci Alliance. 2019;2(1):e201800115.

28. Santarelli S, Zimmermann C, Kalideris G, Lesuis SL, Arloth J, Uribe A, et al. An adverse early life environment can enhance stress resilience in adulthood. Psychoneuroendocrinology. 2017;78:213-21.

29. Bonapersona V, Damsteegt R, Adams ML, van Weert LTCM, Meijer OC, Joels M, et al. Sex-dependent modulation of acute stress reactivity after early life stress in mice: relevance of mineralocorticoid receptor expression. Frontiers in behavioral neuroscience. 2019;13:181.

30. Davies JO, Oudelaar AM, Higgs DR, Hughes JR. How best to identify chromosomal interactions: a comparison of approaches. Nature methods. 2017;14(2):125-34.

3

31. Datson NA, van den Oever JM, Korobko OB, Magarinos AM, de Kloet ER, McEwen BS. Previous history of chronic stress changes the transcriptional response to glucocorticoid challenge in the dentate gyrus region of the male rat hippocampus. Endocrinology. 2013;154(9):3261-72.

32. So AY, Cooper SB, Feldman BJ, Manuchehri M, Yamamoto KR. Conservation analysis predicts in vivo occupancy of glucocorticoid receptor-binding sequences at glucocorticoid-induced genes. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(15):5745-9.

33. Datson NA, Polman JA, de Jonge RT, van Boheemen PT, van Maanen EM, Welten J, et al. Specific regulatory motifs predict glucocorticoid responsiveness of hippocampal gene expression. Endocrinology. 2011;152(10):3749-57.

34. Finotto S, Krieglstein K, Schober A, Deimling F, Lindner K, Bruhl B, et al. Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. Development. 1999;126(13):2935-44.

35. Vockley CM, D'Ippolito AM, McDowell IC, Majoros WH, Safi A, Song L, et al. Direct GR Binding Sites Potentiate Clusters of TF Binding across the Human Genome. Cell. 2016;166(5):1269-81 e19.

36. Meijer OC, Van Oosten RV, De Kloet ER. Elevated basal trough levels of corticosterone suppress hippocampal 5-hydroxytryptamine(1A) receptor expression in adrenally intact rats: implication for the pathogenesis of depression. Neuroscience. 1997;80(2):419-26.

37. Reddy TE, Pauli F, Sprouse RO, Neff NF, Newberry KM, Garabedian MJ, et al. Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. Genome research. 2009;19(12):2163-71.

38. Sharma D, Bhave S, Gregg E, Uht R. Dexamethasone induces a putative repressor complex and chromatin modifications in the CRH promoter. Molecular endocrinology. 2013;27(7):1142-52.

39. Surjit M, Ganti KP, Mukherji A, Ye T, Hua G, Metzger D, et al. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. Cell. 2011;145(2):224-41.

40. Binder EB. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology. 2009;34 Suppl 1:S186-95.

41. Aronheim A, Zandi E, Hennemann H, Elledge SJ, Karin M. Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. Molecular and cellular biology. 1997;17(6):3094-102.

42. Wardell SE, Boonyaratanakornkit V, Adelman JS, Aronheim A, Edwards DP. Jun dimerization protein 2 functions as a progesterone receptor N-terminal domain coactivator. Molecular and cellular biology. 2002;22(15):5451-66.

43. Garza AS, Khan SH, Moure CM, Edwards DP, Kumar R. Binding-folding induced regulation of AF1 transactivation domain of the glucocorticoid receptor by a cofactor that binds to its DNA binding domain. PloS one. 2011;6(10):e25875.

44. Rivers CA, Rogers MF, Stubbs FE, Conway-Campbell BL, Lightman SL, Pooley JR. Glucocorticoid receptor tethered mineralocorticoid receptors increase glucocorticoid-induced transcriptional responses. Endocrinology. 2019.

45. Sasse SK, Zuo Z, Kadiyala V, Zhang L, Pufall MA, Jain MK, et al. Response Element Composition Governs Correlations between Binding Site Affinity and Transcription in Glucocorticoid Receptor Feed-forward Loops. The Journal of biological chemistry. 2015;290(32):19756-69.

46. Pabona JM, Simmen FA, Nikiforov MA, Zhuang D, Shankar K, Velarde MC, et al. Kruppel-like factor 9 and progesterone receptor coregulation of decidualizing endometrial stromal cells: implications for the pathogenesis of endometriosis. The Journal of clinical endocrinology and metabolism. 2012;97(3):E376-92.

47. Villanueva CJ, Vergnes L, Wang J, Drew BG, Hong C, Tu Y, et al. Adipose subtype-selective recruitment of TLE3 or Prdm16 by PPARgamma specifies lipid storage versus thermogenic gene programs. Cell Metab. 2013;17(3):423-35.

48. Guedea AL, Schrick C, Guzman YF, Leaderbrand K, Jovasevic V, Corcoran KA, et al. ERK-associated changes of AP-1 proteins during fear extinction. Molecular and cellular neurosciences. 2011;47(2):137-44.

49. Bruenig D, Morris CP, Mehta D, Harvey W, Lawford B, Young RM, et al. Nitric oxide pathway genes (NOS1AP and NOS1) are involved in PTSD severity, depression, anxiety, stress and resilience. Gene. 2017;625:42-8.

3