Kennedy, C. LM., Carter, S. D., Mifsud, K. R., & Reul, J. M. H. M. (2020). Unexpected effects of metyrapone on corticosteroid receptor interaction with the genome and subsequent gene transcription in the hippocampus of male rats. *Journal of Neuroendocrinology*, *32*(2), [e12820]. https://doi.org/10.1111/jne.12820

Unexpected effects of metyrapone on corticosteroid receptor interaction with the genome and subsequent gene transcription in the hippocampus of male rats

Clare LM Kennedy, Sylvia D Carter, Karen R Mifsud & Johannes MHM Reul*

Neuro-Epigenetics Research Group, Bristol Medical School, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol BS1 3NY, United Kingdom

*Corresponding author
Professor Johannes M.H.M. Reul
Neuro-Epigenetics Research Group,
Bristol Medical School,
University of Bristol,
Dorothy Hodgkin Building,
Whitson Street, Bristol BS1 3NY, United Kingdom
Phone: +44 117 331 3137
E-mail: Hans.Reul@bristol.ac.uk

Acknowledgement

This study was supported by a grant (BB/N015045/1) from the Biotechnology and Biological Sciences Research Council (BBSRC, UK). Furthermore, Clare Kennedy and Sylvia Carter were supported by PhD studentships from the Wellcome Trust Neural Dynamics Programme and the Medical Research Council (MRC, UK), respectively.

Abstract

Glucocorticoid hormones (GCs) play a pivotal role in many stress-related biological processes. In the hippocampus, GCs act through mineralocorticoid (MRs) and glucocorticoid receptors (GRs) to modify gene transcription. Involvement of GCs in biological processes has been studied using the corticosterone (CORT)-synthesis blocker metyrapone. How metyrapone affects GC action at the genomic level is however still unclear. Therefore, we studied the effects of this enzyme blocker on plasma CORT levels and hippocampal MR and GR binding to GC responsive elements (GREs) within the GC target genes *Fkbp5* (FK506-binding protein 5), *Per1* (Period 1) and *Sgk1* (Serum- and glucocorticoid-activated kinase 1), as well as transcriptional responses of these genes under control and acute stress conditions in rats. For comparison, we also studied these endpoints in adrenalectomized (ADX) rats. Although metyrapone had no effect on baseline levels of CORT, the drug increased MR and GR to GRE binding within the GC target genes and the transcriptional activity of these genes. As expected, acute forced swim (FS) stress strongly increased plasma CORT levels, hippocampal MR and GR to GRE binding within *Fkbp5*, *Per1* and *Sgk1*, and transcriptional activity (mainly hnRNA levels) of these genes. Metyrapone attenuated, but not abolished, these effects of stress on plasma CORT and MR and GR to GRE binding. The drug effects on FS-induced transcriptional activity were gene-dependent with a reduction seen in *Fkbp5* hnRNA (but not *Fkbp5* mRNA), an enhancement in *Per1* hnRNA (but not *Per1* mRNA), and no effect on both *Sgk1* hnRNA and mRNA levels. ADX however completely abrogated the effects of FS on plasma CORT as well as hippocampal MR and GR to GRE binding and transcriptional responses.

Thus, in contrast to ADX, metyrapone produced inconsistent effects on GC-sensitive genomic endpoints that question its suitability as a tool in neuroendocrine and other

research.

Keywords

Metyrapone, adrenalectomy, mineralocorticoid receptor, glucocorticoid receptor, gene transcription, hippocampus, stress

Introduction

Glucocorticoid hormones (GCs) are essential for appropriate responses to stressful challenges and thereby support adaptation and survival [1]. They fulfil this vital role by acting on numerous biological processes, including metabolism, the immune system and the central nervous system [2-3]. Dysregulation of GC secretion as a result of hypothalamic-pituitary-adrenal (HPA) axis malfunction, as often seen under chronic stress conditions, is thought to be strongly linked to a number of neuropsychiatric disorders such as major depression, anxiety-related disorders, post-traumatic stress disorder (PTSD) and schizophrenia [4-7]. Presently, the exact role of GCs in the aetiology of these disorders is still unclear. Therefore, insight into the molecular mechanisms underpinning GC action in the brain is of critical importance.

Endogenous GCs (corticosterone (CORT) in rodents like rats and mice) bind to two types of intracellular receptors, the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), which are both found in relatively high concentration in the hippocampus [8, 9] and are known to act as ligand-dependent transcription factors. Recently, we found that an acute stressful challenge results in a substantial rise in the binding of MRs and GRs to glucocorticoid response elements (GREs) in the DNA leading to enhanced transcriptional activity of the GC target genes FK506-binding protein 5 (*Fkbp5*), Period 1 (*Per1*) and Serum- and glucocorticoid-activated kinase 1 (*Sgk1*) [14]. The genomic action of MRs and GRs is thought to underlie many GC-dependent behavioural and physiological changes observed after stress or associated with circadian rhythm [10].

As stress exerts its effects on physiology and behaviour via multiple mediators, an investigation into the role of endogenous GCs in stress-induced responses would require intervention strategies to block or remove GC secretion from the adrenal glands. Metyrapone,

an inhibitor of the enzyme 11-β-hydroxylase (thus, preventing the conversion of deoxycorticosterone to CORT [11]), has been used to block endogenous GC synthesis in behavioural paradigms [12, 13]. Until now, however, the consequences of metyrapone administration on the molecular effects of GCs at the genomic level have not been investigated. Therefore, we decided to study the effects of this enzyme inhibitor on MR and GR binding to GREs within the *Fkbp5*, *Per1* and *Sgk1* genes in the hippocampus under early morning baseline and acute stress condition using chromatin immuno-precipitation (ChIP). In addition, we investigated the effect of metyrapone on baseline and stress-induced transcriptional responses of these genes. In these studies, we could take advantage of our recent characterization of MR/GR GRE-binding and transcriptional responses under baseline and acute stress conditions with regard to these GC target genes [14]. Our experiments revealed effects of metyrapone which were contradictory to the expected, GC-reducing effects of the drug. In light of these findings, we decided to additionally investigate the effects of complete removal of endogenous GCs by bilateral adrenalectomy (ADX). We found that ADX indeed completely abolished the effects of stress on MR/GR GRE-binding and *Fkbp5*, *Per1* and *Sgk1* transcriptional responses. This work shows that when using metyrapone unexpected effects at the genomic level need to be taken into account that may preclude proper interpretation of the data on the paradigm under investigation.

Materials and Methods

Animals

Male Wistar rats (150-175 g) were purchased from Harlan and group-housed (two to three animals per cage). Animals were kept under standard light (lights on 5:00–19:00; 80–100 Lux) and environmentally controlled conditions (temperature 21 ± 1 °C; relative humidity 40–60%) with food and water available ad libitum. All procedures were approved by the University of Bristol Ethical Committee and by the Home Office of the United Kingdom (Animal Scientific Procedures Act, 1986, UK). Except for the day of the experiment, all rats were handled (2 min per rat per day) to reduce any nonspecific stress effects.

Drug Treatment

Rats were intraperitoneally (i.p.) injected either once or twice at 90-min intervals with the 11-β-hydroxylase inhibitor metyrapone (100 mg/kg i.p., as indicated). As a control condition, the same amount of vehicle (40% PEG, 60% 0.9%-saline; 1 ml/kg i.p.) was injected at corresponding intervals. Following the injection protocol, some animals were subjected to forced swimming (see below). Full details of dosing schedule and experimental regimens can be found in Supplementary Figure 1 and in the legends to the figures. All drugs and chemicals were purchased from Sigma-Aldrich (Poole, UK).

Surgical Procedures

Some rats underwent bilateral ADX or sham surgery. Surgeries were performed under isoflurane anaesthesia. Sham surgeries were identical to ADX except that the adrenals were not removed. ADX rats were provided with 0.9% saline containing CORT supplementation (15

mg/l) in their drinking water for 1 week following surgery. One-week post-surgery, CORT supplementation was discontinued 1 day before experimentation.

Animal experimentation and collection of hippocampus tissue and blood

All animal experiments were conducted between 8:00 and 12:00. Rats were acutely stressed by forced swimming (15 min in 25 °C water) in individual glass beakers (height 35 cm, diameter 21.7 cm). Depending on the protocol (see Suppl. Fig. 1 and legends to the figures), rats were either naïve (i.e. untouched), injected once or twice with metyrapone of vehicle, or had been adrenalectomized or sham-adrenalectomized. Rats were killed under baseline (non-FS) conditions or killed 30 min (FS30) or 60 min (FS60) after the start of forced swimming (see legends). Rats that were injected but not forced to swim, were killed at the corresponding time after injection. For ChIP experiments, animals were killed at FS30 as 30 min after the start of FS stress was the time at which MR and GR binding to GREs with Fkbp5, Per1 and Sgk1 was maximal [14]. For RNA experiments, rats were killed at 60 min post-FS, i.e. the time of maximal hnRNA or mRNA responses after stress [14].

Rats were killed by decapitation following brief (<10 s) isoflurane anaesthesia after which trunk blood was collected in EDTA-containing tubes. The hippocampus was rapidly dissected on an ice-filled steel box and snap-frozen in liquid N2. The blood was spun down at 4 °C to prepare plasma. Tissue and plasma samples were stored at -80 °C until analysis.

CORT Radioimmunoassay (RIA)

Plasma CORT concentrations were measured using a commercial CORT RIA Kit (MP Biomedicals) as described previously [14].

Chromatin immuno-precipitation (ChIP)

ChIP was performed as previously described [14, 15]. We added 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) or 0.1 mM PMSF, 5 mM Na+-Butyrate (NaBut), and PhosSTOP Phosphatase Inhibitor Mixture Tablets (Roche) to all solutions unless otherwise stated. Briefly, hippocampus tissues were cross-linked for 10 min in 1% formaldehyde in PBS at room temperature (RT). Cross-linking was terminated by addition of glycine (5 min, RT; final concentration: 200 µM) and centrifugation (5 min at 6,000 × g at 4 °C). Pellets were washed three times with ice-cold PBS. Next, the pellets were resuspended in ice-cold lysis buffer [50 mM Tris HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% (vol/vol) Igepal, 0.5% Na-deoxycholate, 1% SDS, 2mM AEBSF,1 mM Na3VO4, Complete Ultra EDTA-Free Protease Inhibitor Tablets (one per 10 ml; Roche)] and rotated for 15 min at 4 °C. Samples were aliquoted, sonicated (high power; 3 × 10 cycles; 30 s on and 60 s off) using a water-cooled (4°C) Bioruptor (UCD-300; Diagenode), and centrifuged (10 min at 20,000 × g at 4 °C). Supernatants (containing the sheared chromatin) were recombined and re-aliquoted into fresh tubes for subsequent ChIP analysis and assessment of input DNA (i.e., the DNA starting material). For ChIP analysis, aliquots of chromatin were diluted 10-times in ice-cold dilution buffer [50 mM Tris HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, pH 8.0, 1% (vol/vol) Triton, 0.1% Na-deoxycholate, 1 mM AEBSF, Complete Ultra EDTA-Free Protease Inhibitor Tablets (one per 10 mL; Roche)]. Ten microliters MR (MR H-300; sc11412X; Santa Cruz) or GR (GR H-300, sc8992X; Santa Cruz) antibody was added to each sample, and tubes were rotated overnight at 4 °C. The anti-MR and anti-GR antibodies had previously been validated in pre-absorption experiments and Western blotting [14]. Protein A-coated Dynabeads (Life Technologies) were washed once in ice-cold 0.5% BSA/PBS before blocking overnight at 4 °C. Pre-blocked beads were washed once in ice-cold Tris-EDTA buffer. Bound DNA was eluted in two steps at RT: first with 200 µl elution buffer 1 (10 mM Tris HCl, pH 7.4, 50 mM NaCl, 1.5% SDS) and, second,

with 100 µl elution buffer 2 (10 mM Tris HCl, pH 7.4, 50 mM NaCl, 0.5% SDS). Cross-links were reversed by addition of NaCl (final concentration 200 mM) and overnight incubation at 65 °C. The next day, samples were incubated first with RNase A (60 µg/mL, 37 °C, 1 h) followed by incubation with proteinase K (250 µg/mL, 37 °C, 3.5 h). DNA was purified using a QIAquick PCR Purification Kit (Qiagen) as per the manufacturer's instructions. Input samples were incubated overnight at 65 °C in 200 mM NaCl to reverse cross-links and incubated with RNase A and proteinase K (over-night), and DNA was purified using a QIAquick PCR Purification Kit (Qiagen). Total dsDNA content was determined with a High-Sensitivity Qubit DNA Assay Kit (Life Technologies) as per the manufacturer's instructions and quantified using a Qubit 2.0 Fluorometer. All samples (bounds and inputs) were diluted to a standardized concentration with nuclease-free water and analyzed by qPCR as described below. A standard curve, created from serial dilutions of rat brain genomic DNA (Biochain), was included in each qPCR run for sample quantification. Data are expressed as quantity of bound DNA divided by the respective quantity of input DNA (i.e., B/I), which is a measure of the enrichment of steroid receptor bound to specific genomic sequences.

RNA extraction

RNA was extracted as described previously [14] using TRI Reagent (Sigma) following the manufacturer's guidelines and quantified using a NanoPhotometer P300 (Implen). 260/280 absorbance ratios, as an index of RNA purity, were consistently >2 and RNA integrity (RIN numbers) were >7.5 indicating the presence of intact RNA. Total RNA was reverse-transcribed into cDNA as per the manufacturer's instructions using the QuantiTect Reverse Transcription Kit (Qiagen; 15 min, 42 °C; 5 min, 95 °C) or using GoScript™ Reverse Transcription System (Promega; 5 min, 70 C; 5 min, 4 °C; 5 min, 25 °C; 60 min, 42 °C, 15 min, 70 °C) using a BioRad

T1000 Thermal Cycler. cDNA was diluted four-fold in nuclease-free water and 2 µl diluted cDNA was used per reaction in the qPCR analysis detailed below. Expression of hnRNA or mRNA in samples was calculated based on the Pfaffl method of relative quantification [16] using primer/probes as previously published [14] and standardized to the expression of house-keeping genes (HKGs) hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*Ywhaz*). These genes were selected as HKGs due to their stability across experimental groups (Suppl. Fig. 2 (metyrapone experiment) & 3 (ADX experiment)). The data were expressed as fold change relative to the relevant control condition.

qPCR Analysis

Mastermix for qPCR was prepared containing 900 nM forward and reverse primers, 200 nM probe, and 1×TaqMan Fast Mastermix (Life Technologies) and made up to volume with nfH_2O (nuclease-free H_2O). Primers and dual-labelled probe with 6-Carboxyfluorescein (6-FAM) as the fluorescent dye and tetra-methylrhodamine (TAMRA) as the quencher were designed using Primer Express software (version 3.0.1; Life Technologies). Standard curves were performed for each primer pair, and the qPCR efficiency was calculated using the equation E = ((10 − 1/slope) − 1) × 100 (where E is qPCR efficiency, and the slope is the gradient of the standard curve). Only primer pairs with efficiencies greater that 90% were used. qPCR was performed using a StepOne Plus Machine (Life Technologies). TaqMan enzymes were activated at 95 °C for 20 s, and then, 40 cycles of 95 °C (1 s) to 60 °C (20 s) to amplify samples.

Statistical Analysis

Data were statistically analyzed using GraphPad Prism 8 software. Results are presented as group means ± SEM; sample sizes are indicated in the legends to the figures. Data were

analyzed with two-way ANOVA. If significant, post-hoc Bonferroni tests were conducted. Results of the statistical analyses are provided in the legends to the figures. $P<0.05$ was considered statistically significant.

Results

Effect of metyrapone treatment on FS-induced plasma CORT and hippocampal MR and GR to GRE binding levels

Rats were injected once or twice with metyrapone (100mg/kg) or vehicle and killed under non-stress control conditions or at 30 min after the start of a 15min FS session (FS30). Figure 1 shows the changes in plasma CORT and MR and GR to GRE binding to the GC-responsive genes *Fkbp5*, *Per1* and *Sgk1* in the hippocampus. Metyrapone did not affect the control levels of plasma CORT. As expected, FS resulted in a strong hormone response (Fig. 1A; for statistical data, see the legends to the figures) reaching similar levels as previously reported by us in naïve rats [14]. Pre-treatment with metyrapone significantly attenuated the FS-induced CORT response but even after two metyrapone injections a residual stress effect was observed (Fig. 1A).

Overall, FS evoked an increase in the binding of MR and GR to GREs within the three targeted genes in the hippocampus (Fig. 1B-G) which is in agreement with our previous observations [14]. Metyrapone treatment led to significant reductions in stress-induced MR and GR binding to GREs, in particular with regard to receptor binding to GREs within *Fkbp5* and *Per1*. ~~Except for the binding of MR to *Fkbp5* and *Sgk1* (Fig. 1B, F), it appeared that the dual metyrapone injection resulted in a loss of the FS-induced increases of MR and GR to GREs (Fig. 1C, D, E, G).~~ The data in Figure 1 also indicate that metyrapone exerts distinct effects depending on whether rats were killed under control or stress conditions. This observation was statistically supported by the significant interaction terms of the two-way ANOVA analyses on the data in Fig. 1B-G. Whereas metyrapone overall resulted in reductions in stress-induced MR and GR binding, the drug, particularly after two injections, led to significant increases in receptor

binding in the control condition (see Fig. 1B, C, D, F). With regard to GR binding to *Per1* and *Sgk1*, a trend (P=0.09, Bonferroni post-hoc test) towards an increased binding was observed after two drug injection under control conditions (Fig. 1E, G). Thus, it appears that after two metyrapone injections, MR and GR binding reach levels which are indiscriminate between control and stress conditions.

Effect of metyrapone treatment on FS-induced plasma CORT and hippocampal GC target gene expression

In view of the more potent effects of dual metyrapone injections, we decided to use this condition when studying drug and stress effects on the expression of the *Fkbp5*, *Per1* and *Sgk1* genes in the hippocampus. As a control condition for the dual drug treatment, we conducted dual injections of the vehicle. Thus, as described in the Methods and depicted in Suppl. Fig. 1, we injected rats twice with vehicle or metyrapone (100mg/kg i.p.) and killed them under non-stress control conditions or at 60min after start of FS (FS60). The FS60 time point was chosen as this was the peak time for *Fkbp5*, *Per1* and *Sgk1* hnRNA or mRNA [14]. In the vehicle-injected animals, FS resulted in a significant increase in plasma CORT levels at 60min compared with control animals, similar to levels reported by us before [14]. In the metyrapone-treated rats, no significant stress-induced increase in plasma hormone levels at this time point could be observed compared with the drug-treated controls (Fig. 2A). Furthermore, no significant effect of the drug on CORT levels in control animals was found at this time point (Fig. 2A).

FS significantly enhanced the hippocampal hnRNA expression of *Fkbp5*, *Per1* and *Sgk1* (Fig. 2B, D, F), but only the mRNA of *Per1* and *Sgk1* (Fig. 2E, G). *Fkbp5* mRNA is known to respond

to FS at FS120 but not at FS60 [14]. Metyrapone treatment produced differential effects on control and stress-induced RNA levels. In the control rats, the dual drug treatment resulted in significant increases in the levels of all hnRNAs and mRNAs of the investigated GC-target genes (Fig. 2B-G). We observed, however, gene-dependent effects after stress and drug treatment. With regard to *Fkbp5*, metyrapone treatment strongly attenuated the stress-induced increase in hnRNA levels but slightly, albeit significantly, increased the mRNA levels (Fig. 2B, C). In both instances, the attained *Fkbp5* RNA levels were not different from the respective levels in the drug-treated control animals (Fig. 2B, C). *Per1* hnRNA levels were significantly higher in the metyrapone-treated rats after FS when compared with the corresponding vehicle-treated animals whereas there were no differences in the stress-evoked mRNA responses (Fig. 2D, E). FS stress evoked significant increases in both hnRNA and mRNA levels of *Sgk1* in the vehicle-treated rats (Fig. 2F, G). *Sgk1* hnRNA and mRNA levels in the stressed drug-treated animals were similar to those in the stressed vehicle-treated animals but these levels were not different from those in the drug-treated control animals (Fig. 2F, G). Clearly, metyrapone seems to produce distinct effects which are condition-, gene- and transcript-dependent.

Adrenalectomy abolishes stress-evoked MR and GR binding to GREs within GC target genes

The experiments applying the CORT synthesis inhibitor metyrapone showed that there was incomplete inhibition of synthesis as well as unexpected results with regard to MR and GR GRE-binding and RNA responses. Therefore, we decided to study these molecular GC endpoints under conditions of complete surgical removal of the source of CORT, i.e. ADX. We used two control conditions: sham operated and untouched intact rats. As expected, ADX

completely abolished the FS-induced increase in CORT levels (Fig. 3A). Both intact and sham rats showed strong CORT responses to FS and this response was slightly but significantly higher in the sham-operated rats than in the intact animals (Fig. 3A). Baseline AM (BLAM) CORT levels were very low and not significant different between ADX, sham or intact rats (Fig. 3A). The BLAM groups in this set of experiments were not termed 'controls' to distinguish them from the control groups in the metyrapone experiments which had received injections as part of the experimental protocol.

Regarding MR and GR binding to GREs within target genes, significant increases were found after stress at all genes in both intact and sham groups (Fig. 3B-G), except for the stress-induced MR binding to the *Sgk1* GRE in the sham group that just escaped significance (P=0.053, post-hoc Bonferroni test (Fig. 3F). ADX abrogated all stress-evoked increases in MR and GR binding to GREs (Fig. 3B-G). Furthermore, under BLAM conditions, MR binding to GREs within *Per1* and *Sgk1* in ADX rats was significantly lower than levels observed under such conditions in intact and/or sham animals (Fig. 3D, F). As expected, GR binding to GREs under BLAM conditions did not differ at any gene loci between groups.

Together, these results show that ADX results in a consistent abrogation of the effects of stress on MR and GR binding to GREs within the GC target genes *Fkbp5*, *Per1* and *Sgk1*.

Adrenalectomy abrogates stress-induced GC target gene transcriptional responses

Using the same experimental design as in the previous experiment, except for killing the rats at 60min after FS, we studied the effects of ADX on baseline and stress-induced gene transcriptional responses. Also in this experiment, as expected, ADX abolished the plasma

CORT response to stress (Fig. 4A). The stress-induced plasma CORT response was higher in the sham rats than in the intact animals (Fig. 4A) which is consistent with the difference in responses at FS30 (Fig. 3A).

FS stress resulted in significant increases in *Fkbp5* hnRNA, *Per1* hnRNA and mRNA, and *Sgk1* hnRNA and mRNA in the hippocampus of intact and sham rats (Fig. 4B, D-G); thereby, intact and sham animals showing comparable responses to stress. Clearly, ADX completely abolished these stress-evoked RNA responses (Fig. 4B, D-G). Removal of the adrenal glands however had no effect on the BLAM levels of these RNAs. Consistent with previous observations (Fig. 3C; [14]), there was no effect of stress apparent on hippocampal Fkbp5 mRNA levels in the intact and sham rats. ADX resulted in a significant decrease in the BLAM levels of this mRNA, indicating that, in addition to stress, Fkbp5 gene transcription is regulated by very low levels of early morning GC secretion as well (Fig. 4C).

Discussion

This study reveals unexpected genomic effects in the hippocampus following metyrapone treatment which appear to be unrelated to the drug's effects on GC secretion. Despite failing to exert any effect on CORT levels under unstressed control conditions, drug treatment resulted in enhanced MR and GR to GRE binding within the GC target genes and increased transcriptional activity of these genes. Metyrapone reduced stress-induced GC levels, however without leading to full abrogation of this stress response. Under these conditions, drug treatment led to a partial inhibition of MR and GR to GRE binding and transcriptional activation, in particular if responses were compared with the respective vehicle-treated control groups. Thus, under stress conditions, the observed changes in MR and GR binding and transcriptional activity after metyrapone treatment appear to be the combined result of unspecific, GC-unrelated effects (discernible under control conditions) and GC-reducing effects. In particular regarding effects on transcriptional activity, the drug effects were gene-dependent. In contrast, ADX abolished the stress effects on plasma CORT as well as hippocampal MR and GR to GRE binding and transcriptional responses. These clear-cut genomic effects directly correspond with the absence of endogenous GCs as a result of the adrenal extirpation.

Our ChIP data show that vehicle-treated control rats as well as intact and sham rats present very low MR and GR binding to GREs within the classical GC target genes *Fkbp5*, *Per1* and *Sgk1*. FS resulted in significant increases in receptor binding to these GREs. These results are highly consistent with our previously reported findings in (untreated) intact rats [14]. Similarly, the FS-induced RNA responses in the vehicle-treated, intact and sham rats paralleled our previously published data [14]. Our present study shows that ADX completely

abolished the stress-evoked responses in receptor binding and gene transcription indicating that the presence of endogenous GCs is crucial for these responses. Earlier work has shown that CORT is metabolized rapidly, and MRs and GRs become unoccupied within a few hours post-ADX [17]. Thus, similarly, in the present study in the case of ADX, MRs and GRs will have become devoid of ligand shortly after withdrawal of CORT from the drinking water. Accordingly, we show here for the first time that these receptors indeed require ligand binding in order to interact with GREs in vivo after stress.

We started this study on the role of endogenous GCs in baseline and stress-induced genomic responses in GC target genes by using the CORT-synthesis inhibitor metyrapone. Our data show, however, that treatment with this drug produced contradictory results with regard to plasma CORT levels versus MR and GR to GRE binding and RNA responses concerning the genes *Fkbp5*, *Per1* and *Sgk1*. Metyrapone attenuated, albeit not abolished, the stress-induced increases in plasma CORT and GRE-binding of MR and GR. In view of the drug's effect on GC levels, a reduction in stress-induced receptor binding was expected but was not as stark as may have been predicted given the strong reduction in stress-evoked CORT levels. Surprisingly, however, although metyrapone produced no changes in circulating GC levels in the unstressed control rats, drug treatment resulted in significant increases in MR binding and also to some extent GR binding (i.e. *Fkbp5*) to GREs within the target genes. These observations are rather puzzling as CORT levels were unchanged. A possible reason for the elevated MR binding after metyrapone treatment may be 11-deoxycorticosterone (DOC), the CORT precursor known to accumulate after inhibition of 11β-hydroxylase [18]. DOC is a mineralocorticoid with very high affinity for binding to MR [19] and is thought to be responsible for some of the side effects of metyrapone treatment (e.g. hypertension, hypokalemia, edema) observed in humans treated for instance for

Cushing's disease [20]. As hippocampal MRs are at least 75-80% occupied under early morning baseline conditions [17, 21], DOC could bind to the 20-25% rest-capacity in MR binding potentially resulting in the enhanced MR to GRE binding observed after metyrapone. Whilst this perhaps may be an explanation for the increased MR to GRE binding after metyrapone under control conditions, it is very unlikely to explain the elevated GR to GRE binding after drug administration under these conditions. Increased DOC levels after metyrapone are irrelevant for GR as this steroid has very low affinity for binding to GRs. Based on our recent study [14], it may be argued that possibly increased GR to GRE binding may be indirectly brought about through heterodimerization with MR whose GRE-binding is found to be enhanced after metyrapone. This is, however, an unlikely possibility as, under these control conditions, the occupancy of GRs by CORT will be very low and thus the receptor would not be in a GRE-binding state [17, 21]. Thus, presently, the mechanisms underlying the increased MR and GR to GRE binding are unclear. Elevated post-metyrapone DOC levels can partially provide an explanation. Most likely, other, as yet unknown, effects of the enzyme inhibitor are very likely to play a role in the observations made in the drug-treated control rats.

In view of the diverse effects of metyrapone on the binding of MR and GR to *Fkbp5*, *Per1* and *Sgk1* GREs under control and stress conditions, it is difficult to explain the effects of the drug on the responses in hnRNA and mRNA of these genes. The increased hnRNA and mRNA levels observed under control conditions after metyrapone administration appear to correspond with the enhanced MR and GR to GRE binding under these conditions. The stress-induced responses in hnRNA after metyrapone treatment are highly varied. Hence, we found that the drug inhibited, enhanced, or had no effects on the stress-induced changes in *Fkbp5* hnRNA, *Per1* hnRNA, and *Sgk1* hnRNA, respectively. These results appear

not to point to a single, consistent mechanism underpinning the gene transcriptional effects of metyrapone. Factors contributing to these distinct effects may include differences in the relative contribution of MR and GR to gene activity, inherent differences between genes regarding transcriptional regulation (e.g. multiple GREs within genes or in enhancer regions, chromatin looping mechanisms), and differences in the timeline of transcriptional activation and RNA processing. ADX, in contrast, produced highly consistent effects on both receptor to GRE binding as well as gene transcriptional responses.

The aim of the present study was to investigate the role of endogenous GCs in the effects of an acute stressful challenge (FS) on MR and GR binding to GC target genes and the transcriptional responses of these genes. To block endogenous GCs we first used the CORT-synthesis inhibitor metyrapone in order to learn whether this pharmacological approach would be an appropriate alternative to the surgical procedure of ADX. Our data show that the drug produces diverse genomic effects that can only partially be explained by its CORT-reducing activity and accumulation of DOC. In particular, its effects on gene transcriptional activity were highly diverse. In contrast, the effects of ADX on the GC-dependent genomic endpoints were unambiguous. Importantly, the genes *Fkbp5*, *Per1* and *Sgk1* in the hippocampus play important roles in GC sensitivity and negative feedback regulation, circadian regulation of physiological and behavioral activities, and neuroplasticity processes underlying learning and memory, respectively [22-24]. Moreover, these genes as well as many other GC-regulated genes play important roles in many organs throughout the entire body. Therefore, evidently the unexpected genomic effects of metyrapone question its suitability for conducting studies on the GC dependency of physiological and behavioral processes. Our observations indicate that results on such processes may be difficult to

interpret or may be liable to misinterpretation. We conclude that ADX is (still) the method of choice to elucidate the role of endogenous GC secretion in physiology and behavior.

Author Contribution Statement

All authors contributed to the design of the study, the experimental work, the data analysis and the writing of the manuscript.

Conflict of Interest Statement

The authors have no conflicts of interest.

Data Sharing Statement

The data that support the findings of this study are available upon request.

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Legends to the Figures

Fig. 1. Plasma CORT levels (A) and hippocampal MR and GR binding to GREs within GC target genes (B-G) under control and stress conditions following metyrapone treatment. Rats were treated with vehicle or metyrapone (100mg/kg) as described in the Methods section and Suppl. Figure 1. Rats were killed 30 min (FS30) after the start of FS (15 min in 25 °C-water). In parallel, separate groups of rats were killed as unstressed controls. Plasma CORT levels were determined in trunk blood (A) and expressed as ng/ml (mean ± SEM, n = 8 per group). Hippocampus tissues were collected for MR and GR ChIP and qPCR analysis. Tissues of 2 rats were pooled for each chromatin preparation. Enrichment of MR and GR were determined at GREs within *Fkbp5* (B, C), *Per1* (D, E) and *Sgk1* (F, G), as indicated. Enrichment was expressed as Bound/Input; mean ± SEM, n = 3-4. Data were analyzed by two-way ANOVA followed by post-hoc Bonferroni tests, if appropriate. Two-way ANOVA analysis: A: Effect of (drug) treatment: $F(2, 42)=120.2$, $P<0.0001$, Effect of stress: $F(1, 42)=388.4$, $P<0.0001$, Interaction: $F(2, 42)=133.7$, $P<0.0001$; B: Effect of treatment: $F(2, 17)=5.603$, $P=0.0135$, Effect of stress: $F(1, 17)=161.6$, $P<0.0001$, Interaction: $F(2, 17)=22.43$, $P<0.0001$; C: Effect of treatment: $F(2, 16)=4.629$, $P=0.0259$, Effect of stress: $F(1, 16)=92.65$, $P<0.0001$, Interaction: $F(2, 16)=20.54$, $P<0.0001$; D: Effect of treatment: $F(2, 17)=1.363$, $P=0.2825$, Effect of stress: $F(1, 17)=62.50$, $P<0.0001$, Interaction: $F(2, 17)=9.143$, $P=0.0020$; E: Effect of treatment: $F(2, 18)=0.6013$, $P=0.5587$, Effect of stress: $F(1, 18)=36.53$, $P<0.0001$, Interaction: $F(2, 18)=6.187$, $P=0.0090$; F: Effect of treatment: $F(2, 17)=3.297$, $P=0.0616$, Effect of stress: $F(1, 17)=50.47$, $P<0.0001$, Interaction: $F(2, 17)=9.787$, $P=0.0015$; G: Effect of treatment: $F(2, 18)=0.7892$, $P=0.4693$, Effect of stress: $F(1, 18)=48.13$, $P<0.0001$, Interaction: $F(2, 18)=4.058$, $P=0.0351$; $^{*}P < 0.05$ compared with the respective Control group; $^{\$}P < 0.05$ compared with the respective vehicle-

treated group; E: P=0.093, Met 2x100/Control vs Vehicle/Control; F: P=0.085, Met 2x100/FS30 vs Vehicle/FS30; G: P=0.095, Met 2x100/FS30 vs Vehicle/FS30, post-hoc Bonferroni test. Abbreviations: Met 100, a single injection of 100 mg/kg metyrapone; Met 2x 100, two injections of 100 mg/kg metyrapone

Fig. 2. Plasma CORT levels (A) and hippocampal GC target gene expression (B-G) under control and stress conditions following metyrapone treatment. Rats were treated with vehicle or metyrapone (100mg/kg) as described in the Methods section and Suppl. Figure 1. Rats were killed 60 min (FS60) after the start of FS (15 min in 25 °C-water). In parallel, separate groups of rats were killed as unstressed controls. Plasma CORT levels were determined in trunk blood (A) and expressed as ng/ml (mean ± SEM, n = 6 per group). Hippocampus tissues were collected for RNA extraction and qPCR analysis. Levels of RNA expression (*Fkbp5* hnRNA and mRNA, B, C, respectively; *Per1* hnRNA and mRNA, D, E; *Sgk1* hnRNA and mRNA, F, G) were expressed as fold change relative to the vehicle-treated group (mean ± SEM, n = 6 per group). For more information, see Methods section. Data were analyzed by two-way ANOVA followed by post-hoc Bonferroni tests, if appropriate. Two-way ANOVA analysis: A: Effect of (drug) treatment: $F(1, 19)=10.31$, $P=0.0046$, Effect of stress: $F(1, 19)=38.39$, $P<0.0001$, Interaction: $F(1, 19)=18.75$, $P=0.0004$; B: Effect of (drug) treatment: $F(1, 20)=28.42$, $P<0.0001$, Effect of stress: $F(1, 20)=212.9$, $P<0.0001$, Interaction: $F(1, 20)=199.6$, $P<0.0001$; C: Effect of (drug) treatment: $F(1, 20)=27.48$, $P<0.0001$, Effect of stress: $F(1, 20)=0.07551$, $P=0.7863$, Interaction: $F(1, 20)=1.527$, $P=0.2309$; D: Effect of (drug) treatment: $F(1, 20)=56.49$, $P<0.0001$, Effect of stress: $F(1, 20)=52.48$, $P<0.0001$, Interaction: $F(1, 20)=1.027$, $P=0.3229$; E: Effect of (drug) treatment: $F(1, 20)=21.13$, $P=0.0002$, Effect of stress: $F(1, 20)=29.65$, $P<0.0001$, Interaction: $F(1, 20)=2.762$, $P=0.1122$; F: Effect of (drug) treatment: $F(1, 20)=33.13$, $P<0.0001$, Effect of

stress: F(1, 20)=46.63, P<0.0001, Interaction: F(1, 20)=28.89, P<0.0001; G: Effect of (drug) treatment: F(1, 20)=32.04, P<0.0001, Effect of stress: F(1, 20)=81.45, P<0.0001, Interaction: F(1, 20)=48.42, P<0.0001; *$P < 0.05$ compared with the respective Control group; $^{\$}P < 0.05$ compared with the respective vehicle-treated group, post-hoc Bonferroni test

Fig. 3. Plasma CORT levels (A) and hippocampal MR and GR binding to GREs within GC target genes (B-G) under control and stress conditions after ADX. Rats were ADX, sham-ADX or left untouched as described in the Methods section and Suppl. Figure 1. Rats were killed 30 min (FS30) after the start of FS (15 min in 25 °C-water). In parallel, separate groups of rats were killed under baseline AM (BLAM) conditions. Plasma CORT levels were determined in trunk blood (A) and expressed as ng/ml (mean ± SEM, n = 8 per group). Hippocampus tissues were collected for MR and GR ChIP and qPCR analysis. Tissues of 2 rats were pooled for each chromatin preparation. Enrichment of MR and GR were determined at GREs within *Fkbp5* (B, C), *Per1* (D, E) and *Sgk1* (F, G), as indicated. Enrichment was expressed as Bound/Input; mean ± SEM, n = 3-4. Data were analyzed by two-way ANOVA followed by post-hoc Bonferroni tests, if appropriate. Two-way ANOVA analysis: A: Effect of treatment: F(2, 18)=87.11, P<0.0001, Effect of stress: F(1, 18)=325.8, P<0.0001, Interaction: F(2, 18)=85.40, P<0.0001; B: Effect of treatment: F(2, 18)=69.81, P<0.0001, Effect of stress: F(1, 18)=177.3, P<0.0001, Interaction: F(2, 18)=38.41, P<0.0001; C: Effect of treatment: F(2, 18)=32.31, P<0.0001, Effect of stress: F(1, 18)=108.0, P<0.0001, Interaction: F(2, 18)=26.47, P<0.0001; D: Effect of treatment: F(2, 18)=31.32, P<0.0001, Effect of stress: F(1, 18)=18.77 P=0.0004, Interaction: F(2, 18)=3.366, P=0.0573; E: Effect of treatment: F(2, 18)=54.47, P<0.0001, Effect of stress: F(1, 18)=169.0, P<0.0001, Interaction: F(2, 18)=34.12, P<0.0001; F: Effect of treatment: F(2, 17)=22.11,

P<0.0001, Effect of stress: F(1, 17)=10.79, P=0.0044, Interaction: F(2, 17)=1.990, P=0.1673; G: Effect of treatment: F(2, 18)=36.01, P<0.0001, Effect of stress: F(1, 18)=101.3, P<0.0001, Interaction: F(2, 18)=18.86, P<0.0001; *$P < 0.05$ compared with the respective BLAM group; $^{\$}P < 0.05$ compared with the respective Intact group; $^{\#}$, P<0.05 compared with the respective Sham group; F: P=0.083, BLAM/ADX vs BLAM/Intact, P=0.053, FS30/Sham vs BLAM/Sham, post-hoc Bonferroni test

Fig. 4. Plasma CORT levels (A) and hippocampal GC target gene expression (B-G) under control and stress conditions after ADX. Rats were ADX, sham or left untouched as described in the Methods section and Suppl. Figure 1. Rats were killed 60 min (FS60) after the start of FS (15 min in 25 °C-water). In parallel, separate groups of rats were killed under baseline AM (BLAM) conditions. Plasma CORT levels were determined in trunk blood (A) and expressed as ng/ml (mean ± SEM, n = 6 per group). Hippocampus tissues were collected for RNA extraction and qPCR analysis. Levels of RNA expression (*Fkbp5* hnRNA and mRNA, B, C, respectively; *Per1* hnRNA and mRNA, D, E; *Sgk1* hnRNA and mRNA, F, G) were expressed as fold change relative to the vehicle-treated group (mean ± SEM, n = 6 per group). For more information, see Methods section. Data were analyzed by two-way ANOVA followed by post-hoc Bonferroni tests, if appropriate. Two-way ANOVA analysis: A: Effect of treatment: F(2, 29)=5.196, P=0.0118, Effect of stress: F(1, 29)=4.540, P=0.0417, Interaction: F(2, 29)=1.874, P=0.1717; B: Effect of treatment: F(2, 29)=32.80, P<0.0001, Effect of stress: F(1, 29)=126.4, P<0.0001, Interaction: F(2, 29)=25.47, P<0.0001; C: Effect of treatment: F(2, 29)=21.02, P<0.0001, Effect of stress: F(1, 29)=2.809, P=0.1045, Interaction: F(2, 29)=0.300, P=0.7431; D: Effect of treatment: F(2, 29)=13.42, P<0.0001, Effect of stress: F(1, 29)=34.27, P<0.0001, Interaction:

F(2, 29)=6.069, P=0.0063; E: Effect of treatment: F(2, 29)=13.29, P<0.0001, Effect of stress: F(1, 29)=91.85, P<0.0001, Interaction: F(2, 29)=13.26, P<0.0001; F: Effect of treatment: F(2, 29)=17.45, P<0.0001, Effect of stress: F(1, 29)=49.30, P<0.0001, Interaction: F(2, 29)=15.18, P<0.0001; G: Effect of treatment: F(2, 29)=60.00, P<0.0001, Effect of stress: F(1, 29)=189.7, P<0.0001, Interaction: F(2, 29)=51.26, P<0.0001; *P < 0.05 compared with the respective BLAM group; $^{\$}P$ < 0.05 compared with the respective Intact group; $^{\#}$, P<0.05 compared with the respective Sham group, post-hoc Bonferroni test.

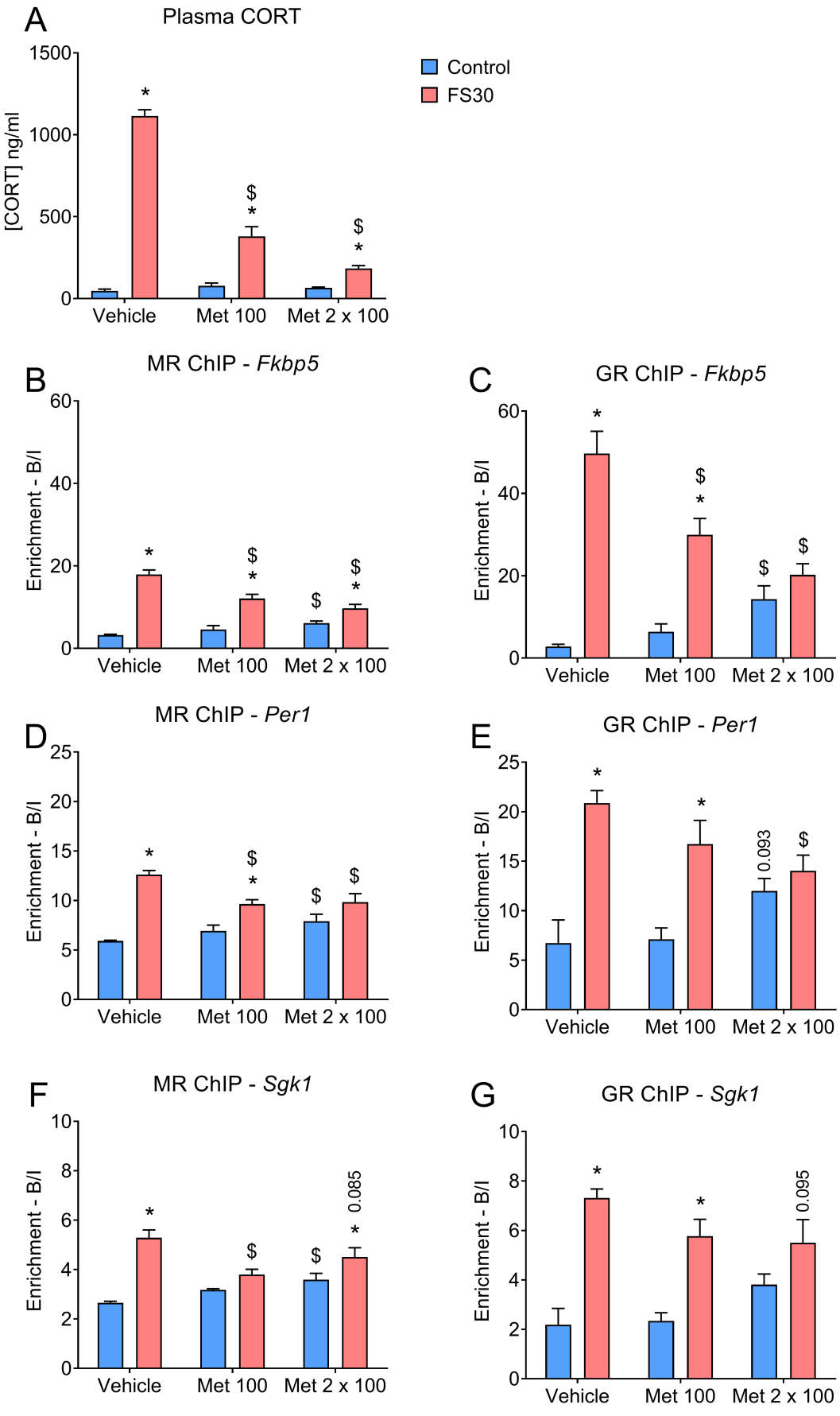
Supplementary Fig. 1. Experimental schedule of the experiments involving metyrapone treatment. Rats were injected once (injection at 0 min) or twice (injections at 0 min and 90 min) with 100mg/kg metyrapone or vehicle which was followed by a FS challenge or not (home cage). After FS, the rats were returned to their home cage until they were killed for blood and tissue collection, as indicated. The data of the experiment described in the top aspect of the table are depicted in Figure 1 whereas the data of the experiment described in the bottom part are shown in Figure 2.

Supplementary Fig. 2. Hippocampal HKG gene expression under control and stress conditions following metyrapone treatment. Rats were treated with vehicle or metyrapone (100mg/kg) as described in the Methods section and Suppl. Figure 1. Rats were killed 60 min (FS60) after the start of FS (15 min in 25 °C-water). In parallel, separate groups of rats were killed as unstressed controls. Hippocampus tissues were collected for RNA extraction and qPCR analysis. Expression of the housekeeping genes *Hprt1* (A) and *Ywhaz* (B), based on qPCR cycle threshold (CT) values, remained stable across treatment groups (mean ± SEM, n =

6 per group). Stability was confirmed by a lack of statistical difference in the two-way ANOVA. Two-way ANOVA analysis: A: Effect of (drug) treatment: F(1, 20)=1.894, P=0.1840, Effect of stress: F(1, 20)=1.643, P=0.2145, Interaction: F(1, 20)=1.094, P=3082; B: Effect of (drug) treatment: F(1, 20)=1.128, P=0.3009, Effect of stress: F(1, 20)=1.608, P=0.2194, Interaction: F(1, 20)=1.045, P=0.3189.

Supplementary Fig. 3. Hippocampal HKG gene expression under control and stress conditions after ADX. Rats were ADX, sham or left untouched as described in the Methods section. Rats were killed 60 min (FS60) after the start of FS (15 min in 25 °C-water). In parallel, separate groups of rats were killed under baseline AM (BLAM) conditions. Hippocampus tissues were collected for RNA extraction and qPCR analysis. Expression of the housekeeping genes *Hprt1* (A) and *Ywhaz* (B), based on qPCR cycle threshold (CT) values, remained stable across treatment groups (mean ± SEM, n = 5-6 per group). Stability was confirmed by a lack of statistical difference in the two-way ANOVA. Two-way ANOVA analysis: A: Effect of treatment: F(2, 29)=0.8653, P=0.4315, Effect of stress: F(1, 29)=0.06723, P=0.7973, Interaction: F(2, 29)=0.5806, P=0.5659; B: Effect of treatment: F(2, 29)=0.6436, P=0.5327, Effect of stress: F(1, 29)=0.3685, P=0.5485, Interaction: F(2, 29)=0.2234, P=0.8011

Figure 1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1000
500
0
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FS30
Vehicle
Met 100
Met 2 x 100
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Enrichment - B/I
60
40
20
0
C
GR ChIP - Fkbp5
D
MR ChIP - Per1
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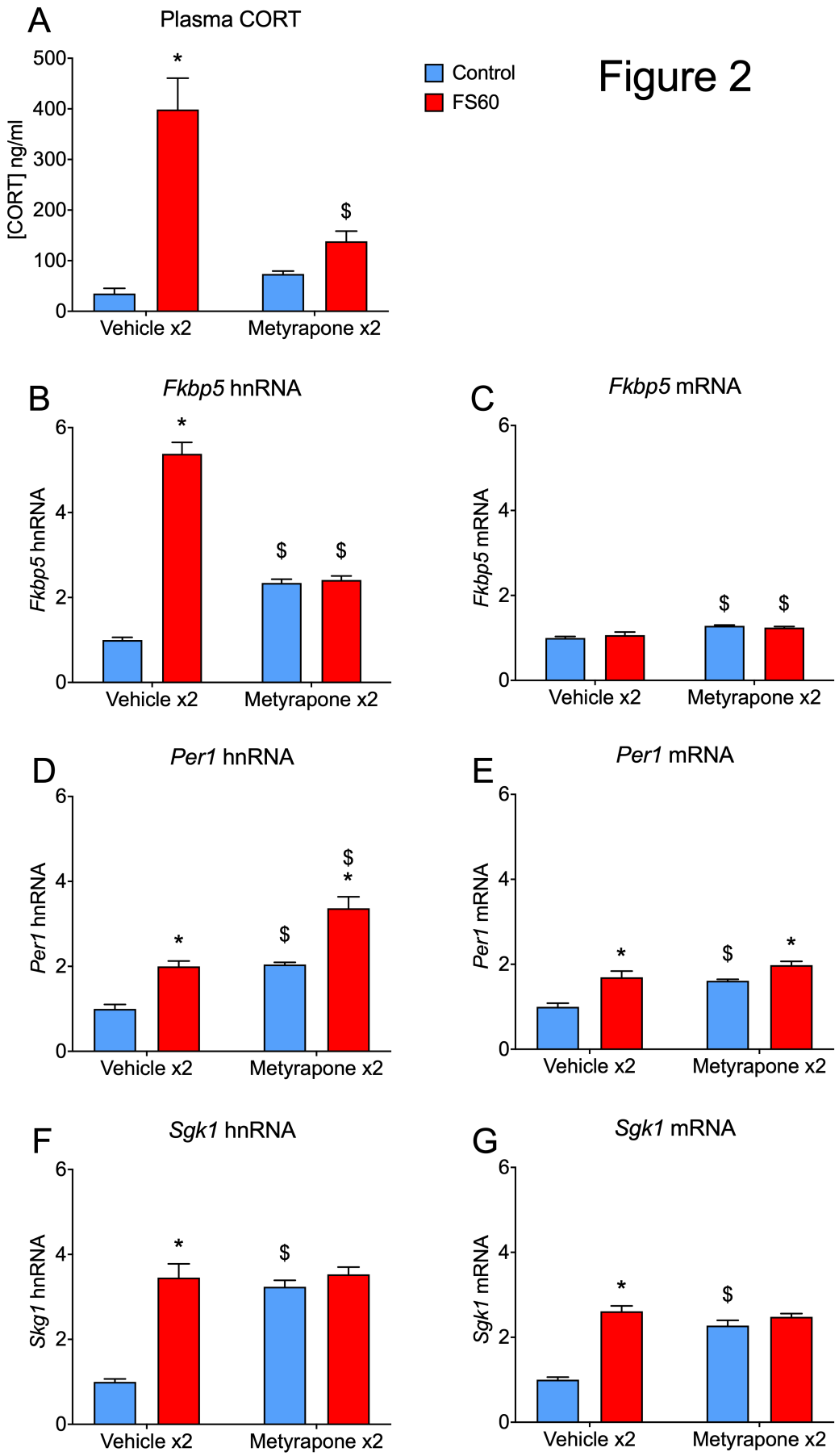
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Plasma CORT
[CORT] ng/ml
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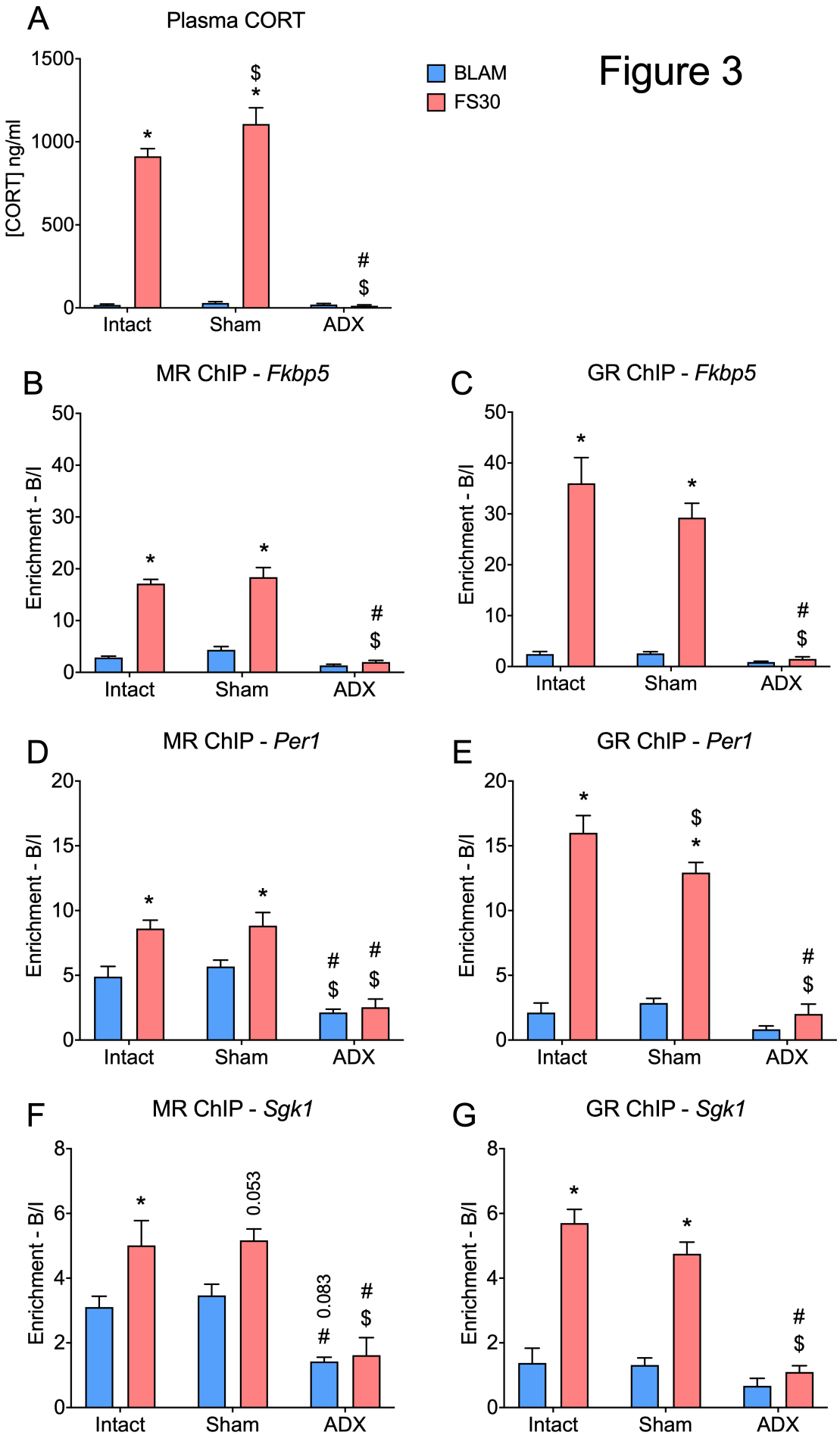
400
300
200
100
0
Vehicle x2
Metyrapone x2
Control
FS60
Figure 2
B
Fkbp5 hnRNA
C
Fkbp5 mRNA
D
Per1 hnRNA
E
Per1 mRNA
F
Skg1 hnRNA
G
Sgk1 mRNA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1000
500
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Intact
Sham
ADX
BLAM
FS30
Figure 3
B
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Enrichment - B/I
50
40
30
20
10
0
C
GR ChIP - Fkbp5
D
MR ChIP - Per1
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F
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6
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2
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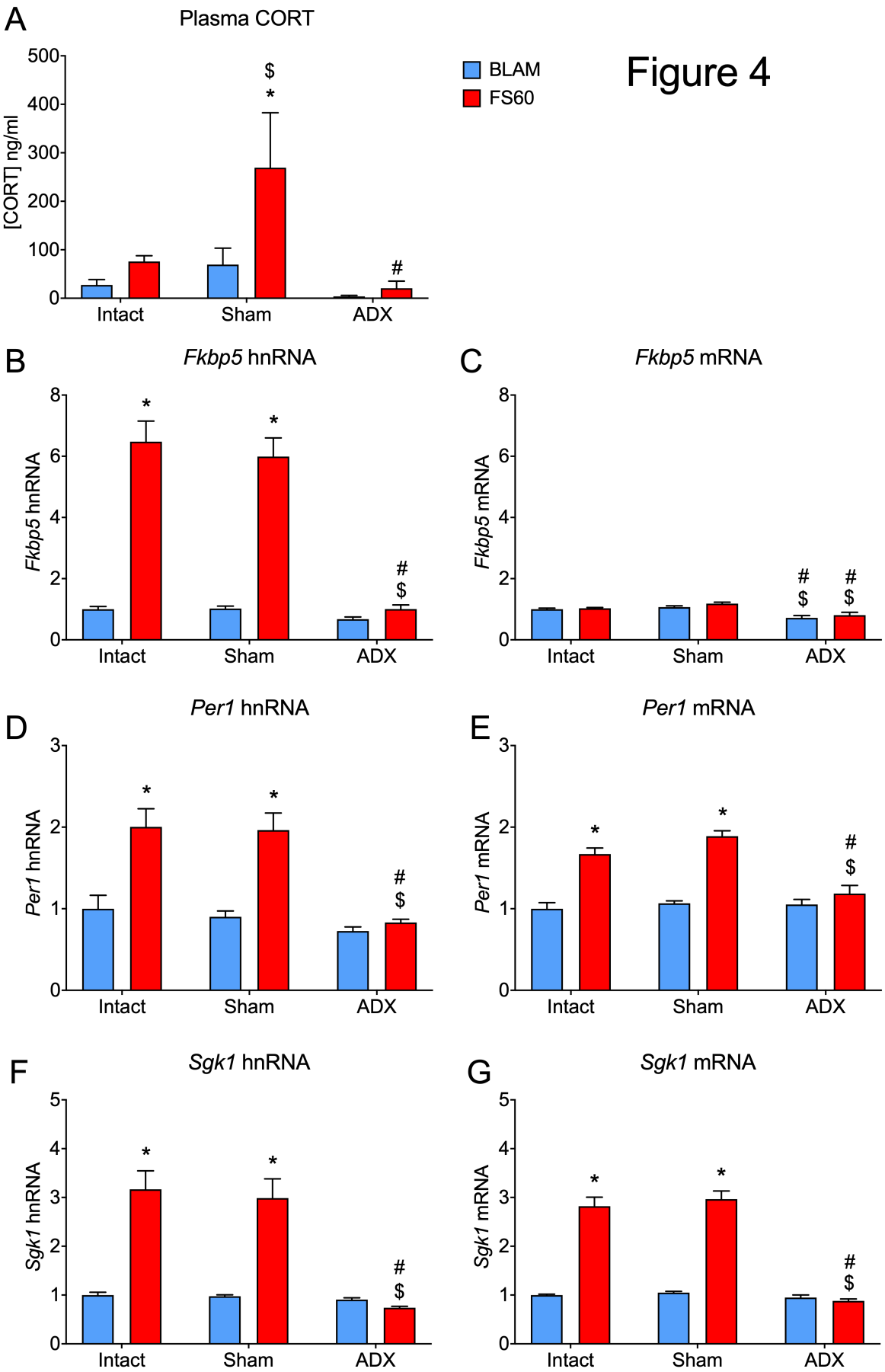
G
GR ChIP - Sgk1


A
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200
100
0
Intact
Sham
ADX
BLAM
FS60
Figure 4
B
Fkbp5 hnRNA
C
Fkbp5 mRNA
D
Per1 hnRNA
E
Per1 mRNA
F
Sgk1 hnRNA
G
Sgk1 mRNA

Supplemental Figure 1

<table>
<tr><td colspan="2">Metyrapone experiments</td><td colspan="49">Time (min)</td></tr>
<tr><td>Experiment 1 (Figure 1)</td><td>Time of 1st injection</td><td>0</td><td>5</td><td>10</td><td>15</td><td>20</td><td>25</td><td>30</td><td>35</td><td>40</td><td>45</td><td>50</td><td>55</td><td>60</td><td>65</td><td>70</td><td>75</td><td>80</td><td>85</td><td>90</td><td>95</td><td>100</td><td>105</td><td>110</td><td>115</td><td>120</td><td>125</td><td>130</td><td>135</td><td>140</td><td>145</td><td>150</td><td>155</td><td>160</td><td>165</td><td>170</td><td>175</td><td>180</td><td>185</td><td>190</td><td>195</td><td>200</td><td>205</td><td>210</td><td>215</td><td>220</td><td>225</td><td>230</td><td>235</td><td>240</td></tr>
<tr><td>Vehicle Control</td><td>~8-11 am</td><td colspan="18">Injection (i.p.)</td><td colspan="6">Home cage</td><td>Kill</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Vehicle FS30</td><td>~8-11 am</td><td colspan="18">Injection (i.p.)</td><td colspan="3">FS test</td><td colspan="3">Home cage</td><td>Kill</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Met (100 mg/kg) Control</td><td>~8-11 am</td><td colspan="18">Injection (i.p.)</td><td colspan="6">Home cage</td><td>Kill</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Met (100 mg/kg) FS30</td><td>~8-11 am</td><td colspan="18">Injection (i.p.)</td><td colspan="3">FS test</td><td colspan="3">Home cage</td><td>Kill</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Met (2*100 mg/kg) Control</td><td>~8-11 am</td><td colspan="18">Injection (i.p.)</td><td colspan="18">Injection (i.p.)</td><td colspan="6">Home cage</td><td>Kill</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Met (2*100 mg/kg) FS30</td><td>~8-11 am</td><td colspan="18">Injection (i.p.)</td><td colspan="18">Injection (i.p.)</td><td colspan="3">FS test</td><td colspan="3">Home cage</td><td>Kill</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
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<table>
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<tr><td>Vehicle Control</td><td>~7-10 am</td><td colspan="18">Injection (i.p.)</td><td colspan="18">Injection (i.p.)</td><td colspan="12">Home cage</td><td>Kill</td></tr>
<tr><td>Vehicle FS30</td><td>~7-10 am</td><td colspan="18">Injection (i.p.)</td><td colspan="18">Injection (i.p.)</td><td colspan="3">FS test</td><td colspan="9">Home cage</td><td>Kill</td></tr>
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<tr><td>Met (2*100 mg/kg) FS30</td><td>~7-10 am</td><td colspan="18">Injection (i.p.)</td><td colspan="18">Injection (i.p.)</td><td colspan="3">FS test</td><td colspan="9">Home cage</td><td>Kill</td></tr>
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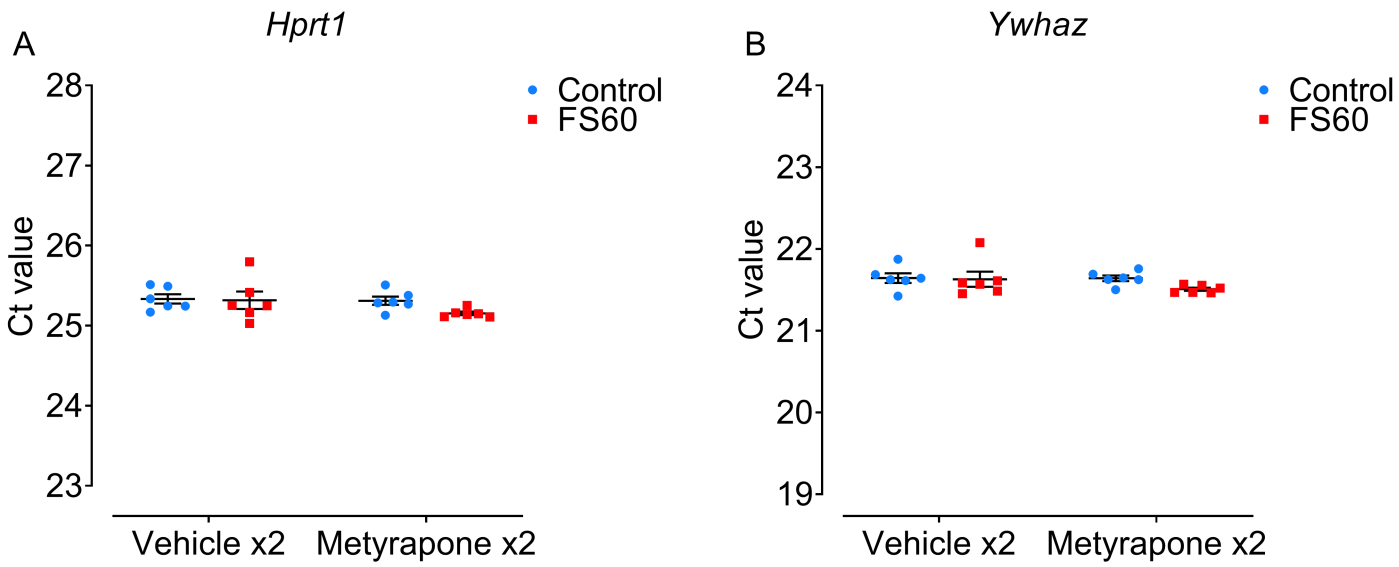
Suppl. Fig. 2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FS60
Vehicle x2
Metyrapone x2
B
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Ct value
24
23
22
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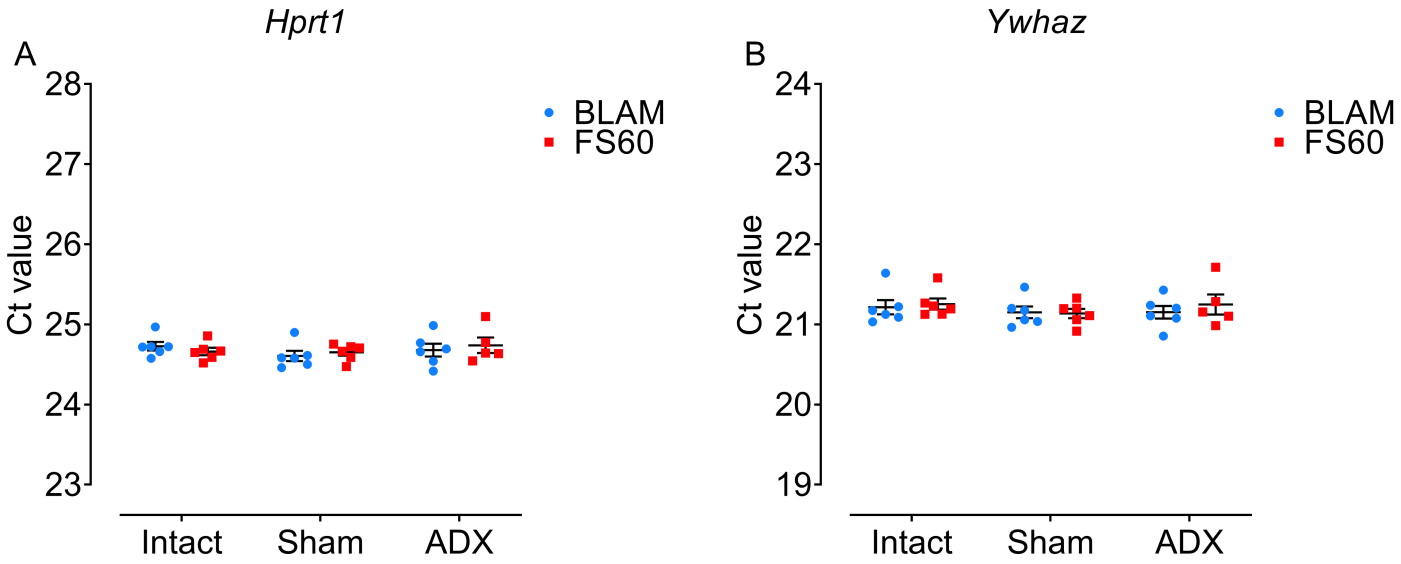
20
19
Control
FS60
Vehicle x2
Metyrapone x2

Suppl. Fig. 3


A
Hprt1
Ct value
28
27
26
25
24
23
Intact
Sham
ADX
BLAM
FS60
B
Ywhaz
Ct value
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22
21
20
19
Intact
Sham
ADX
BLAM
FS60