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Leiden
The Netherlands

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

Koorneef, L.L.

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Dexamethasone-associated metabolic effects are partially caused by depletion of endogenous corticosterone

Lisa L. Koorneef
Sander Kooijman
Jari F. Scheerstra
Maaïke C. Voorhoeve
Ajith Narayanan Nadamuni Ramesh
Patrick C. N. Rensen
Jan Kroon*
Onno C. Meijer*

**Authors contributed equally*

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ABSTRACT

Dexamethasone is commonly used in the clinic to treat auto-immune disease and inflammation. Despite being relatively safe at a low dose, high-dose dexamethasone causes side effects such as obesity and insulin resistance in the majority of patients. Dexamethasone has a high affinity for the glucocorticoid receptor (GR) and completely suppresses endogenous glucocorticoid secretion. Since dexamethasone has a very low affinity for the cortisol-binding mineralocorticoid receptor (MR), (side) effects of dexamethasone may be the result of GR hyperactivation as well as MR hypoactivation. We therefore hypothesized that reactivation of MR, via co-administration of a low dose of corticosterone, can reduce part of the metabolic (side) effects of dexamethasone treatment. The 8-week old male C57Bl/6J mice received fructose water and a high-fat diet (HFD) mixed with dexamethasone or vehicle, next to subcutaneously implanted vehicle or low-dose corticosterone pellets. Dexamethasone strongly reduced body weight and fat mass gain, while corticosterone add-on (partially) normalized this. Dexamethasone-induced hyperglycaemia and hyperinsulinemia was exacerbated by corticosterone add-on. In subcutaneous white adipose tissue (WAT), corticosterone add-on prevented dexamethasone effects on lipolysis, including increased expression of rate-limiting enzyme *Atgl*. Dexamethasone also upregulated expression of brown adipose tissue identity marker genes, lipid transporters and *Atgl*, which was prevented by corticosterone add-on. Aldosterone add-on during dexamethasone treatment did not affect the dexamethasone effects on metabolism. In conclusion, corticosterone add-on treatment prevents some, and enhances other metabolic effects of dexamethasone. While the exact role of MR remains inconclusive, this study suggests that corticosterone suppression by dexamethasone is relevant to its effects.

INTRODUCTION

Glucocorticoids (GCs) coordinate a wide range of metabolic processes to support the body's needs during basal conditions and during stress. Adrenal GC secretion is under control of the hypothalamus-pituitary-adrenal (HPA)-axis, which, upon activation, releases corticotropin releasing factor (CRF) from the hypothalamus to stimulate pituitary ACTH secretion, which eventually leads to GC release [1]. GCs, such as corticosterone in mice, bind to the high-affinity mineralocorticoid receptor (MR), and the lower affinity glucocorticoid receptor (GR). Given the 10-fold difference in receptor affinities, the majority of MRs are already largely occupied under basal, non-stressed conditions [2]. Since the GR is only activated at higher GC levels, it regulates distinct aspects of the stress response and mediates negative feedback to suppress endogenous GC production [1, 2].

Dexamethasone is a synthetic GC that is used in the clinic to treat inflammatory and auto-immune disease. While dexamethasone is relatively safe at low dosages, high doses cause side effects such as obesity, insulin resistance and neuropsychological disturbances in more than 50% of subjects [3-5]. In rodents, the effects of dexamethasone on body composition are controversial, as both loss and gain of total fat mass have been reported [6-8]. Dexamethasone has a higher affinity for GR than for MR [9]. Since even moderate doses of dexamethasone completely suppress endogenous GC production, it has been postulated that part of the (side) effects of dexamethasone may result from MR hypoactivation rather than GR hyperactivation alone [10]. We therefore hypothesized that reactivation of MR, via co-administration of a low dose of corticosterone, can prevent part of the dexamethasone-induced (side) effects [10].

Various studies have reported beneficial effects of corticosterone or cortisol add-on treatment on mood, memory and sleep quality in humans and rodents treated with high doses of dexamethasone [11-13]. While these studies were mainly focussed on the brain, the MR is also expressed and available for GC binding in peripheral tissues such as brown adipose tissue (BAT) and white adipose tissue (WAT) [14, 15]. In this study, we therefore evaluated the metabolic effects of corticosterone add-on treatment in male mice treated with a high dose of dexamethasone.

MATERIALS AND METHODS

Animals

All studies involving animals were approved by the institutional ethics committee on animal care and experimentation at the Leiden University Medical Center. Throughout

all experiments, body weight, food intake and body composition (EchoMRI-100-analyzer; EchoMRI, Frankfurt, Germany) was monitored.

The 8-week old male C57Bl/6J mice (Charles River, Sulzfeld, Germany) were housed in cages with a 12:12 light-dark cycle with lights on at 11:00 AM, *i.e.*, Zeitgeber Time (ZT) 0 and *ad libitum* access to food and water. After 2 weeks of adaptation to the new light-dark cycle, mice received fructose water (10% D-(-)-fructose, Sigma-Aldrich, Zwijndrecht, the Netherlands) and a high-fat diet (HFD, 60%, Research Diets, New Brunswick, NJ) mixed with vehicle or dexamethasone (1 mg/kg/day, Sigma-Aldrich) for 23 days. In addition, mice were subcutaneously implanted with pellets containing vehicle (100 mg cholesterol, PanReac AppliChem, Meppel, the Netherlands), low-dose corticosterone (5 mg corticosterone, 95 mg cholesterol, Sigma-Aldrich) or high-dose corticosterone (20 mg corticosterone, 80 mg cholesterol). Pellets were implanted while mice were under isoflurane anesthesia and buprenorphine analgesia (0.03 mg/kg, Reckitt Benckiser Healthcare, Hull, 95 United Kingdom), and were replaced after two weeks. At day 0-7, mice were housed in metabolic cages (TSE systems, Bad Homburg, Germany) for indirect calorimetry measurements. At day 8, blood was collected at ZT1 and ZT11 within 2 min after a tail incision, *i.e.*, before corticosterone levels start to rise due to the sampling procedure, for corticosterone measurements using a HS EIA kit (Immunodiagnostic systems, Boldon Business Park, UK). At day 9, mice were fasted for 4 h and a triglyceride tolerance test was performed at ZT1. At day 23, mice were fasted for 6 h and at ZT1, blood was collected for glucose and insulin measurements with appropriate kits (Instruchemie, Delfzijl, Netherlands and Crystal Chem, Zaandam, Netherlands). Immediately afterwards, uptake of [³H]triglyceride-derived fatty acids and [¹⁴C]-glucose was determined.

To evaluate aldosterone add-on treatment, 8-week old male C57Bl/6J mice were housed in individually ventilated cages with a 12:12 light-dark cycle with lights on at 7:00 AM, *i.e.*, ZT0 and *ad libitum* access to food and water. Mice received fructose water (10%, Sigma-Aldrich) and a high-fat diet (60%, Research Diets) mixed with vehicle or dexamethasone (1 mg/kg/day, Sigma-Aldrich). Mice were subcutaneously implanted with pellets containing vehicle (100 mg cholesterol, PanReac AppliChem) or aldosterone (0.3 mg aldosterone, 100 mg cholesterol, Sigma-Aldrich) for 10 days. At day 9, blood was collected within 2 min after a tail incision for determination of plasma corticosterone levels (Immunodiagnostic systems) at ZT1 and ZT11. At day 10, mice were fasted for 6 h and at ZT1, blood was collected for measurement of plasma glucose, insulin, free fatty acid and triglyceride levels (Roche, Almere, the Netherlands, Instruchemie, Crystal Chem) after which mice were euthanised.

For all experiments, mice were killed by CO₂ inhalation and perfused with ice-cold PBS for 5 min, after which several tissues were harvested to measure tissue weights and for histological and/or molecular analyses.

Triglyceride tolerance test

To measure the post-prandial lipid response, a baseline blood sample was collected in paraoxon-coated capillaries via a tail incision, after which mice received an oral olive oil bolus (200 μ l, Carbonell, Cordoba, Spain). Additional blood samples were collected after 1, 2, 4 and 6 h and plasma triglyceride and free fatty acid levels were determined using commercially available kits (Roche).

Plasma clearance and organ uptake of radiolabeled triglyceride-derived fatty acids

Radiolabeled, triglyceride-rich lipoprotein (TRL)-like particles were prepared as previously described [16]. Mice were intravenously injected with 200 μ L particles containing 1 mg glycerol tri[3 H]oleate and a tracer amount of [14 C]-glucose. Additional blood samples were collected after 2, 5, 10 and 15 min to evaluate the plasma decay of the radiolabels. After collection of various metabolic active tissues, uptake of [3 H] triglyceride-derived fatty acids and [14 C]-glucose was determined by liquid scintillation.

RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA was isolated from snap frozen tissues using TriPure isolation reagent (Roche). After reverse transcription to cDNA (Promega, Leiden, the Netherlands), RT-qPCR was performed using IQ SYBR-Green supermix (Promega) and MyIQ thermal cycler (Bio-RAD CFX96, Veenendaal, Netherlands). Primers were tested for high efficiency (90%-110%) and for amplification of a single PCR product. All primer sequences are listed in Sup. Table 1, and fold change expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot

Western blot was performed using the Wes apparatus (ProteinSimple, Wiesbaden, Germany), according to the manufacturer's protocol. For BAT samples, 0.2 μ g/mL lysate was used. The following primary and secondary antibodies were used: UCP1 (Sigma-Aldrich, U6382, 1:20), GAPDH (Santa Cruz Biotechnology, sc25778, 1:50), HRP anti-rabbit (ProteinSimple, DM001) and HRP anti-sheep (Abcam, Amsterdam, the Netherlands, ab6900, 1:100).

Histology

Intrascapular BAT (iBAT), gonadal WAT (gWAT), subcutaneous WAT (sWAT) and liver tissue were fixed in 4% paraformaldehyde for 24 h and stored in 70% ethanol until further processing. Tissues were dehydrated, embedded into paraffin, and cut into 5 μ m sections. Paraffin-waxed tissues were dewaxed and dehydrated before staining with Mayers Haematoxylin (Merck, Netherlands) and eosin (Sigma-Aldrich). Adipocyte size and lipid content was quantified using ImageJ software [National Institutes of Health, Bethesda, MD [17]]. The scoring system for hepatic steatosis and characteristics was adapted from [18].

Statistics

Statistical analyses were performed with (version 8.1.1.330, GraphPad Software, La Jolla, CA, USA). All data are expressed as mean \pm SEM. All p-values are two-tailed and $p < 0.05$ was considered as statistically significant; $p < 0.1$ as statistical trend. Balanced data with one, two or three factors were analysed with a one-way, two-way or three-way ANOVA respectively, and a Tukey post-hoc test. Unbalanced data were analysed with linear mixed models. All statistical tests were performed in GraphPad Prism for Windows, version 8.1.1.

RESULTS

Corticosterone add-on treatment partially prevents the dexamethasone-induced loss of body weight and fat mass

To investigate to what extent corticosterone depletion mediates the metabolic effects of dexamethasone during HFD, male mice were treated with dexamethasone or vehicle in combination with low-dose corticosterone (5 mg) or vehicle pellets for 3.5 weeks. We first evaluated HPA-axis suppression by measuring corticosterone levels at the trough (AM, ZT1) and peak (PM, ZT11). Vehicle-treated mice displayed a normal circadian rhythmicity of corticosterone levels, while corticosterone-treated animals had constant corticosterone levels throughout the day, which were comparable to the PM levels of vehicle-treated mice (**Fig. 1A**). Dexamethasone completely suppressed endogenous corticosterone levels. (**Fig. 1A**). Corticosterone add-on treatment restored plasma corticosterone levels, although levels were lower than those of animals treated with corticosterone alone (**Fig. 1A**). All steroid-treatment groups had a strongly reduced weight of the GC-sensitive adrenal and spleen compared to vehicle-treated animals, although spleen weight was higher after corticosterone add-on treatment than after dexamethasone alone (**Fig. 1B-C**). The body weight and fat mass gain in vehicle-treated mice were increased by corticosterone treatment (**Fig. 1D-E**). Dexamethasone reduced body weight and fat mass gain (**Fig. 1D-E**). Strikingly, corticosterone add-on treatment counteracted the dexamethasone-induced effects on fat mass and (partially) on body weight (**Fig. 1D-E**). Dexamethasone and dexamethasone with corticosterone add-on treatment reduced lean mass to a similar extent (**Fig. 1F**). Metabolic cage data during the first week of treatment showed that dexamethasone decreased fructose water intake and locomotor activity, but not food intake (**Sup. Fig. 1A-C**). Dexamethasone, irrespective of corticosterone add-on treatment, increased total and light phase energy expenditure and respiratory quotient, indicating a higher carbohydrate oxidation in these groups (**Sup. Fig. 1D-I**). Corticosterone treatment tended to increase fat oxidation during the light phase (**Sup. Fig. 1J-K**). All significant effects on energy expenditure,

carbohydrate and fat oxidation only occurred after the lean mass correction (Sup. Fig. 1L-N).

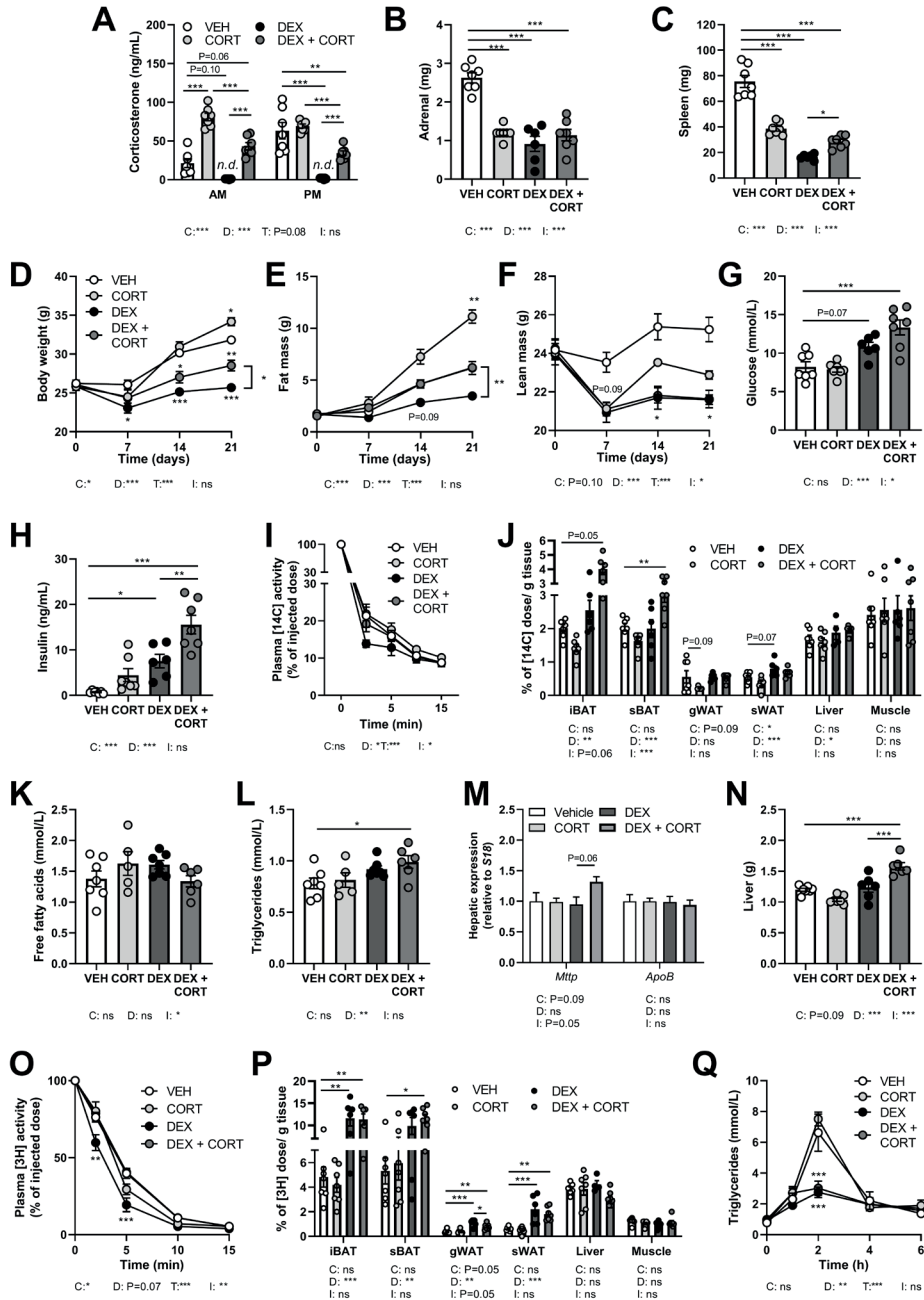
Corticosterone add-on treatment aggravates the dexamethasone-induced elevation of plasma glucose and insulin levels

Given that insulin resistance is a common side effect of dexamethasone [19, 20], we determined treatment effects on glucose metabolism. Dexamethasone increased fasted glucose levels and corticosterone add-on aggravated this effect (Fig. 1G). Insulin levels were increased by dexamethasone and corticosterone treatments alone, and even higher after the combined treatment (Fig. 1H). Dexamethasone led to a more rapid plasma clearance of [¹⁴C]-glucose, while corticosterone add-on prevented this (Fig. 1I). Dexamethasone tended to increase the uptake of [¹⁴C]-glucose in sWAT, irrespective of corticosterone (Fig. 1J). In contrast, the combined dexamethasone with corticosterone add-on treatment led to increased [¹⁴C]-glucose uptake in intrascapular BAT (iBAT) and suprascapular BAT (sBAT) relative to all other groups (Fig. 1J). In liver, [¹⁴C]-glucose uptake was slightly increased by DEX, irrespective of corticosterone. In quadriceps muscle, uptake of [¹⁴C]-glucose was unaffected by all treatments.

Dexamethasone increases the uptake of triglyceride-derived fatty acids in BAT

With respect to lipid metabolism, corticosterone and dexamethasone did not alter fasted free fatty acid levels (Fig. 1K). Fasted plasma triglyceride levels were increased after dexamethasone treatment, and were further increased after corticosterone add-on treatment (Fig. 1L). Because increased plasma triglycerides can reflect an elevation of very-low-density-lipoproteins (VLDL) levels, we next measured hepatic expression of genes involved in VLDL production, i.e., mitochondrial transfer protein (*Mttp*) and apoprotein B (*ApoB*). Dexamethasone with corticosterone add-on treatment (but neither single treatment) tended to increase *Mttp* expression, while none of the treatments affected *ApoB* expression (Fig. 1M). Dexamethasone alone did not alter liver weight, but dexamethasone combined with corticosterone add-on treatment increased liver weight (Fig. 1N). Histological examination of the liver showed that vehicle-treated, HFD-fed animals already had a mild to moderate microvesicular steatosis, and corticosterone additionally induced macrovesicular steatosis (Sup. Fig. 2A-C). Dexamethasone treatment caused hepatocellular ballooning, while the combined dexamethasone with corticosterone add-on treatment induced both ballooning and macrovesicular steatosis (Sup. Fig. 2C-D).

The clearance of [³H]-labeled TRL-like particles from the circulation was more rapid after dexamethasone treatment compared to vehicle treatment, while corticosterone add-on normalized this effect (Fig. 1O). Dexamethasone increased the uptake of [³H]



← **Figure 1: Corticosterone add-on treatment reverses dexamethasone-induced fat mass loss.** Mice received a high-fat diet mixed with dexamethasone (DEX) or vehicle (VEH) and were subcutaneously implanted with corticosterone (CORT) or vehicle pellets for 3.5 weeks. A) After DEX, plasma CORT levels were not detected (n.d.) at AM (ZT1) and PM (ZT11) of the light phase at day 8. B-C) DEX strongly reduced spleen and adrenal weight. D-F) DEX decreased body weight, fat and lean mass, while DEX + CORT prevented the effects on body weight and fat mass. G-H) DEX increased 6 h-fasted glucose and insulin levels after 23 days, while DEX + CORT aggravated this effect. I) At AM, DEX induced a more rapid plasma clearance of intravenously injected [¹⁴C]-glucose, which was prevented by DEX + CORT. J) DEX + CORT increased [¹⁴C]-glucose uptake in brown adipose tissue (BAT). K) 4 h-fasted plasma free fatty acid levels were unaffected in all treatment groups. L-M) DEX + CORT increased 4 h-fasted plasma triglyceride levels and hepatic expression of *Mtgp* but not *Apob*. N) DEX + CORT increased liver weight. O) DEX induced a more rapid plasma clearance of intravenously injected triglyceride-rich lipoprotein-like particles containing [³H]labelled triglycerides. P) DEX and DEX + CORT similarly increased the uptake [³H] triglyceride-derived fatty acids in BAT and white adipose tissue (WAT) depots. Q) Upon an oral lipid bolus, DEX strongly reduced peak plasma triglyceride levels. Statistical significance was calculated using 2-way ANOVA analysis in B-C, G-H, J-N and P; with 3-way ANOVA analysis in A, I and O; with linear mixed models in D, E, F and Q. Depicted below the graphs are the P-values of the ANOVA tests for either time (T), CORT (C), DEX (D) or the interaction between CORT and DEX (I). *P < 0.05, ** P < 0.01, ***P < 0.001.

triglyceride-derived fatty acids in iBAT, sBAT and sWAT, irrespective of corticosterone (Fig. 1P). In gWAT, dexamethasone increased the uptake of [³H]triglyceride-derived fatty acids, but corticosterone counteracted the dexamethasone effect (Fig. 1P). Uptake of [³H]triglyceride-derived fatty acids in liver and quadriceps muscle was unaffected by all treatments. The dexamethasone stimulation of triglyceride and glucose uptake in BAT suggests a higher metabolic activity of the tissue, which may reduce post-prandial plasma triglyceride levels [21]. To investigate this hypothesis, mice received an oral bolus of triglycerides at AM (ZT1), when BAT is least active [21]. Dexamethasone strongly reduced peak plasma triglyceride levels compared to the vehicle-treated animals, irrespective of corticosterone (Fig. 1Q).

Corticosterone add-on treatment counteracts the dexamethasone-induced expression of *Atgl*, but not browning marker genes in sWAT

In line with the decrease in total fat mass, dexamethasone decreased tissue weights of gWAT and sWAT and reduced adipocyte size (Fig. 2A-F). Corticosterone increased gWAT and sWAT weight and adipocyte size (Fig. 2A-F). To investigate whether the decreased lipid content in WAT after dexamethasone was a result of enhanced lipolysis, we evaluated gene expression of the lipolysis enzymes hormone sensitive lipase (*Hsl*) and adipose triglyceride lipase (*Atgl*), the latter being rate-limiting and also a GR target gene [22]. Interestingly, *Atgl* expression was elevated after dexamethasone treatment alone, and corticosterone add-on treatment prevented this (Fig. 2G). *Hsl* expression was unaffected by dexamethasone and corticosterone treatments. As the elevated influx of lipoprotein-derived triglycerides into sWAT may indicate a browning of the tissue [23], we evaluated expression of browning markers genes and genes involved in lipid transport. Neither dexamethasone nor corticosterone affected expression of lipid transporters *Cd36/FAT* and lipoprotein lipase (*Lpl*) (Fig. 1G). However, dexamethasone

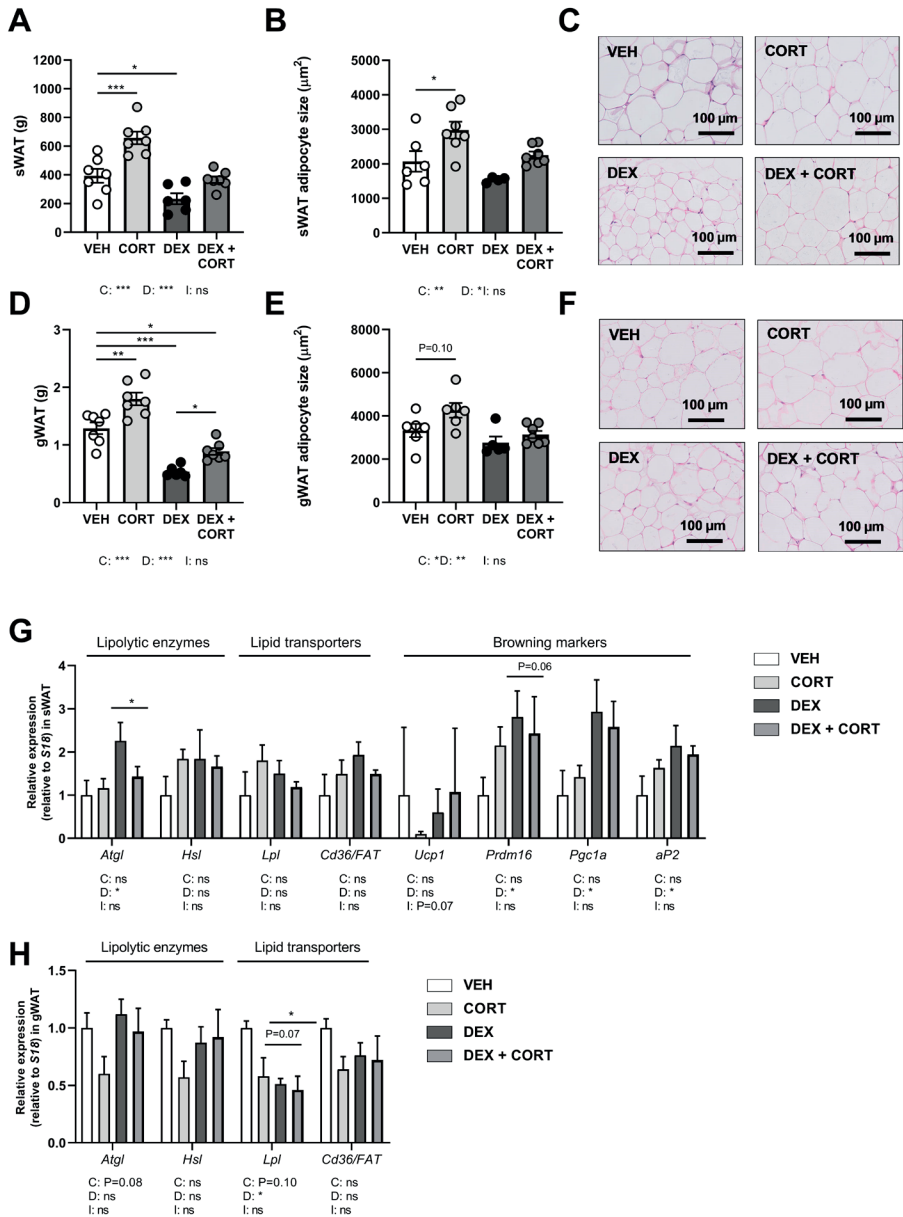


Figure 2: Dexamethasone stimulates expression of lipolytic enzyme *Atgl* in WAT, which is prevented by corticosterone add-on treatment. Mice received a high-fat diet mixed with dexamethasone (DEX) or vehicle (VEH) and were subcutaneously implanted with corticosterone (CORT) or vehicle pellets for 3.5 weeks. DEX decreased tissue weight and adipocyte size, as determined by H&E staining, in A-C) subcutaneous white adipose tissue (sWAT) and in D-F) gonadal white adipose tissue (gWAT). G) In sWAT, DEX increased the expression of lipolytic enzyme *Atgl* and browning markers *Prdm16*, *Pgc1a* and *aP2*. DEX + CORT prevented the DEX-induced upregulation of *Atgl* expression. H) In gWAT, DEX did not affect *Atgl*, *Hsl*, or *Cd36/FAT* expression, but decreased *Lpl* expression. Statistical significance was calculated using 2-way ANOVA analysis. Depicted below the graphs are the P-values of the ANOVA tests for either CORT (C) or DEX (D) treatment or the interaction between CORT and DEX (I). *P < 0.05, ** P < 0.01, ***P < 0.001.

induced browning markers PR domain containing 16 (*Prdm16*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1a*) and adipocyte protein 2 (*aP2*) expression, while corticosterone add-on treatment did not significantly affect these (Fig. 2G). Dexamethasone and corticosterone did not influence uncoupling protein 1 (*Ucp1*) expression (Fig. 2G). In gWAT, *Atgl*, *Hsl* and *Cd36/FAT* expression were unaffected by dexamethasone and corticosterone treatment (Fig. 2H). *Lpl* expression was decreased by dexamethasone and corticosterone (Fig. 2H).

Dexamethasone activates BAT, which is prevented by corticosterone add-on treatment.

BAT activation might contribute to the observed fat mass loss upon dexamethasone treatment. Since dexamethasone increased energy expenditure and influx of lipids into the BAT, we investigated additional markers of BAT activity. Corticosterone and dexamethasone increased the weight and lipid content of iBAT (Fig. 3A-C). Dexamethasone treatment also increased expression of BAT identity marker genes *Ucp1*, *Prdm16*, *Pgc1a*

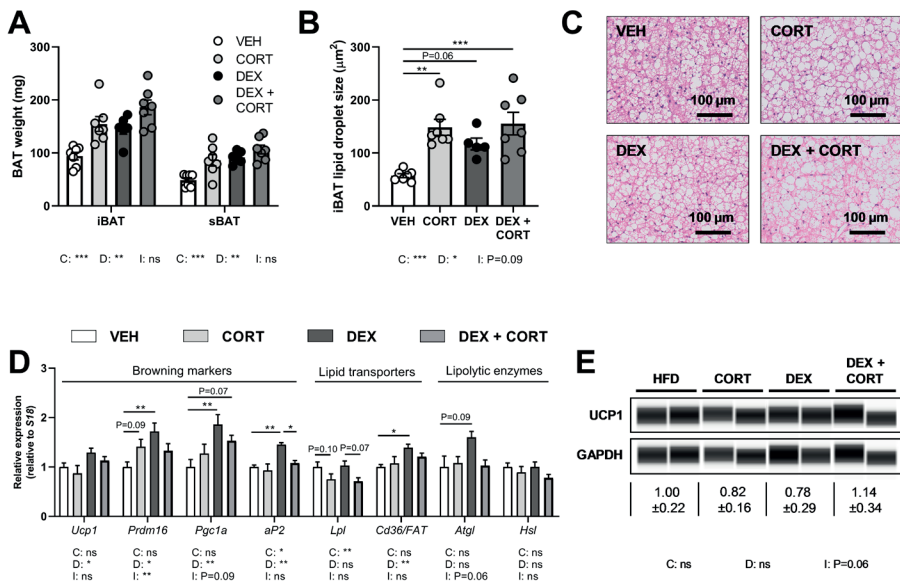


Figure 3: Dexamethasone increases expression of BAT activation markers genes, which is counteracted by corticosterone add-on treatment. Mice received a high-fat diet mixed with dexamethasone (DEX) or vehicle (VEH) and were subcutaneously implanted with corticosterone (CORT) or vehicle pellets for 3.5 weeks. A-C) CORT and DEX increased the weight of interscapular brown adipose tissue (iBAT) and suprascapular brown adipose tissue (sBAT), and increased the lipid content of iBAT, as determined by H&E staining. D) In iBAT, DEX increased expression of *Ucp1*, *Prdm16*, *Pgc1a*, *aP2*, *Cd36/FAT* and *Atgl*, which was (partially) reversed by CORT add-on treatment. E) None of the treatments affected UCP1 protein expression in sBAT. Statistical significance was calculated using 2-way ANOVA analysis. Depicted below the graphs are the P-values of the ANOVA tests for either CORT (C), DEX (D) or the interaction between CORT and DEX (I). *P < 0.05, **P < 0.01, ***P < 0.001.

and *aP2*, which was prevented by corticosterone add-on treatment (Fig. 3D). A similar pattern was observed for *Cd36/FAT* and *Atgl*, which were increased after dexamethasone treatment but normalised by corticosterone add-on (Fig. 3D). In both corticosterone groups, lipoprotein lipase (*Lpl*) expression was decreased. The increase in *Ucp1* expression after dexamethasone was not observed at the protein level, and UCP1 levels were also not influenced by corticosterone (Fig. 3E). Together these data show that, despite the elevated intracellular storage of triglycerides in BAT, dexamethasone seemed to increase BAT activity, which was counteracted by corticosterone add-on treatment.

Corticosterone add-on enhances dexamethasone-induced GR and MR target gene expression.

To evaluate effects on corticosteroid receptor activity, we compared stimulation of GR target genes between corticosterone and dexamethasone treated groups by expression analysis of metallothionein 2 (*Mt2a*), FK506-binding protein 51 (*Fkbp5*) and glucocorticoid-induced leucine zipper (*Gilz/ Tsc22d3*) in iBAT, sWAT, gWAT, liver and hippocampus. *Fkbp5* and *Gilz* are also known to be regulated by MR [24, 25]. In all peripheral tissues, dexamethasone stimulated *Fkbp5* and *Mt2a* expression more strongly than corticosterone treatment (Fig. 4A-D). The low dose of corticosterone enhanced the already substantial dexamethasone induction of *Fkbp5* and *Mt2a* in liver, sWAT, gWAT and iBAT (Fig. 4A-D). A different pattern was observed in the hippocampus, where dexamethasone did not influence the expression of *Gilz* and *Mt2a* (Fig. 4E). This suggests that the dose of dexamethasone via the food was insufficient to reach the brain. Dexamethasone decreased hippocampal *Fkbp5* expression (Fig. 4E), and given the very low corticosterone levels after dexamethasone, this suggests that the brain was in a hypocorticosteroid state [26]. In contrast, corticosterone, which easily penetrates the blood-brain-barrier, upregulated *Mt2a* expression and did not affect *Gilz* expression (Fig. 4E). Corticosterone add-on prevented the reduction of *Fkbp5* expression by dexamethasone (Fig. 4E). Since we hypothesized that the MR may mediate the effects of corticosterone add-on treatment, we additionally evaluated expression of putative MR-responsive target gene *Jdp2* in the hippocampus [24] (Fig. 4E). Dexamethasone did not affect *Jdp2* expression, while corticosterone induced *Jdp2* expression (Fig. 4E). Taken together, these results show that in peripheral tissues dexamethasone increased glucocorticoid target gene expression stronger than corticosterone, and that corticosterone add-on further increased GR target gene expression on top of that induced by dexamethasone.

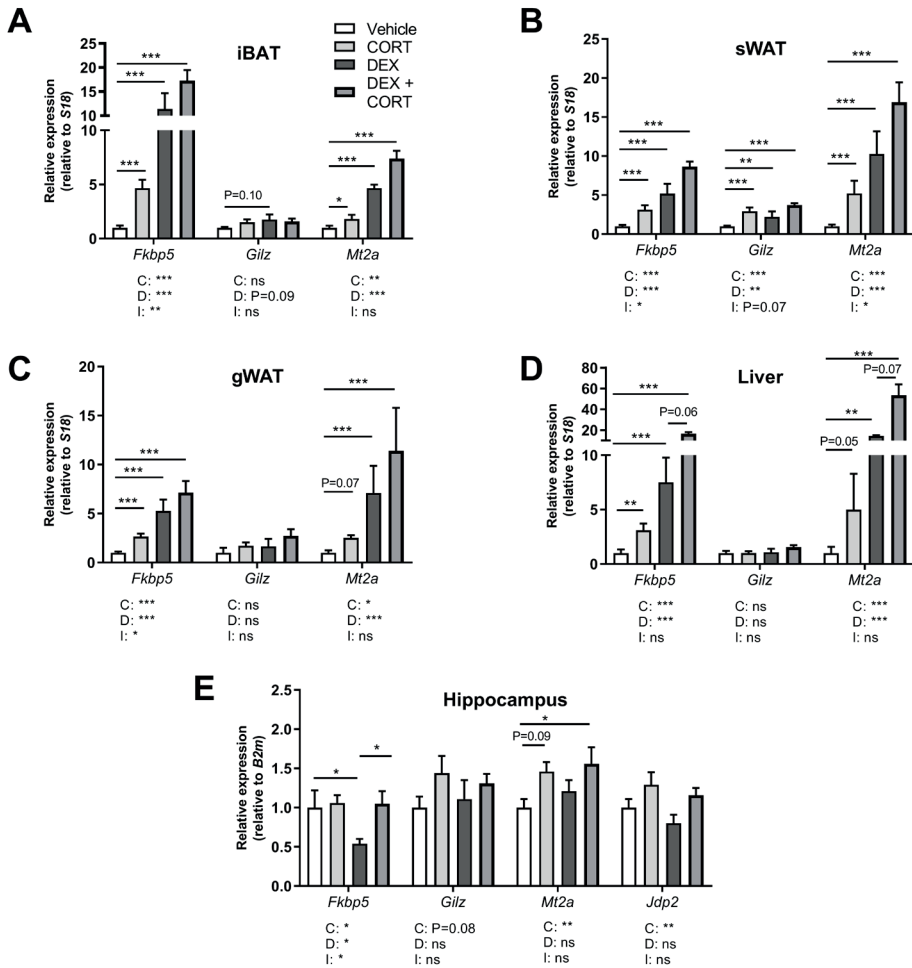


Figure 4: Corticosterone adds to dexamethasone-induced gene expression. Mice received a high-fat diet mixed with dexamethasone (DEX) or vehicle (VEH) and were subcutaneously implanted with corticosterone (CORT) or vehicle pellets for 3.5 weeks. A-D) DEX increased expression of GR target genes *Fkbp5*, *Gilz* and *Mt2a* stronger than CORT in interscapular brown adipose tissue (iBAT), subcutaneous white adipose tissue (sWAT), gonadal white adipose tissue (gWAT) and liver, while the induction after DEX + CORT was highest. E) In the hippocampus, DEX decreased *Fkbp5* expression and did not regulate *Gilz* and *Mt2a* expression. DEX + CORT normalized *Fkbp5*, and induced *Gilz*, *Mt2a* and *Jdp2* expression. Statistical significance was calculated using 2-way ANOVA analysis. Depicted below the graphs are the P-values of the ANOVA tests for either CORT (C), DEX (D) or the interaction between CORT and DEX (I). *P < 0.05, ** P < 0.01, ***P < 0.001.

High-dose corticosterone causes different metabolic effects than dexamethasone

Given that corticosterone has a higher affinity for MR compared to dexamethasone, we hypothesized that a high dose of corticosterone would have metabolic effects more comparable to dexamethasone with corticosterone add-on, than with dexamethasone

treatment alone. We therefore investigated mice that received a higher dosage of corticosterone (20 mg rather than 5 mg), and compared these to vehicle- and dexamethasone-treated animals. Corticosterone levels were strongly elevated throughout the day in mice treated with high-dose corticosterone (Sup. Fig. 3A). High-dose corticosterone caused a strong reduction of adrenal weight, but, unlike dexamethasone, only a mild reduction of spleen weight ($p=0.11$, Sup. Fig. 3B-C). High-dose corticosterone induced a rapid drop of body weight during the first week of treatment, due to a rapid reduction of lean mass, which normalized during the rest of the experiment (Sup. Fig. 3D, E). In contrast to the fat mass loss after dexamethasone, high-dose corticosterone increased total fat mass (Sup. Fig. 3F). Although high-dose corticosterone did not influence glucose levels, it did induce insulin levels stronger than dexamethasone (Sup. Fig. 3G-H). High-dose corticosterone impaired plasma glucose clearance but did not affect peripheral glucose uptake, plasma free fatty acid levels, plasma triglycerides, liver weight, uptake of [³H]triglyceride-derived fatty acids in peripheral tissues or postprandial triglyceride levels (Sup. Fig. 3K-P). To estimate corticosteroid receptor activation, *Fkbp5* and *Mt2a* expression was measured in iBAT, sWAT, gWAT, liver and hippocampus (Sup. Fig. 3Q-R). High-dose corticosterone and dexamethasone similarly induced *Fkbp5* expression in all peripheral tissues (Sup. Fig. 3Q). In the hippocampus, *Fkbp5* expression was increased by high-dose corticosterone, but decreased by dexamethasone (Sup. Fig. 3Q). *Mt2a* expression was similarly induced by high-dose corticosterone and dexamethasone in gWAT (Sup. Fig. 3R). In iBAT, sWAT, liver and hippocampus, *MT2a* mRNA levels tended to be higher after high-dose corticosterone than after dexamethasone (Sup. Fig. 3R). In conclusion, effects of high-dose corticosterone were different from those of dexamethasone, and were more comparable to those of dexamethasone with corticosterone add-on treatment.

Aldosterone add-on therapy does not influence metabolic effects of dexamethasone

To investigate whether MR contributes to the effects of corticosterone add-on treatment, we next evaluated the effect of dexamethasone treatment combined with MR-specific ligand aldosterone, under the assumption that high aldosterone can occupy receptors no longer activated by corticosterone. Aldosterone did not influence corticosterone levels, while dexamethasone again strongly diminished them (Fig. 5A). Aldosterone treatment non-significantly reduced body weight gain, and decreased fat mass, but not lean mass (Fig. 5B-D). Dexamethasone again reduced body weight and lean mass, irrespective of aldosterone add-on treatment, and tended to reduce body weight (Fig. 5C-D). Glucose levels were unaffected by both aldosterone and dexamethasone treatment (Fig. 5E). Aldosterone, both alone and as add-on treatment did not influence insulin levels, while dexamethasone caused hyperinsulinemia (Fig. 5F). Aldosterone alone or as add-on

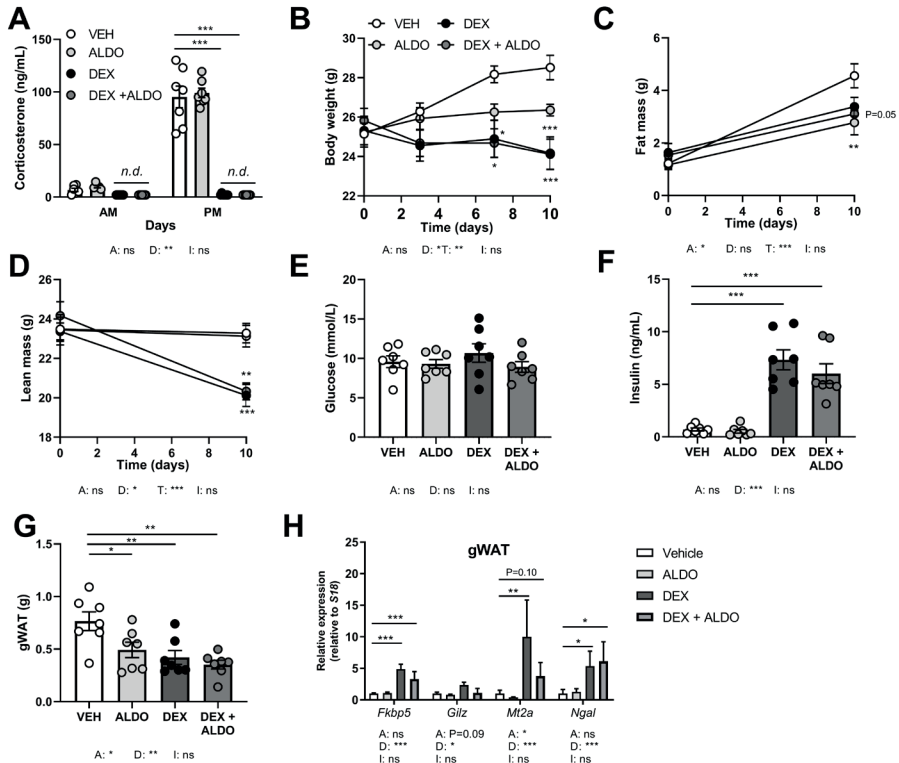


Figure 5: Aldosterone add-on treatment did not influence the metabolic effects of dexamethasone. Mice received a high-fat diet mixed with dexamethasone (DEX) and were subcutaneously implanted with aldosterone (ALDO) or vehicle (VEH) for 10 days. A) ALDO did not affect corticosterone levels at AM (ZT1) and PM (ZT11) of the light phase, while CORT levels were not detected (n.d.) after DEX and DEX + ALDO treatment. B-D) DEX + ALDO did not influence the DEX-induced reduction of body weight, fat mass and lean mass. E-F) 6 h-fasted plasma glucose or insulin levels were unaffected by ALDO and DEX + ALDO. G) ALDO, DEX and DEX + ALDO reduced gonadal white adipose tissue (gWAT) weight. H) DEX + ALDO did not affect the expression of GR target genes *Fkbp5* and *Gilz* and of MR-responsive target gene *Ngal* in gWAT. ALDO and DEX + ALDO downregulated *Mt2a* expression. Statistical significance was calculated using 3-way ANOVA in A-D) and 2-way ANOVA analysis in E-H). Depicted below the graphs are the P-values of the ANOVA tests for either ALDO (A), DEX (D), or the interaction between ALDO and DEX (I). *P < 0.05, ** P < 0.01, ***P < 0.001.

treatment did not affect other metabolic parameters, such as plasma triglycerides, free fatty acid levels, and tissue weights of liver and iBAT (data not shown). In line with the decreases in total fat mass, aldosterone and dexamethasone independently reduced gWAT weight (Fig. 5G). In gWAT, aldosterone alone did not affect expression of *Fkbp5* and *Gilz*, but reduced *Mt2a* expression. Dexamethasone increased *Fkbp5*, *Gilz* and *Mt2a* expression, while aldosterone add-on treatment tended to reduce *Gilz* and *Mt2a* expression, but not *Fkbp5* (Fig. 5H). Remarkably, aldosterone did not influence expression of putative MR target gene *Ngal* in gWAT (Fig. 5H), or *Jdp2* in the hippocam-

pus (data not shown). In iBAT, sWAT, liver, and hippocampus, aldosterone, both alone and as add-on treatment, did not affect glucocorticoid target genes (data not shown).

DISCUSSION

Synthetic GC are widely used in the clinic and often present with metabolic side effects [3, 27]. Although it is widely assumed that these side effects are caused by hyperactivation of the GR, an alternative and non-mutually exclusive mode of action might be via hypoactivation of the MR. In this study we investigated the metabolic effects of corticosterone add-on treatment during dexamethasone treatment, in an attempt to understand whether or not there could be a role for MR hypoactivation. We showed that corticosterone add-on treatment attenuated some, and aggravated other metabolic effects of dexamethasone. Dexamethasone reduced fat mass, likely in part mediated via enhanced WAT lipolysis and increased BAT activation, while corticosterone add-on treatment prevented this. In sharp contrast, corticosterone add-on treatment exacerbated the dexamethasone-induced hyperglycaemia and hyperinsulinemia. The increased fat mass and aggravated hyperinsulinemia after corticosterone add-on treatment are in line with known metabolic effects of MR activation. Blockade of MR activity, by MR antagonists, aldosterone synthase deletion or adipose-specific MR deletion, was shown to improve glucose tolerance and to reduce fat mass in humans and rodents [28-33]. Conversely, MR overexpression and aldosterone treatment was reported to increase fat mass and to induce hyperinsulinemia and hyperglycaemia in mice [32-34]. Other studies reported no effects of adipose-MR deletion on body weight and body composition [35, 36]. In BAT, MR activation inhibits thermogenic capacity and stimulates ‘whitening’ of BAT [37, 38], which is in line with our findings that corticosterone add-on treatment counteracts the increased BAT activity upon dexamethasone treatment.

Corticosterone add-on treatment enhanced the dexamethasone-induced expression of some glucocorticoid target genes (e.g. *Fkbp5*, *Mt2a*) while it reduced the expression of others (e.g. *Atgl*). We cannot fully exclude that corticosterone add-on treatment enhanced dexamethasone-induced *Fkbp5* and *Mt2a* expression via additional GR activation, but this is unlikely given the 25-fold higher potency of dexamethasone relative to corticosterone [39]. Therefore, we interpret the corticosterone effects on gene expression in presence of dexamethasone as indicative for MR activation. Additional MR occupancy may even be necessary for the maximal induction of shared target genes, as high-dose corticosterone increased *Mt2a* expression stronger than dexamethasone alone. Additional effects of MR activation on top of GR activation involve the binding to common GR response elements (GREs) in target genes, based on the very similar DNA binding domain of MR and GR. *Fkbp5* expression is an extensively described GR target

gene, and was found to be reduced in the hippocampus of MR knockout mice. The *Fkbp5* GRE-containing regulatory region is bound by both MR and GR in vivo [24, 40, 41]. The apparent additive effects of MR and GR may therefore involve sequential binding of homodimers, heterodimers or even higher order mixed MR/GR complexes [42, 43]. Genes like *Atgl* that show reversal of effects of dexamethasone after corticosterone may contain separate GR and MR sensitive regulatory regions, or have GREs where the receptors functionally compete. Indeed, different configurations of corticosteroid receptor complexes can enhance [44] or inhibit the homodimer-mediated transcriptional response [45, 46].

The metabolic effects of aldosterone add-on treatment were different than those of corticosterone. Our reasoning for add-on of aldosterone was purely pharmacological, rather than physiological. The occupancy of MRs by aldosterone in absence of dexamethasone is hard to predict in intact animals, given the presence of corticosterone which will compete with aldosterone in cells not expressing 11 β HSD type 2. Given the opposite effects of aldosterone on fat mass between our study and a previous study in adrenalectomized animals, it is indeed likely that MR occupancy by additional aldosterone is suboptimal in intact animals [47]. However, dexamethasone causes a state of ‘chemical adrenalectomy’, and aldosterone as add-on treatment should thus quite easily compete with dexamethasone for MR binding. The differences between aldosterone and corticosterone add-on treatment may be explained by the different pharmacokinetic properties of aldosterone compared to corticosterone (e.g. poor brain penetrance [48]) or haemodynamic changes induced by aldosterone that influence the metabolic response [49]. Alternatively, the aldosterone-MR complex may induce other downstream pathways compared to the corticosterone-MR complex, by recruiting a distinct transcriptional complex [50]. The opposite effects of aldosterone and corticosterone on dexamethasone-induced *Mt2a* expression in WAT support this notion.

The strong reduction of fat mass after dexamethasone seems to be mediated by a redistribution of lipids from WAT towards BAT. In BAT, the lipids were most likely utilized for thermogenesis, but we did not measure this. Elevated FFA release from WAT via increased ATGL activity seems crucial in this process [22]. FFAs also stimulate pancreatic insulin release and could thereby contribute to the insulin resistance [51-53]. Corticosterone add-on treatment prevented the lipid redistribution that was observed upon dexamethasone treatment, by reducing both WAT lipolysis and BAT activity. Corticosterone add-on treatment aggravated the hyperinsulinemia and hyperglycaemia, which may be attributed to increased hepatic gluconeogenesis, hepatic steatosis or to direct effects on the pancreas itself [54]. Despite the high insulin levels and presumed insulin resistance, corticosterone add-on treatment increased the uptake of glucose in WAT and BAT. This may point towards insulin-independent regulation of GLUT4 trans-

porters by GC, as has previously been observed in cultured brown adipocytes treated with dexamethasone [55].

We found that dexamethasone stimulated ‘futile cycling’ of nutrients, especially in the WAT, where it simultaneously increased the uptake and release of triglycerides. This is a well-established effect of glucocorticoids: by simultaneously promoting catabolic and anabolic pathways, the overall flux of lipids and glucose is increased which promotes metabolic flexibility during stress, and replenishes nutrient stores during stress recovery [56]. However, we found that the overall effect of dexamethasone on fat metabolism was catabolic, given the loss of fat mass, while effects of corticosterone were more anabolic, given the increased fat mass. This disparity between dexamethasone and corticosterone may be driven by a differential balance between GR and MR activation, as in full-body GR and MR knock-out zebrafish the MR was found to be essential for triglyceride accumulation, while GR was required for catabolic processes [57].

To conclude, this study shows that metabolic effects of dexamethasone are in part mediated via suppression of endogenous corticosterone. The effects of corticosterone add-on treatment were similar to known metabolic effects of MR, although future studies should further confirm the role of MR. To reduce psychological and metabolic side effects of synthetic GC treatment, we propose as a novel treatment strategy a combination of a mixed GR/MR agonist that easily penetrates the brain, with an MR antagonist that acts in peripheral tissues (e.g. finerenone [58]).

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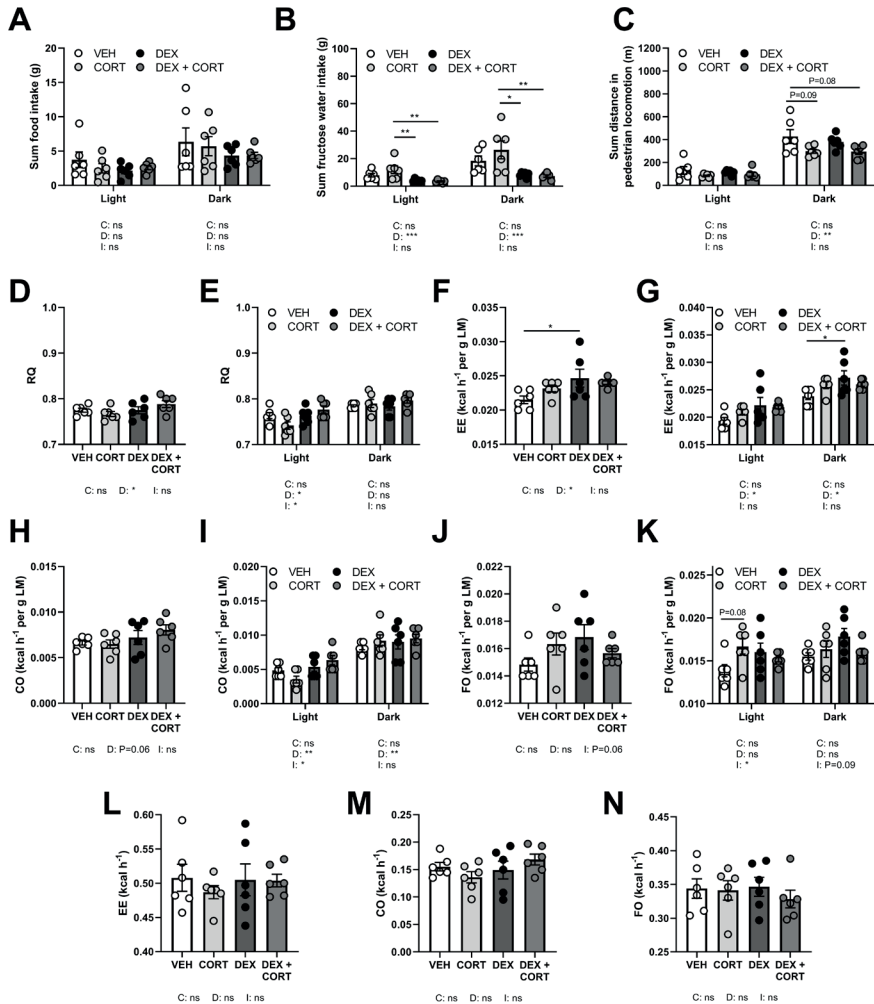
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APPENDIX

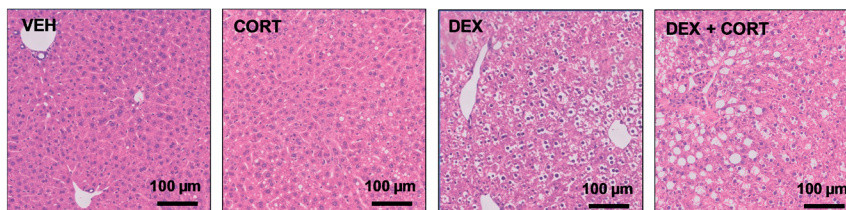
Supplementary Table 1- Primer sequences that were used for RT- qPCR analysis

Gene	Forward primer sequence	Reverse primer sequence
<i>aP2</i>	ACACCGAGATTTCTTCAAACCTG	CCATCTAGGGTTATGATGCTCTTCA
<i>ApoB</i>	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
<i>Atgl</i>	ACAGTGTCCTTCTCAGG	TTGGTTCAGTAGGCCATTCC
<i>Cd36/FAT</i>	GCAAAGAACAGCAGCAAAATC	CAGTGAAGGCTCAAAGATGG
<i>Fkbp5</i>	GCCGACTGTGTGTGTAATGC	CACAATACGCACTTGGGAGA
<i>Gilz</i>	TGGCCCTAGACAACAAGATTGAGC	CCACCTCCTCTCTCACAGCAT
<i>Hsl</i>	AGACACCAGCCAACGGATAC	ATCACCTCGAAGAAGAGCA
<i>Jdp2</i>	TACGCTGACATCCGCAACAT	CGTCTAGCTCACTTTCACGG
<i>Lpl</i>	CCCTAAGGACCCCTGAAGAC	GGCCCGATACAACCAGTCTA
<i>Mt2a</i>	ACGTCCTGAGTACCTTCTCCT	GGAGGTGCACCTTGCAGTTCTTG
<i>MtTp</i>	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGATAGCCGCTCATT
<i>Pgc1a</i>	TGCTAGCGTTCTCACAGAG	AGTGCTAAGACCGCTGCATT
<i>Prdm16</i>	ACTTTGGATGGGAGCAGATG	CTCCAGGCTCGATGTCCTTA
<i>Ptgds</i>	GCTCCTTCTGCCAGTTTTCTCT	GCCCCAGGAAGTTGTCTTGTT
<i>Ucp1</i>	TCAGGATTGGCCTCTACGAC	TGCATTCTGACCTTCACGAC

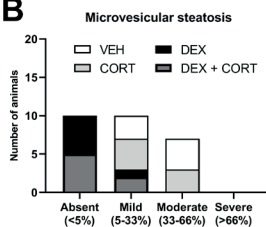


Supplementary Figure 1: Dexamethasone treatment increases energy expenditure. Mice received a high-fat diet mixed with dexamethasone (DEX) or vehicle (VEH) and were subcutaneously implanted with corticosterone (CORT) or vehicle pellets for 3.5 weeks. At day 0-7, mice were housed in metabolic cages. Results are the sum of a 72 h measurement taken between day 3 and 5. A-C) DEX reduced fructose water intake and locomotor activity, but did not affect food intake. D-G) DEX increased the light and total respiratory quotient (RQ) and energy expenditure (EE). H-I) DEX increased carbohydrate oxidation (CO) during the light phase. J-K) CORT tended to increase fat oxidation (FO) in the light phase. L-N) Effects on EE, CO and DO only occurred after the lean mass correction. Statistical significance was calculated using 2-way ANOVA analysis in each light phase (Light: ZT0 - ZT12; Dark: ZT12 - ZT24) separately. Depicted below the graphs are the P-values of the ANOVA tests for either CORT (C), DEX (D) or the interaction between CORT and DEX (I). *P < 0.05, ** P < 0.01, ***P < 0.001.

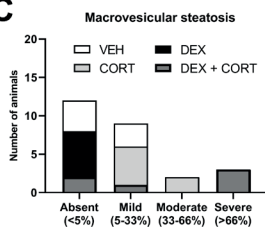
A



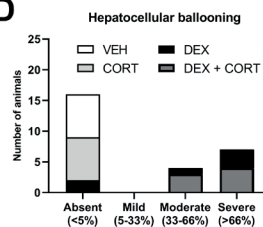
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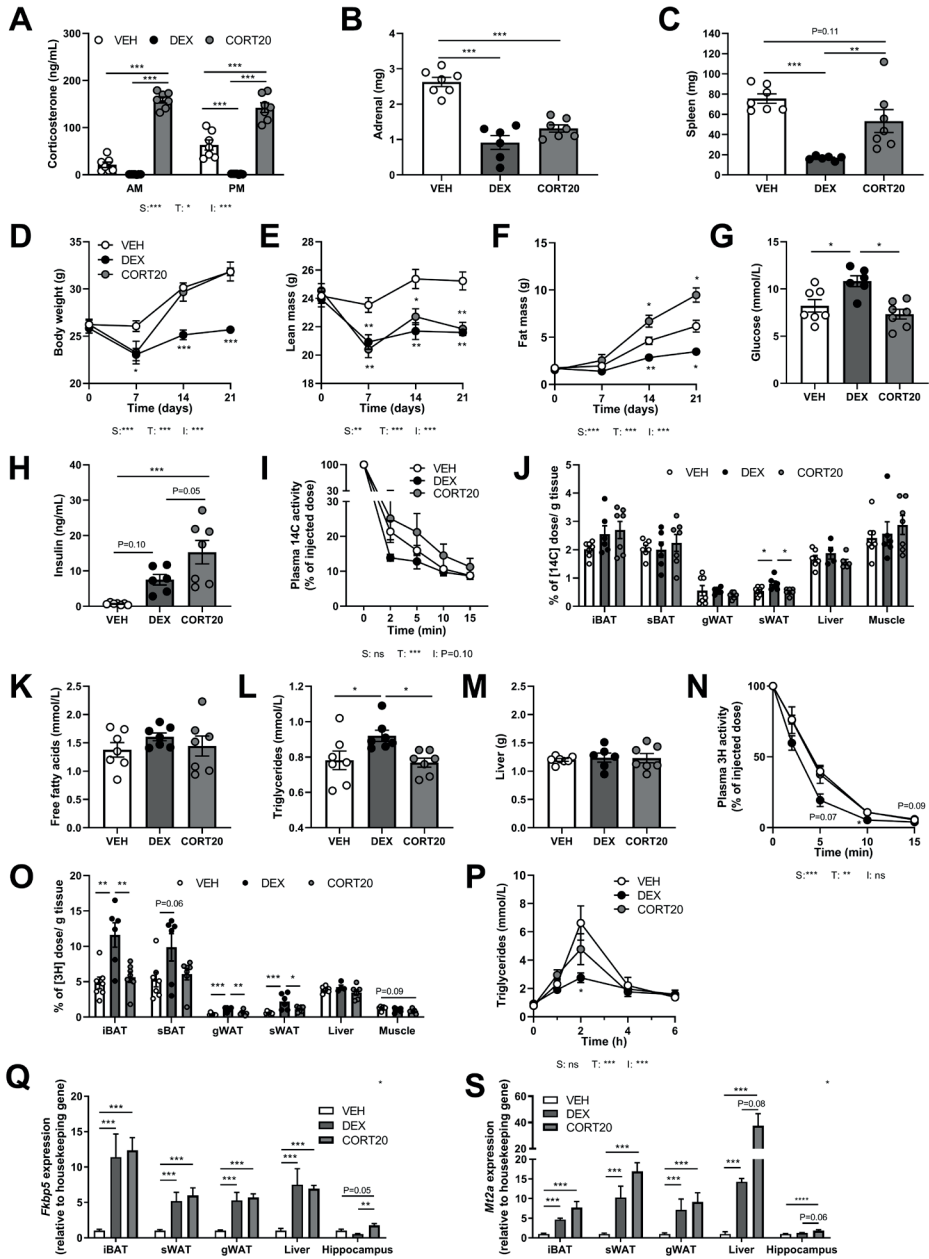
C



D



Supplementary Figure 2: Corticosterone causes hepatic macrosteatosis, while dexamethasone causes hepatocellular ballooning. Mice received a high-fat diet mixed with dexamethasone (DEX) or vehicle (VEH) and were subcutaneously implanted with corticosterone (CORT) or vehicle pellets for 3.5 weeks. A) Livers were examined by H&E-staining. B) A mild degree of microvesicular steatosis was observed in VEH-treated mice, a mild-to-moderate degree in CORT-treated mice. C) CORT and DEX + CORT induced macrovesicular steatosis. D) Hepatocellular ballooning was observed in both DEX and DEX + CORT treatment groups.



← **Supplementary Figure 3: High-dose corticosterone causes different metabolic effects than dexamethasone.** Mice received a high-fat diet mixed with dexamethasone (DEX) or vehicle (VEH) and were subcutaneously implanted with high-dose corticosterone (CORT20) or vehicle pellets for 3.5 weeks. The VEH and DEX groups are identical as those depicted in figures 1-4. A-C) CORT20-treated mice had strongly elevated CORT levels, mildly reduced spleen weight and decreased adrenal weight. D-F) CORT20 increased body weight and fat mass, but decreased lean mass. G-H) CORT20 did not affect 6 h-fasted glucose levels, but increased insulin levels. I-J) After 6 h-fasting at ZT1, CORT20 did not affect the plasma clearance of [¹⁴C]-glucose, or [¹⁴C]-glucose uptake in peripheral tissues. K-M) CORT20 did not influence 4 h-fasted plasma free fatty acids and triglycerides or liver weight. N-O) After 6 h-fasting at ZT1, CORT20 did not affect plasma clearance of triglyceride-rich lipoprotein-like particles. P) Upon an oral olive oil bolus at ZT1, CORT20 non-significantly reduced peak plasma triglyceride levels. Q) DEX and CORT20 similarly induced *Fkbp5* expression in iBAT, sWAT, gWAT and liver. In hippocampus, *Fkbp5* expression was increased by CORT20, and decreased by DEX. S) *Mt2a* expression tended to be higher after CORT20 than after DEX in iBAT, sWAT, liver and hippocampus, and was similarly induced by DEX and CORT20 in gWAT. Statistical significance was calculated using 2-way ANOVA analysis in A, I; with one-way ANOVA analysis in B-C, G-H, J-M, O, Q-S and with linear mixed models in D-F, N and P. Depicted below the graphs are the P-values of the ANOVA tests for either steroid treatment (S), Time (T) or the interaction between treatment and time (I). *P < 0.05, ** P < 0.01, ***P < 0.001.

