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

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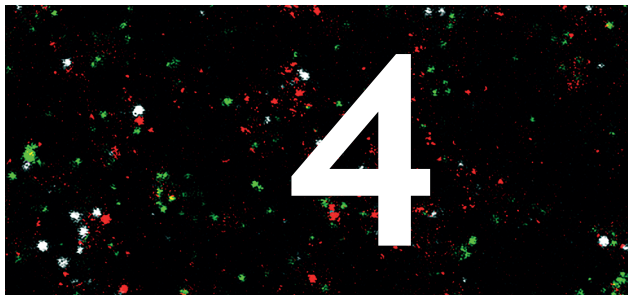

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Selective glucocorticoid receptor antagonist CORT125281 activates brown adipose tissue and alters lipid distribution in male mice

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ABSTRACT

Glucocorticoids influence a wide range of metabolic processes in the human body and excessive glucocorticoid exposure is known to contribute to the development of metabolic disease. We evaluated the utility of the novel glucocorticoid receptor (GR) antagonist CORT125281 for its potential to overcome adiposity, glucose intolerance and dyslipidaemia, and compared this head-to-head with classic GR antagonist RU486 (mifepristone). We show that, although RU486 displays cross-reactivity to the progesterone and androgen receptor, CORT125281 selectively inhibits GR transcriptional activity. In a mouse model for diet-induced obesity, rhythmicity of circulating corticosterone levels was disturbed. CORT125281 restored this disturbed rhythmicity, in contrast to RU486 that further inhibited endogenous corticosterone levels and suppressed adrenal weight. Both CORT125281 and RU486 reduced body weight gain and fat mass. In addition CORT125281, but not RU486, lowered plasma levels of triglycerides, cholesterol and free fatty acids, and strongly stimulated triglyceride-derived fatty acid uptake by brown adipose tissue depots. In combination with reduced lipid content in brown adipocytes, this indicates that CORT125281 enhances metabolic activity of brown adipose tissue depots. CORT125281 was also found to increase liver lipid accumulation. Taken together, CORT125281 displayed a wide range of beneficial metabolic activities, that are in part distinct from RU486, but clinical utility may be limited due to liver lipid accumulation. This warrants further evaluation of GR antagonists or selective modulators that are not accompanied by liver lipid accumulation, while preserving their beneficial metabolic activities.

INTRODUCTION

Obesity and dyslipidaemia constitute major problems in modern society [1, 2], and it is increasingly being recognized that glucocorticoid (GC) stress hormones contribute to such metabolic abnormalities [3]. GC are produced in the adrenal cortex and bind to the glucocorticoid receptor (GR) or the mineralocorticoid receptor (MR), thereby regulating a wide range of processes in the human body, including lipid and glucose mobilization and disposal. Circulating GC levels display a diurnal rhythmicity and GC are released in response to stress. Hypothalamus-pituitary-adrenal (HPA)-axis activity regulates GC secretion by a cascade of hormonal processes, initiated by release of corticotropin-releasing hormone (CRH) and vasopressin by the paraventricular nucleus (PVN) of the hypothalamus which results in secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary. ACTH subsequently stimulates GC production and secretion by the adrenals. HPA-axis activity is controlled by GC-mediated negative feedback on multiple levels including the inhibition of ACTH release [4, 5]. Hyperactivity of the HPA-axis (e.g. in Cushing's syndrome) causes a myriad of metabolic adverse effects, and GR antagonists were shown to be effective in counteracting this [6]. Despite being extensively used in the clinic, GR antagonist RU486 (mifepristone) lacks receptor selectivity [7], and may in certain settings also exhibit partial agonist activity [8]. Therefore, the use of a selective GR antagonist that lacks partial agonistic properties may be of value.

Brown adipose tissue (BAT) is a relevant metabolic target tissue of GC, that has been actively pursued to combat obesity and related disorders after its discovery in humans [9]. BAT effectively combusts glucose and fatty acids into heat, contributing to energy expenditure [10]. BAT is activated by cold via enhanced sympathetic outflow. The norepinephrine (NE) released from sympathetic nerve terminals binds to the ß3-adrenergic receptor on brown adipocytes and strongly enhances activity and expression of uncoupling protein-1 (UCP-1), the main effector protein involved in thermogenesis [11]. Therapeutic targeting of BAT, e.g. with a ß3-adrenergic receptor agonist, may provide an effective strategy to improve metabolic health, as it alleviates dyslipidaemia, lowers blood glucose, prevents weight gain and protects from atherosclerosis development in mice [12, 13]. Accumulating evidence indicates that chronic exposure to elevated endogenous GC [14, 15] or synthetic GR agonists [16] inhibits the activity of brown adipocytes and hampers the browning of white adipose tissue (WAT) [17], although acute effects may differ between mouse and man [18]. Vice versa, the classic GR antagonist RU486 was shown to acutely stimulate BAT activity [8, 15, 19].

In this study, we characterized novel GR antagonist CORT125281 [20] and evaluated its effects on energy metabolism and lipid distribution in male C57BL/6J mice fed a high fat diet (HFD). CORT125281 effectively inhibited GR activity in several cell culture models, whereas mineralocorticoid receptor (MR), progesterone receptor (PR) and

androgen receptor (AR) activity was unaffected. CORT125281 inhibited weight gain and lowered plasma lipids in a model for diet-induced obesity, accompanied by robust activation of BAT in comparison with RU486. CORT125281 adversely affected hepatic lipid metabolism in mice, warranting further search for selective GR modulators that efficiently antagonize GR in BAT without adversely affecting the liver.

MATERIALS AND METHODS

Animals

All animal studies reported here have been approved by the ethical committee of Leiden University Medical Center. Mice were housed in conventional cages with a 12:12 h light-dark cycle with ad libitum access to food and water. Ten-week old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were fed a control chow diet versus chow diet supplemented with CORT125281 (60 mg/kg/day) for 3 weeks (N=8 per group), or animals received water containing 10% fructose in combination with high fat diet (HFD) (60% lard, Research Diets) supplemented with vehicle, CORT125281 (60 mg/kg/day) or RU486 (also known as mifepristone; 60 mg/kg/day) for 3 weeks (N=8 per group), or animals were treated with different dosages of CORT125281 by diet supplementation (6, 20 or 60 mg/kg/day) for 3 weeks (N=7-8 per group).

Body weight, body composition and indirect calorimetry measurements

Body weight and composition (EchoMRI-100, Houston, Texas, USA) were determined throughout all experiments. Indirect calorimetry was performed in fully automatic metabolic cages (LabMaster System, TSE Systems, Bad Homburg, Germany) from day 1 until day 6. Oxygen consumption, carbohydrate production and caloric intake were measured and used to calculate energy expenditure and fat and carbohydrate oxidation [21].

Stress-free blood collection and corticosterone measurement

Stress-free blood samples, i.e. drawn within two minutes before plasma corticosterone levels rise, were collected after 13 days at AM (8h30, Zeitgeber time (ZT) 1.5) and PM (17h30, ZT 10.5), and corticosterone levels were determined using a ^{125}I RIA kit (ImmuChem, MP Biochemicals, Orangeburg, NY, USA).

Plasma lipid determination

After 3 weeks of treatment, blood was collected from 4 h-fasted mice to determine plasma triglycerides (TG), plasma total cholesterol (TC) (both with enzymatic kits from

Roche Diagnostics, Mannheim, Germany) and plasma free fatty acids (FFA) (NEFA C kit, Wako Diagnostics, Instruchemie).

Intravenous glucose tolerance test

After 2 weeks, a glucose tolerance test was performed. Mice were fasted for 6 h and at T=0 minutes blood was collected. After this, mice were intravenously injected with glucose (2 g/kg) and blood was collected at t=5, 15, 30, 60 and 120 minutes. In all samples, plasma glucose was measured using an enzymatic kit (Instruchemie, Delfzijl, the Netherlands).

Triglyceride clearance experiment

At the end of the experiment, the clearance of triglycerides (TG) was determined. Glycerol tri[^{3}H]oleate ([^{3}H]TO)-labeled lipoprotein-like emulsion particles (1.0 mg TG in 200 µL PBS) were injected intravenously in the tail vein of the mice and blood was collected at t=2, 5, 10 and 15 minutes [22]. Mice were euthanized by cervical dislocation directly after the last blood sample, were perfused with ice-cold PBS for 5 minutes and organs were harvested, weighted and divided in pieces for either mRNA analysis, histology or analysis of ^{3}H activity.

Histology

Metabolic organs, i.e., interscapular BAT (iBAT), gonadal WAT (gWAT) and liver, were fixated in 4% paraformaldehyde for 1 day and stored in 70% ethanol (EtOH) until further processing. Tissues were dehydrated and embedded in paraffin, and 5 µm sections were stained for haematoxylin-eosin (HE) and Oil Red O as previously described [23]. Intracellular lipid droplet size and lipid content were quantified using Image J software (version 1.47).

Cell culture HEK293T cells

Human HEK293T cells were transfected using Fugene HD transfection reagent (Promega, Leiden, the Netherlands) with 25 ng TAT3-luciferase (TAT3-luc), 1 ng CAGGS-renilla, 100 ng pcDNA and 10 ng human GR, MR, AR or PR expression vector. Cells were pretreated with different concentrations of RU486 or CORT125281 for 1 h before exposure to 50 nM cortisol (=hydrocorticosone) (for GR, 74-fold induction of GR signalling, data not shown), 10 nM cortisol (for MR, 6-fold induction of MR signalling, data not shown), 10 nM progesterone (for PR, 6-fold induction of PR signalling, data not shown) or 100 nM dihydrotestosterone (DHT, for AR, 4-fold induction of AR signalling, data not shown). After 24 h, firefly and renilla luciferase signals were measured using a Dual Luciferase assay (Promega).

Cell culture murine brown adipocytes

Brown preadipocytes from murine BAT depots were isolated from 5 week old male C57BL6/J mice. Cells were reversibly immortalized by using a lentiviral vector conferring doxycylin-controlled expression of simian virus large T antigen, and expanded in maintenance medium (DMEM/F12 medium supplemented with heat-inactivated fetal bovine serum (FBS), 4.5 g/L glucose, penicillin/streptomycin (P/S) and 0.1 µg/mL doxycycline). Adipogenic differentiation was induced by culturing the cells for 13-15 days in differentiation medium (DMEM/F12 supplemented with 4.5 g/L glucose, 10% heat-inactivated FBS, P/S, 4 nM bovine insulin, 10 mM HEPES, 25 µg/mL ascorbate and 1 µM rosiglitazone). During the last 2 days of differentiation and during the experiments GC-free charcoal-stripped serum was used and the effects on GR transcriptional activity and BAT activity were examined in fully differentiated brown adipocytes. BAT activity was stimulated with 1 µM NE and cells were simultaneously exposed to a combination of 10-1000 nM corticosterone, 10-1000 nM CORT125281 and/or 10-1000 nM RU486. After an incubation period of 8 h, cells were lysed using TriPure (Roche, Mijdrecht, the Netherlands).

RNA isolation, cDNA synthesis and RT-PCR analysis

Total RNA was isolated using TriPure (Roche) according to the manufacturer's protocol and 500-1000 ng RNA was reverse-transcribed using M-MLV reverse-transcriptase (Promega). RT-PCR was performed on a CFX96 PCR machine using IQ SYBR-Green (BioRad, Veenendaal, the Netherlands) and expression levels were normalized to housekeeping genes ß2-microglobulin (B2M) or 36B4. Primer sequences are shown in **Sup. Table 1**.

Statistical analysis

All data are presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism 7 software, and for mixed model analysis IBM SPSS 23 software was used. Statistical differences were calculated with a one-way ANOVA with Tukey's multiple comparison test, with a two-way ANOVA with Tukey's multiple comparison test, with a linear mixed model with time as covariate or with an unpaired t-test, as appropriate. $P < 0.05$ was considered significant for all analyses. IC50 values were calculated using GraphPad Prism 7 software, using a nonlinear fit model.

RESULTS

CORT125281 selectively inhibits GR whereas RU486 exhibits cross-reactivity for the PR and AR

The effect of the novel GR antagonist CORT125281 on GR transcriptional activity was examined and compared to classic GR antagonist RU486. Human HEK293T cells were transfected with GR and a TAT3-luc reporter, and this revealed the expected inhibition of cortisol-induced GR activity by both GR antagonists [20], in which RU486 was significantly more potent than CORT125281 (IC50 of 43 nM and 427 nM respectively, p<0.0001) (**Fig. 1A**). To investigate receptor selectivity, HEK293T cells were transfected

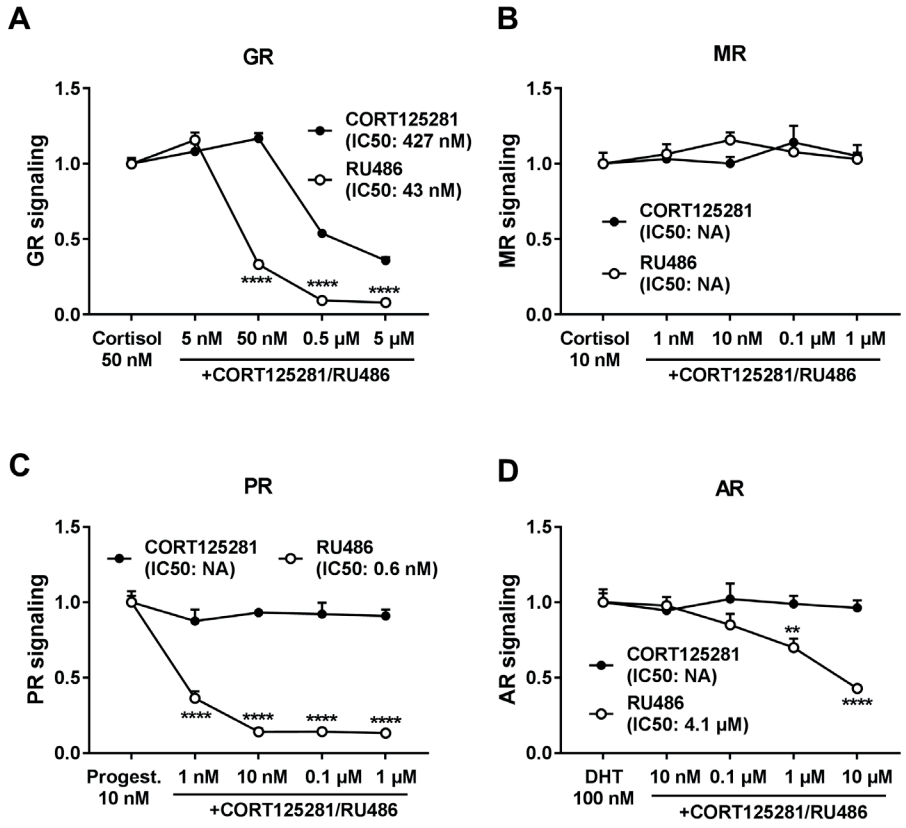
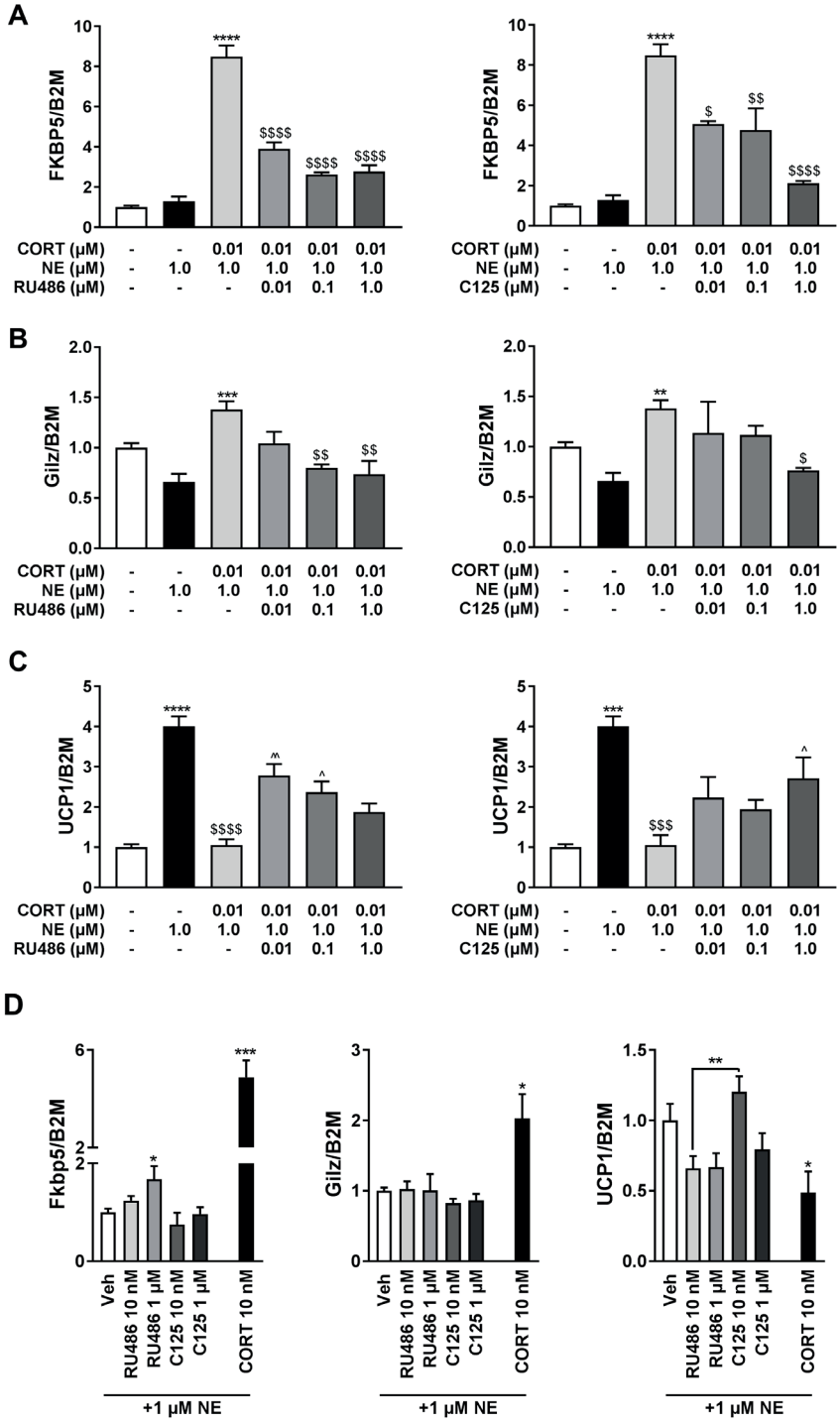


Figure 1: The effect of GR antagonists on nuclear receptor signaling *in vitro*. HEK293T cells transfected with a TAT3-luciferase reporter were utilized to determine the antagonistic effects of CORT125281 and RU486 on (A) corticosterone-induced (10 nM) GR signaling, (B) corticosterone-induced (50 nM) MR signaling, (C) progesterone-induced (10 nM) PR signaling and (D) DHT-induced (100 nM) AR signaling. Statistical significance was calculated using two-way ANOVA with Bonferroni's multiple comparisons test. ** p<0.01 vs. CORT125281, **** p<0.0001 vs. CORT125281.


A
FKBP5/B2M
CORT (μM) - - 0.01 0.01 0.01 0.01
NE (μM) - 1.0 1.0 1.0 1.0 1.0
RU486 (μM) - - - 0.01 0.1 1.0
FKBP5/B2M
CORT (μM) - - 0.01 0.01 0.01 0.01
NE (μM) - 1.0 1.0 1.0 1.0 1.0
C125 (μM) - - - 0.01 0.1 1.0
B
Gilz/B2M
CORT (μM) - - 0.01 0.01 0.01 0.01
NE (μM) - 1.0 1.0 1.0 1.0 1.0
RU486 (μM) - - - 0.01 0.1 1.0
Gilz/B2M
CORT (μM) - - 0.01 0.01 0.01 0.01
NE (μM) - 1.0 1.0 1.0 1.0 1.0
C125 (μM) - - - 0.01 0.1 1.0
C
UCP1/B2M
CORT (μM) - - 0.01 0.01 0.01 0.01
NE (μM) - 1.0 1.0 1.0 1.0 1.0
RU486 (μM) - - - 0.01 0.1 1.0
UCP1/B2M
CORT (μM) - - 0.01 0.01 0.01 0.01
NE (μM) - 1.0 1.0 1.0 1.0 1.0
C125 (μM) - - - 0.01 0.1 1.0
D
Fkbp5/B2M
Gilz/B2M
UCP1/B2M
Veh
RU486 10 nM
RU486 1 μM
C125 10 nM
C125 1 μM
CORT 10 nM
+1 μM NE

← **Figure 2: The effects of GR antagonists on murine BAT cells *in vitro*.** RU486 and CORT125281 antagonistic properties on 10 nM corticosterone-regulated gene expression (A) *Fkbp5* and (B) *Gilz*. ** p<0.01 vs. NE, *** p<0.001 vs. NE, **** p<0.0001 vs. NE, $ p<0.05 vs. CORT + NE, $$ p<0.01 vs. CORT + NE, $$$$ p<0.0001 vs. CORT + NE. (C) The effect of CORT125281 and RU486 on *Ucp1* expression in murine brown adipocytes after 8 hours of exposure. *** p<0.001, **** p<0.0001, $$$ p<0.001 vs. CORT, $$$$ p<0.0001 vs. CORT, ^ p<0.05 vs. CORT + NE, ^^ p<0.01 vs. CORT + NE. (D) Assessment of partial agonism by RU486 and CORT125281 compared to 10 nM corticosterone on the expression of *Fkbp5*, *Gilz* and *UCP1* in murine brown adipocytes after 8 hours of exposure. Statistical signficance was calculated using a one-way ANOVA with Tukey's multiple comparisons test. * p<0.05, ** p<0.01, *** p<0.001, Abbreviations: CORT = corticosterone, NE = norepinephrine.

with MR, PR or AR in combination with TAT3-luc, and treated with their respective agonists around their estimated EC_{90} concentration [24]. Neither CORT125281 nor RU486 affected cortisol-induced MR signalling (**Fig. 1B**). Although RU486 potently inhibited PR signalling (IC50: 0.6 nM) (**Fig. 1C**) and also displayed moderate inhibitory actions on AR signalling (IC50: 4.1 μM) (**Fig. 1D**), CORT125281 did not affect progesterone-induced PR signalling and DHT-induced AR signalling (**Fig. 1C-D**). Taken together, this supports the notion that CORT125281 is a selective GR antagonist while RU486 exhibits cross-reactivity for PR and AR.

CORT125281 reverses corticosterone-mediated GR activity in murine brown adipocytes *in vitro*

To assess whether CORT125281 influences the activity of brown adipocytes, we used cell lines derived from murine BAT depots. Preadipocytes were differentiated into mature brown adipocytes and treated with corticosterone in combination with RU486 or CORT125281. To determine the effect of both compounds on GR transcriptional activity, we measured the expression of the well-known GR target genes *Fkbp5* and *Gilz* [25, 26]. Murine brown adipocytes were responsive to corticosterone, as treatment with 10 nM corticosterone significantly upregulated expression of *Fkbp5* and *Gilz* (**Fig. 2A-B**). Treatment with either RU486 or CORT125281 effectively inhibited corticosterone-induced GR transcriptional activity (**Fig. 2A-B**). Also for GR transcriptional activity induced by 1 μM corticosterone, both GR antagonists significantly inhibited GR-target gene expression, although GR inhibition by 1 μM RU486 was stronger compared with 1 μM CORT125281, likely reflecting differences in binding affinity (**Sup. Fig. 1A-B**) [20]. As expected, activity of murine brown adipocytes was inhibited by corticosterone, i.e. decreased NE-induced UCP-1 expression upon 10 nM (**Fig. 2C**) and 1 μM corticosterone exposure (**Sup. Fig. 1C**). Both CORT125281 and RU486 were able to (partially) prevent corticosterone-induced inhibition of BAT activity, as coincubation with the GR antagonists results in enhanced UCP1 expression (**Fig. 2C, Sup. Fig. 1C**). Of note, RU486 did not dose-dependently reverse corticosterone-inhibited UCP-1 expression (**Fig. 2C**), and was not able, even at high doses, to fully prevent corticosterone-inhibited UCP-1 expression (**Sup. Fig. 1C**). These findings may be explained by partial agonistic properties of RU486 on the GR.

To test this, mature brown adipocytes were treated with different doses of RU486 or CORT125281, and this was compared to the agonistic effect of 10 nM corticosterone. Treatment with RU486 resulted in upregulation of the GR-target gene *Fkbp5* ($p<0.05$) but not *Gilz*, whereas CORT125281 did not influence *Fkbp5* or *Gilz* expression (**Fig. 2D**). RU486 treatment tended to reduce NE-induced *Ucp1* expression (-40% versus vehicle), which is significant compared to 10 nM CORT125281 ($p<0.01$, **Fig. 2D**). Taken together, these data suggest partial agonistic properties of RU486, which could limit BAT activating capacity by RU486, whereas CORT125281 showed only antagonistic properties on brown adipocytes.

CORT125281 reduces body weight, fat mass and plasma lipids in HFD-fed mice

To evaluate the effects of CORT125281 on metabolism in a whole organism, a mouse model for diet-induced obesity was used. HFD-fed mice were treated with either CORT125281 (60 mg/kg/d) or the classic GR antagonist RU486 (60 mg/kg/d). For comparison, we also performed an experiment in which chow-fed mice were treated by diet-supplementation with CORT125281 (60 mg/kg/d). At equal dosage, CORT125281 and RU486 similarly reduced HFD-induced body weight gain with approximately 10% ($p<0.001$ for CORT125281 at d21, **Fig. 3A**), while body weight was not altered in chow-fed mice (**Sup. Fig. 2A**). Both GR antagonists significantly reduced fat mass, but not lean mass, in HFD-fed mice (-23% for RU486 and -32% for CORT125281 at day 21, $p<0.01$ and $p<0.0001$ respectively) (**Fig. 3B-C**), while CORT125281 did not affect fat mass or lean mass in chow-fed mice (**Sup. Fig. 2B-C**). In the HFD condition, treatment with CORT125281 significantly lowered plasma TG (-56%, $p<0.0001$) (**Fig. 3D**) and cholesterol levels (-30%, $p<0.05$) (**Fig. 3E**) compared to vehicle and RU486-treated mice, as well as significantly lowered plasma FFA compared with RU486-treated mice (-23%, $p<0.05$) (**Fig. 3F**). Similar plasma lipid lowering activities of CORT125281 were found in chow-fed mice, i.e. significant reduction of plasma TG and cholesterol and a near-significant reduction of FFA (**Sup. Fig. 2D-F**). In a subsequent experiment, different dosages of CORT125281 (6, 20 or 60 mg/kg/d) were evaluated in HFD-fed mice, which revealed that CORT125281 seemed to reduce body weight, fat mass, plasma TG, cholesterol and FFA in a dose-dependent manner, with no effect on lean mass (**Sup. Fig. 3A-F**). In addition to lipid metabolism, we investigated the effects of CORT125281 on glucose metabolism. This revealed that CORT125281 did not affect basal glucose levels or intravenous glucose tolerance (**Fig. 3G-H**), whereas RU486 significantly improved glucose tolerance in HFD-fed mice, to a similar degree as previously described [27]. Altogether, these data suggest that CORT125281 treatment reduces diet-induced weight gain and body fat mass and that CORT125281 effectively lowers plasma lipids.

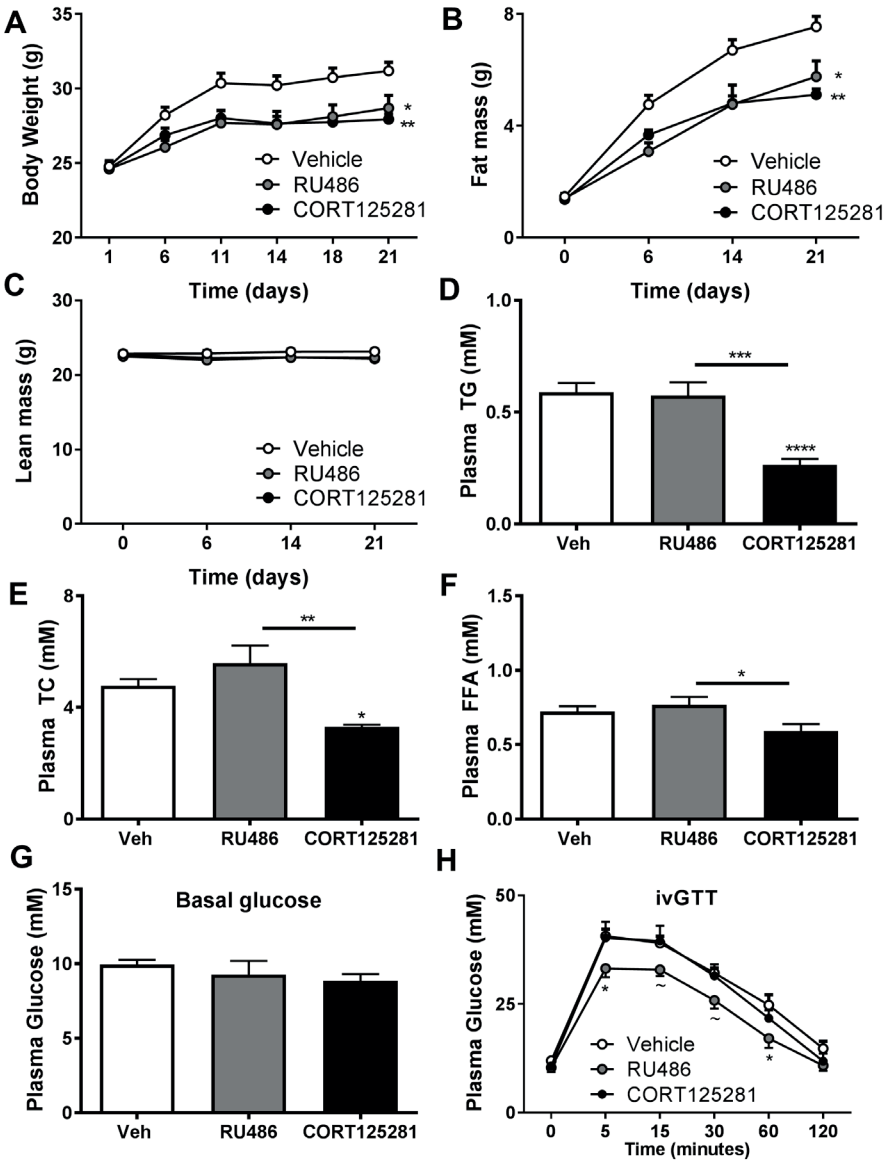


Figure 3: The effect of GR antagonists on body weight, body composition and plasma lipids and glucose of high fat diet-fed C57BL/6J mice. The effect of the classic GR antagonist RU486 and the novel GR antagonist CORT125281 on (A) body weight, (B) body fat mass and (C) lean mass. The effect on (D) plasma triglycerides, (E) plasma total cholesterol and (F) plasma free fatty acids after 3 weeks of treatment. The effect on (G) basal glucose levels and (H) intravenous glucose tolerance after 2 weeks of treatment. Statistical significance was calculated using a mixed model analysis (A-C), an one-way ANOVA with Tukey's multiple comparisons test (D-G) or a two-way ANOVA with Tukey's multiple comparisons test (H). ~ $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

CORT125281 restores HFD-disturbed HPA axis activity

Under chow-fed conditions, mice display a circadian rhythm in circulating corticosterone with peak levels before the dark phase (i.e. start of the active period, data not shown). Three weeks of HFD resulted in dampening of the circadian corticosterone rhythm, as morning (AM) and evening (PM) circulating corticosterone levels were similar (**Fig. 4A**). RU486 treatment resulted in lower circulating corticosterone levels at PM, suggesting an agonistic effect on the HPA-axis resulting in enhanced negative feedback under this dose regimen (-80%, $p<0.01$) (**Fig. 4A**). Strikingly, CORT125281 restored the HFD-disturbed circadian corticosterone rhythm ($p<0.0001$ AM vs. PM), and substantially higher circulating corticosterone levels at PM were found compared to vehicle HFD fed mice ($p<0.0001$) (**Fig. 4A**). The lack of agonistic activity of CORT125281 is further supported by the observation that CORT125281 did not influence adrenal weight, whereas RU486 induced adrenal atrophy (-51% organ weight, $p<0.05$) (**Fig. 4B, Sup. Fig. 4A**) indicating continuous negative feedback on the HPA-axis. At all evaluated dosages, CORT125281 did not influence the weight of the thymus and spleen (**Fig. 4C-D, Sup. Fig. 4B-C**), organs that involute after chronic GC exposure, whereas RU486 was found to reduce thymus weight (-47%, $p<0.05$) (**Fig. 4C**).

CORT125281 stimulates fatty acid uptake and combustion by interscapular BAT

To monitor energy expenditure (EE), mice were housed in fully automated metabolic cages during the first week of treatment. RU486 significantly increased total EE, while CORT125281 did not (**Fig. 5A**). Treatment with RU486 and CORT125281 both resulted in increased fat oxidation (**Fig. 5B**) and decreased carbohydrate oxidation (**Fig. 5C**), as evident from a lowered respiratory exchange ratio (**Fig. 5D**). We next investigated the fate of intravenously injected lipoprotein-like particles labelled with [^{3}H]TO lipids. In mice treated with CORT125281, but not RU486, plasma decay of [^{3}H]TO was more rapid ($p<0.01$, **Fig. 5E**), indicating enhanced TG uptake from plasma. Uptake of [^{3}H]TO-derived activity by iBAT and dorso-cervical (dc)BAT was significantly increased in the CORT125281 group (+115%, $p<0.0001$ and +61%, $p<0.05$, respectively) (**Fig. 5F**), indicating enhanced metabolic activity of these BAT depots. This is further supported by a tendency toward reduced iBAT weight (**Fig. 5G, Sup. Fig. 5A**), accompanied by reduced lipid content in CORT125281-treated mice compared to RU486-treated mice (- 51%, $p<0.05$) (**Fig. 5H-I**). In addition, CORT125281- and RU486-treated mice showed decreased gWAT weight and smaller average cell size (**Fig. 5J-L, Sup. Fig. 5B**). Collectively these data suggest that CORT125281 activated BAT to stimulate fatty acid uptake and combustion whereas RU486 did not.

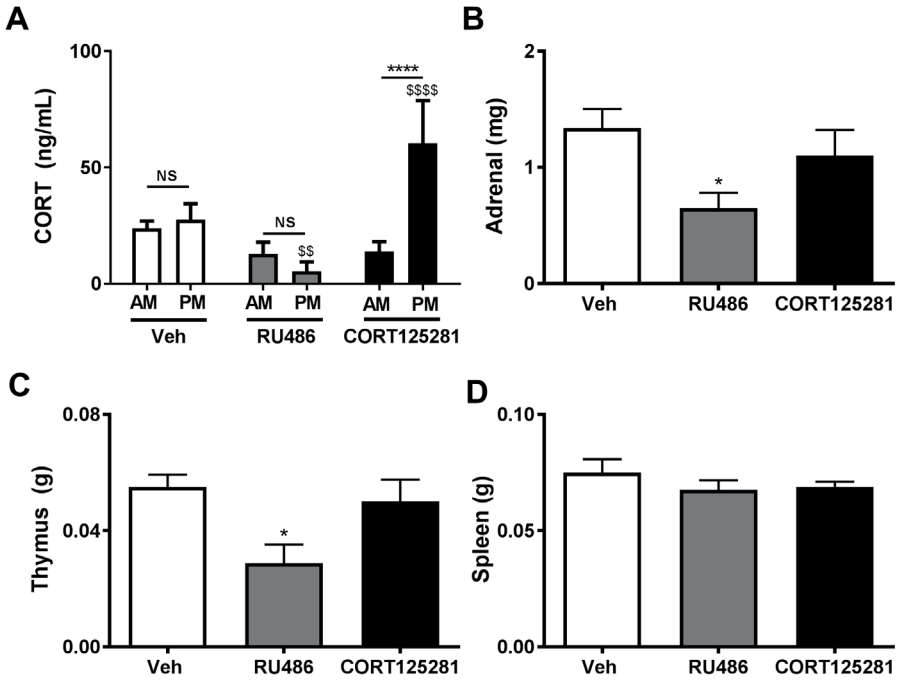


Figure 4: The effect of GR antagonists on endogenous corticosterone and GC-sensitive organ weights in high fat diet-fed C57BL/6J mice. The effect of classic GR antagonist RU486 and the novel GR antagonist CORT125281 on circulating corticosterone levels at (A) AM and PM and on (B) adrenal, (C) thymus and (D) spleen weight. Statistical significance was calculated using a two-way ANOVA with Tukey's multiple comparisons test (A) or a one-way ANOVA with Tukey's multiple comparisons test (B-D). * $p<0.05$, **** $p<0.0001$, $$ $p<0.01$ vs. vehicle PM, $$$$ $p<0.0001$ vs. vehicle PM.

CORT125281 increases liver lipid content

As GR activity is known to influence hepatic function (e.g. lipid uptake, VLDL-production, de novo lipogenesis) [14], we next analysed the livers of mice treated with RU486 and CORT125281. In HFD-fed mice, CORT125281 significantly increased liver weight (+51%, $p<0.01$) (**Fig. 6A, Sup. Fig. 5C**) and treatment with CORT125281 was accompanied by increased liver TG (+88%, $p<0.05$) (**Sup. Fig. 5D**) and total liver lipids (visualized by an Oil Red O staining, **Fig. 6B-C**). Also in chow-fed mice, CORT125281 seemed to increase liver weight and significantly increased liver TG (**Sup. Fig. 2G-H**). Of note, both RU486 and CORT125281 strongly upregulated expression of *Cyp3a11*, suggestive of induction of the pregnane X receptor (PXR) (**Fig. 6D**). PXR agonism is known to cause hepatic lipid accumulation, at least partly via increased hepatic expression of fatty acid transporter *Cd36* [28], which we also observed for both CORT125281 and RU486 (**Fig. 6E**). Although RU486 induced *Cyp3a11* expression most strongly, this was not accompanied by the highest hepatic lipid content (**Fig. 6B-E**), suggesting additional, differential effects of RU486 and CORT125281 on liver lipid metabolism.

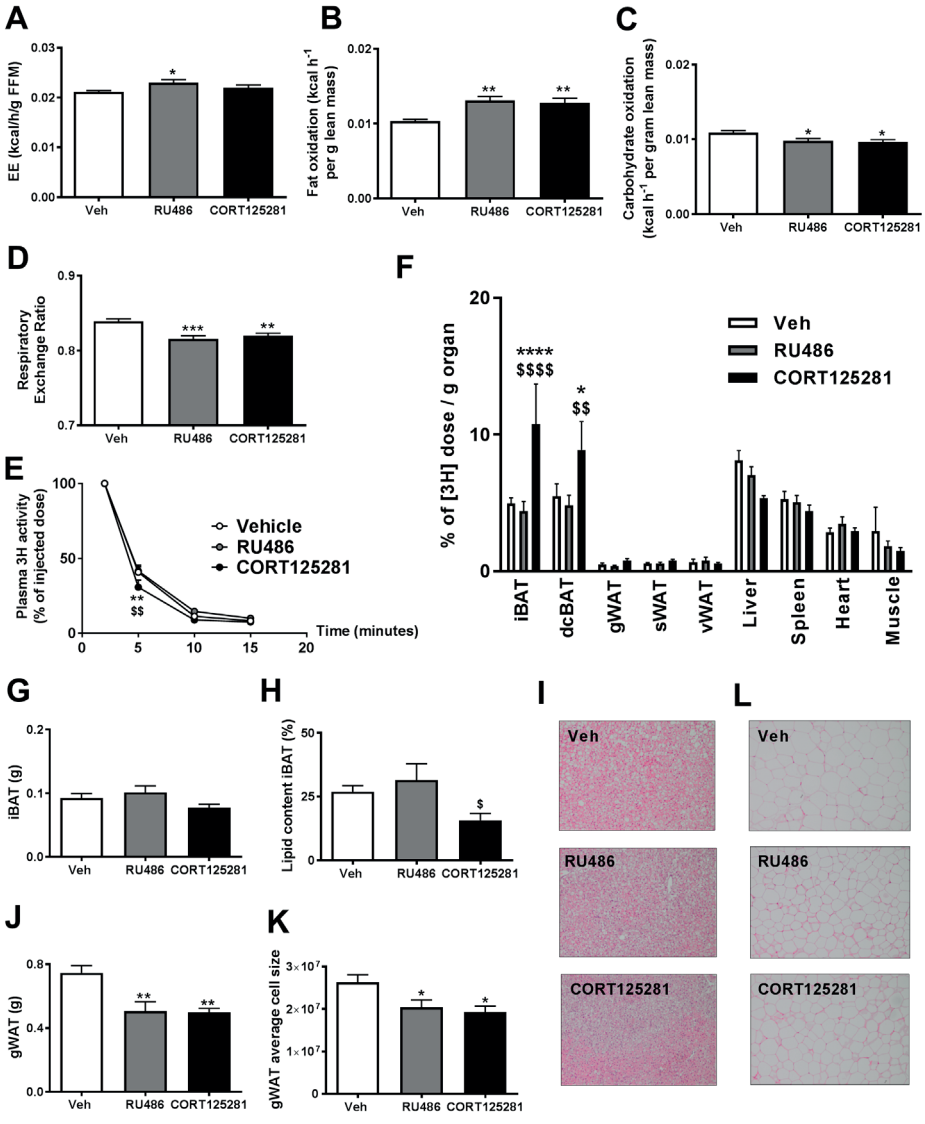


Figure 5: The effect of GR antagonists on the activity of metabolic organs. The effect of classic GR antagonist RU486 and the novel GR antagonist CORT125281 on (A) energy expenditure, (B) fat oxidation, (C) carbohydrate oxidation and (D) respiratory exchange ratio. The effect on (E) plasma decay and (F) uptake of lipoprotein-triglyceride-derived free fatty acid by metabolic tissues. The effect on (G) iBAT weight and (H) lipid content, and (I) representative images of H&E-stained iBAT slices. The effect on (J) gWAT weight and (K) average cell size, and (L) representative images of H&E stained gWAT slices. Statistical significance was calculated using a one-way ANOVA with Tukey's multiple comparisons test (A-D, G-H, J-K) or two-way ANOVA with Tukey's multiple comparisons test (E-F). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ vs. Veh, $ $p<0.05$ vs. RU486, $$ $p<0.01$ vs RU486, $$$$ $p<0.0001$ vs RU486.

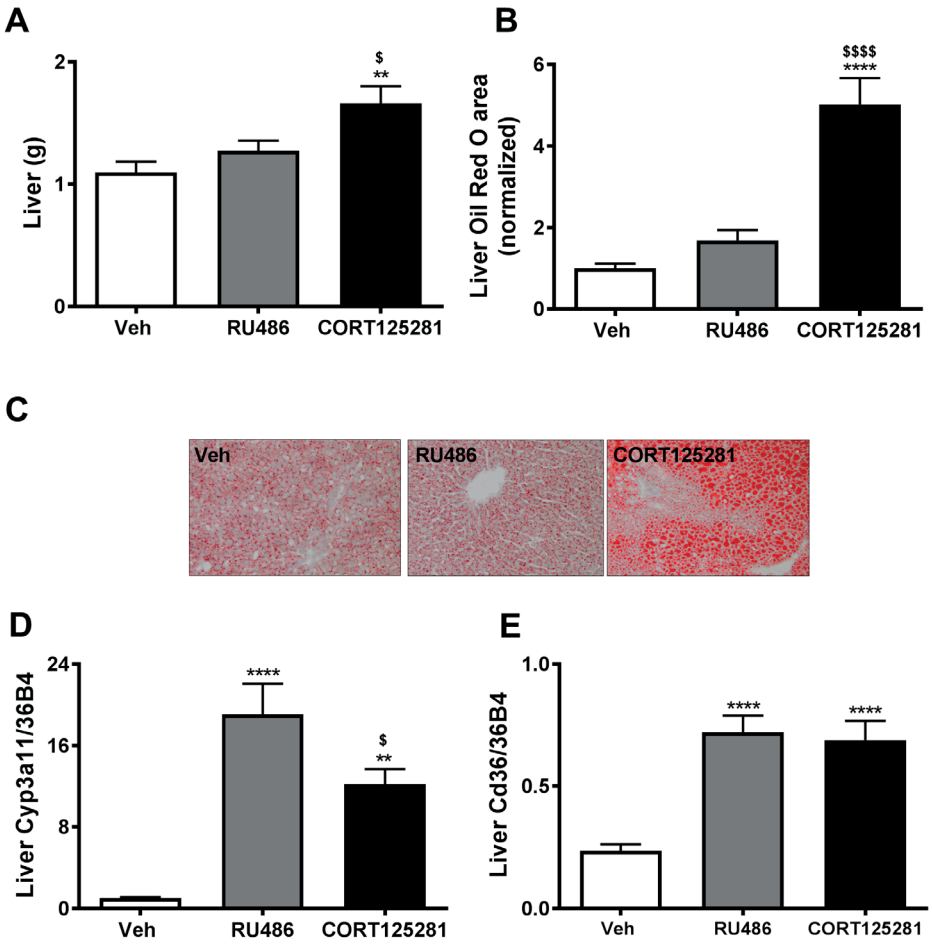


Figure 6: The effect of GR antagonists on the liver. The effect of the novel GR antagonist CORT125281 and the classic GR antagonist RU486 on (A) liver weight and (B) liver Oil Red O. (C) Representative images of Oil Red O-stained liver slices. Hepatic expression of (D) *Cyp3a11* and (E) *Cd36*. Statistical significance was calculated using a one-way ANOVA with Tukey's multiple comparisons test. ** p<0.01 vs. Veh, **** p<0.0001 vs. Veh. $ p<0.05 vs. RU486, $$$$ p<0.0001 vs. RU486.

DISCUSSION

In this study, we describe the effects of novel GR antagonist CORT125281 on metabolism and HPA-axis activity in a model for diet-induced obesity in male mice. In our studies, we compared CORT125281 head-to-head with the classic GR antagonist RU486. First, we characterized the GR specificity of CORT125281, and luciferase reporter experiments clearly show that CORT125281 selectively inhibits the GR, while classic GR antagonist RU486 also inhibits PR and AR transcriptional activity, as expected [7, 29]. In a model

for diet-induced obesity, we have shown that CORT125281 and RU486 equally reduce body weight gain in HFD-fed mice, similar to previous observations for RU486 [30]. In addition, both GR antagonists reduce total fat mass without adversely affecting the lean mass. CORT125281, but not RU486, significantly lowered plasma lipids, restored circadian corticosterone rhythmicity and induced fatty acid uptake and combustion by iBAT. Both GR antagonists effectively reversed corticosterone-suppressed UCP1 expression in brown adipocytes *in vitro*, and this reversal of corticosterone-inhibitory actions on BAT could partially underlie the CORT125281-induced BAT activity observed *in vivo*. Although RU486 may activate BAT in specific contexts [8], in the present setting RU486 did not seem to activate BAT *in vivo*, and this discrepancy between RU486 and CORT125281 may be explained by differential effects on PR or AR activity. Alternatively, because ACTH was shown to stimulate BAT activity [15], the restored HPA-axis activity in CORT125281-treated mice may augment circulating ACTH levels and thereby enhance ACTH-induced BAT activation. The lack of partial agonism on the HPA-axis by CORT125281 may thus underlie the differential effects of RU486 and CORT125281 on BAT activity *in vivo*. Additional partial agonistic activities of RU486 (as evident from reduced NE-induced UCP-1 expression in RU486 treated brown adipocytes *in vitro*, **Fig. 2D**) may also explain the lower BAT activating capacity of RU486.

In our study, we observed disturbed corticosterone rhythmicity upon HFD, which is in line with a previous study [31]. Nutrient sensors influence the peripheral clock [32], and HFD was shown to alter diurnal patterns of leptin and insulin, as well as to reduce circadian patterns of clock genes in metabolic tissues [33]. Thus, the flattened corticosterone rhythm observed in our study could be a consequence of HFD-disturbed circadian rhythm, which is supported by the observation that the HFD fed mice in our study eat throughout the whole day rather than mainly in the dark period (data not shown). Alternatively, HFD could influence the HPA-axis and its hormones directly, as decreased 11ß-HSD1 expression (which converts inactive into active GC) and altered CRH and GR expression in the PVN were observed upon HFD feeding [31]. Fatty acids were also shown to regulate circulating corticosterone levels, and fatty acid sensors are known to be present in hypothalamus [34]. FFA lowering strategies (e.g. insulin administration) were shown to increase plasma ACTH and corticosterone levels [35] and based on this, the decreased plasma FFA levels upon CORT125281 treatment could contribute to the restored corticosterone rhythmicity observed in our study. In addition, the lack of peripheral negative GC feedback on the HPA-axis, due to the continuous presence of CORT125281, could contribute to the restored corticosterone rhythmicity. Although acute RU486 treatment can interfere with GR-mediated negative feedback and disinhibit the HPA-axis [36], in the present setting (continuous administration of high dose via the food) both corticosterone levels and adrenal weights were strongly reduced, suggesting suppression of ACTH release rather than classic disinhibition.

Although the reduced plasma lipids upon CORT125281 treatment can partially be attributed to enhanced BAT activity, it seems likely that enhanced lipid uptake by the liver is also involved. HFD induces hepatic expression of the cellular fatty acid transporter CD36 [37]. CD36 mediates hepatic lipid uptake and is critically involved in the pathogenesis of liver steatosis as its upregulation induces lipid accumulation in the liver [38] and hepatic deletion of CD36 prevents this [39]. Both endogenous GC [14, 40] and synthetic GC agonists [41, 42] have been shown to increase hepatic CD36 expression, thereby aggravating liver steatosis [14]. *Vice versa*, GR knockout decreases hepatic CD36 expression and subsequently lowers liver lipids [43]. Surprisingly, treatment with CORT125281 and RU486 also increased hepatic *Cd36* expression, which is likely attributed to activation of the xenobiotic sensor PXR [44]. This subsequently enhances fatty acid uptake resulting in lipid accumulation and enhanced liver weight in mice. Currently, it is unknown if CORT125281 induces similar hepatic lipid accumulation in humans. Remarkably, in our studies RU486 treatment is not associated with lipid accumulation, while RU486 is a known PXR-ligand [28] and enhanced hepatic *Cd36* and *Cyp3a11* expression. This suggests additional lipid lowering activities of RU486 (e.g. VLDL production, beta-oxidation) that prevent hepatic lipid uptake and accumulation and the development of steatosis. The differential effects of RU486 and CORT125281 may therefore be a consequence of the partial agonistic features of RU486 that are lacking in CORT125281.

To date, the utility of GR antagonists could be further improved for the treatment of metabolic disease. Based on our current study, GR antagonism with RU486 affects only body weight and fat mass but does not display additional beneficial metabolic activities, while the potential of CORT125281 may be limited due to adverse liver steatosis-inducing effects that we observe in mice. This may call for GR ligands that selectively act on BAT, or that exhibit mixed agonistic and antagonistic features [45-48], to exploit the beneficial metabolic effects of both GR agonism and GR antagonism.

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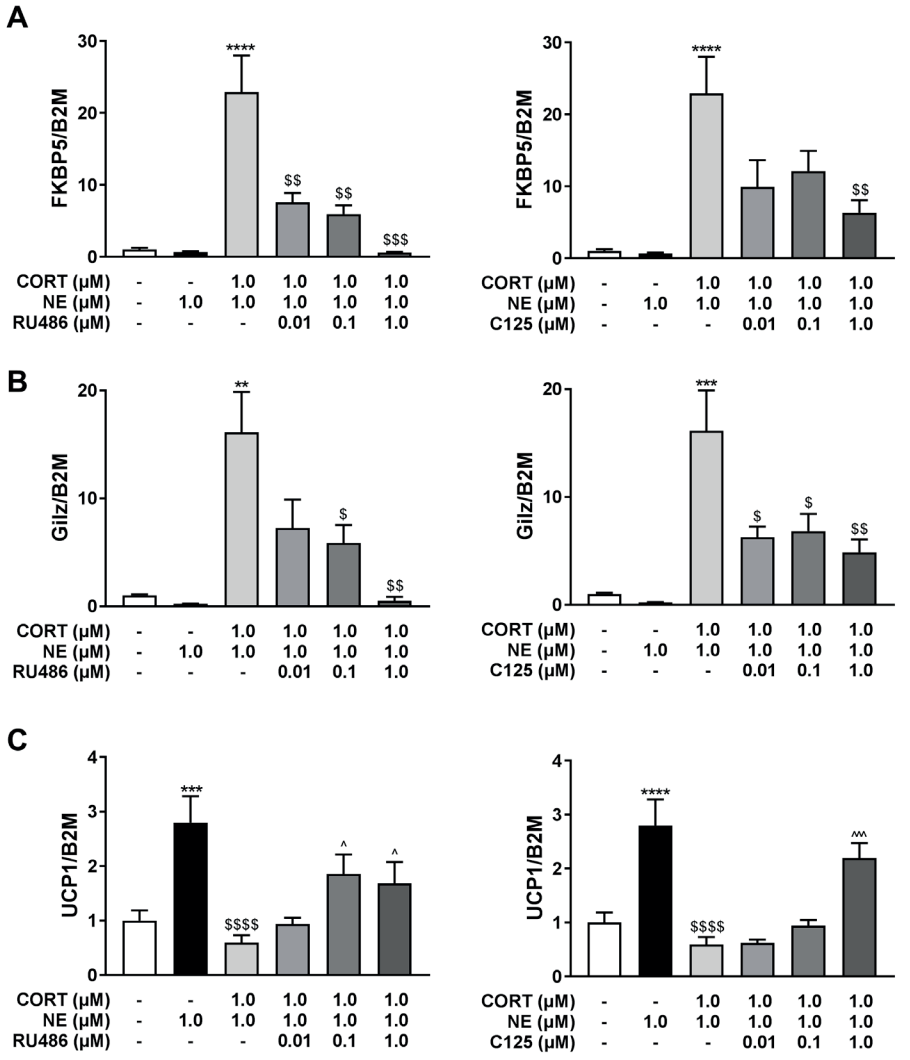
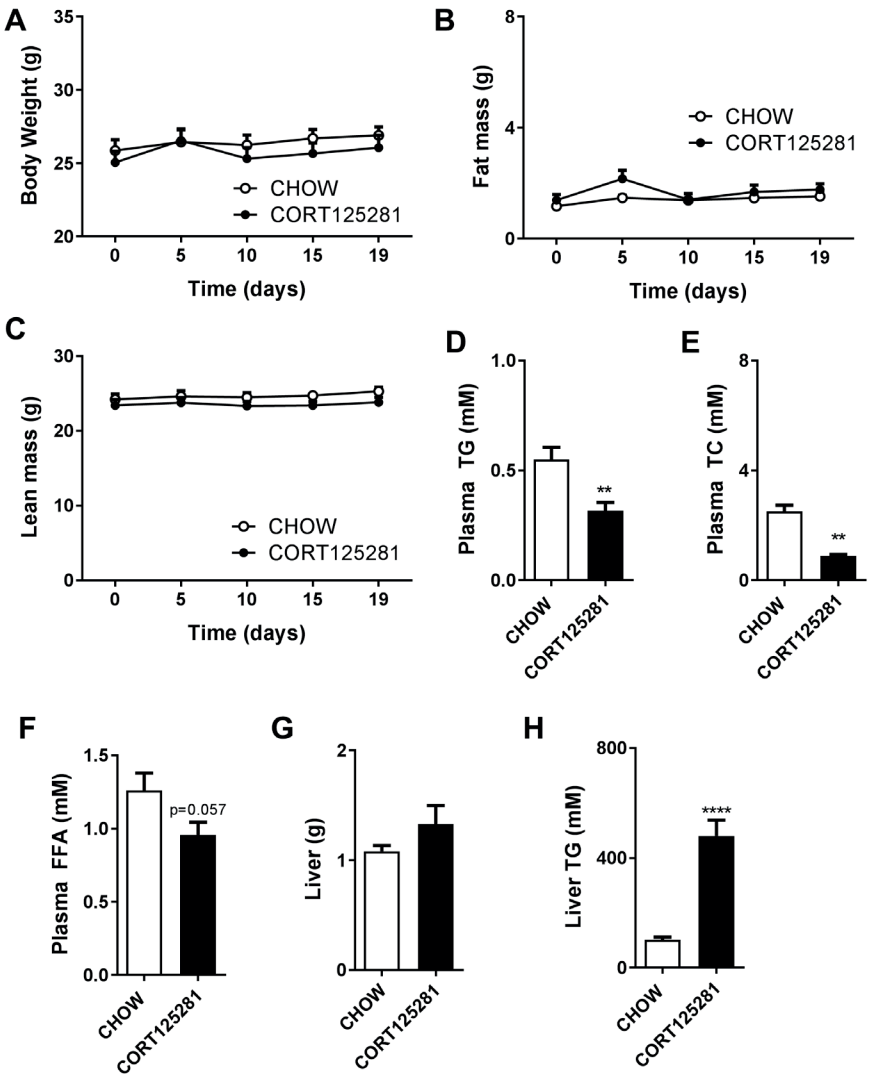
APPENDIX

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

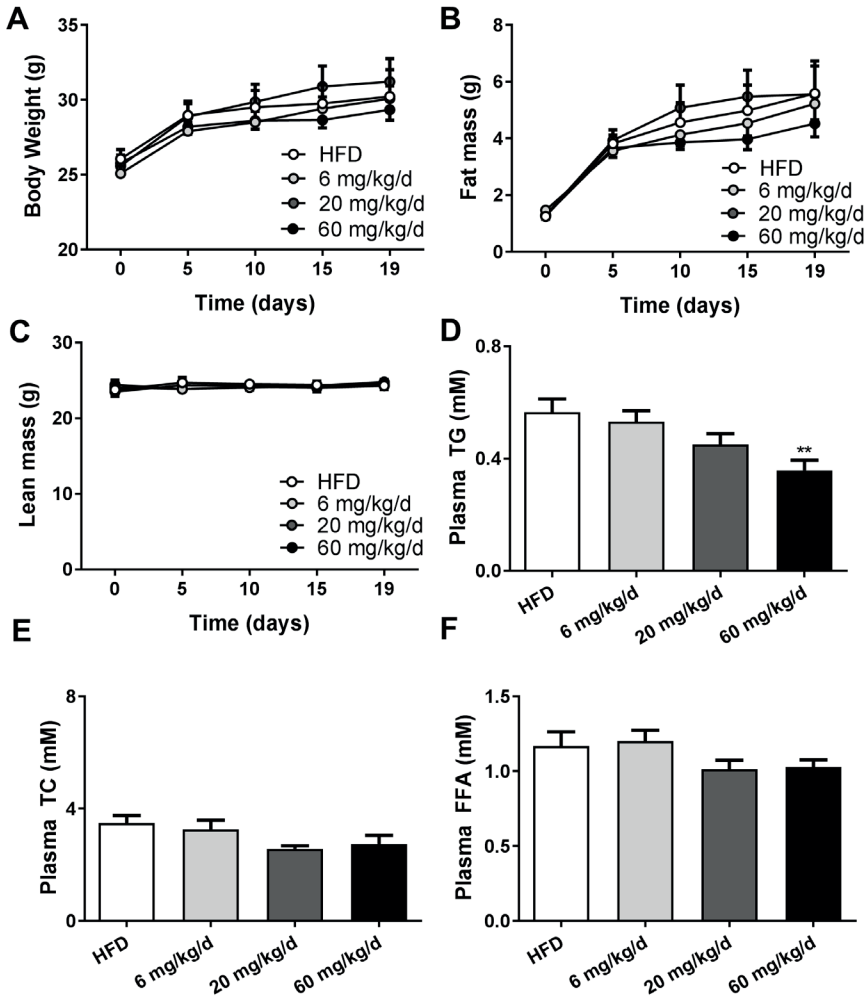
Gene	Forward primer sequence	Reverse primer sequence
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
Cd36	GCAAAGAACAGCAGCAAAATC	CAGTGAAGGCTCAAAGATGG
Cyp3a11	CTTTCCTTCACCCTGCATTCC	CTCATCCTGCAGTTTTTTCTGGAT
Fkbp5	GCCGACTGTGTGTGTAATGC	CACAATACGCACTTGGGAGA
Gilz	TGGCCCTAGACAACAAGATTGAGC	CCACCTCCTCTCTCACAGCAT
Ucp1	TCAGGATTGGCCTCTACGAC	TGCATTCTGACCTTCACGAC
B2-microglobulin	TGACCGGCTTGTATGCTATC	CAGTGTGAGCCAGGATATAG



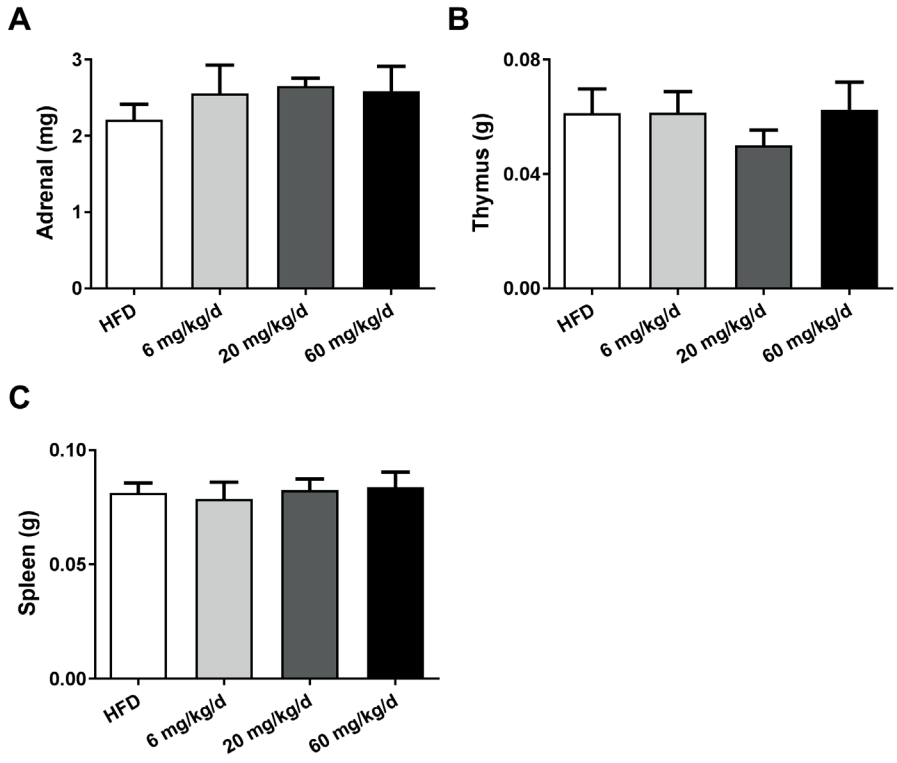
Supplementary Figure 1: The effects of GR antagonists on murine BAT cells *in vitro*. The effect of 1 μM corticosterone and different doses RU486 or CORT125281 on expression of (A) *Fkbp5* and (B) *Gilz*. ** p<0.01, *** p<0.001, **** p<0.0001, $ p<0.05 vs. CORT + NE, $$ p<0.01 vs. CORT + NE, $$$ p<0.001 vs. CORT + NE. (C) The effect of RU486 and CORT125281 on the expression of *Ucp1* in murine brown adipocytes after 8 hours of exposure. *** p<0.001, **** p<0.0001, $$$$ p<0.0001 vs. NE, ^ p<0.05 vs. CORT + NE, ^^^ p<0.001 vs. CORT + NE. Statistical significance was calculated using a one-way ANOVA with Tukey's multiple comparisons test. Abbreviations: CORT = corticosterone, NE = norepinephrine.



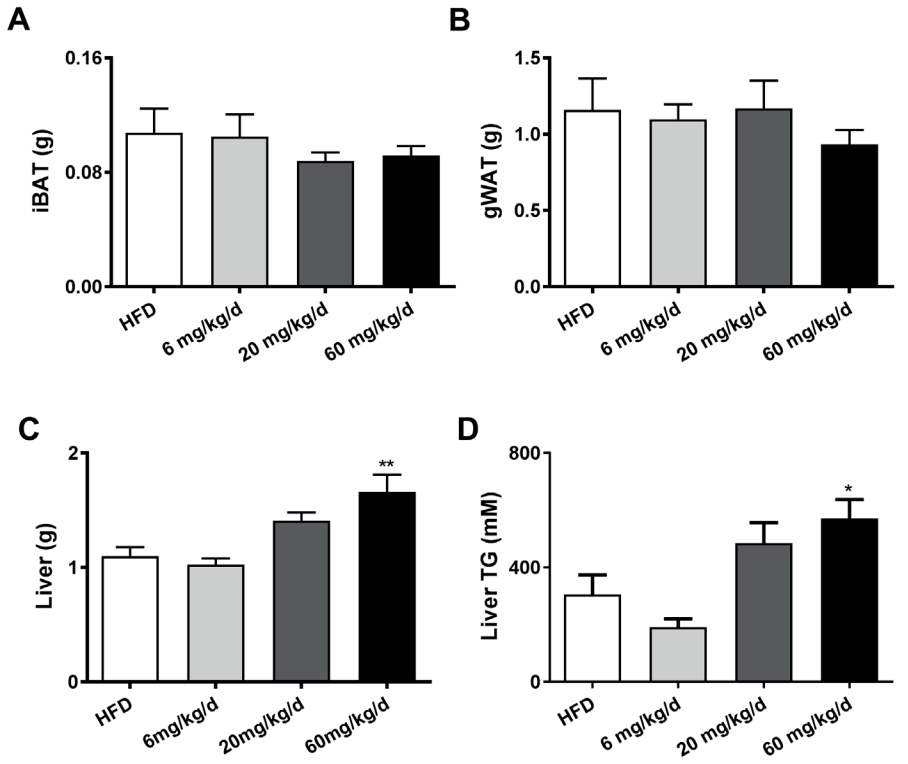
Supplementary Figure 2: The effect of CORT125281 on body weight, body composition, plasma lipids and the liver of chow-fed C57BL/6J mice. The effect of novel GR antagonist CORT125281 on (A) body weight, (B) body fat mass and (C) lean mass. The effect on (D) plasma triglycerides, (E) cholesterol and (F). The effect on (G) liver weight and (H) triglyceride content. Statistical significance was calculated using a mixed model analysis (A-C) or an unpaired t-test (D-H). ** $p<0.01$, **** $p<0.0001$.



Supplementary Figure 3: The effect CORT125281 on body weight, body composition and plasma lipids of HFD-fed C57BL/6J mice. The effect of different doses of the novel GR antagonist CORT125281 (6, 20 and 60 mg/kg/d) was examined on (A) whole body weight, (B) fat mass, (C) lean mass, (D) plasma triglycerides, (E) plasma cholesterol and (F) plasma free fatty acids. Statistical significance was calculated using a mixed model analysis (A-C) or a one-way ANOVA with Tukey's multiple comparisons test (D-F). ** $p<0.01$ vs. HFD.



Supplementary Figure 4: The effect CORT125281 on the weight of GC-sensitive organs of HFD-fed C57BL/6J mice. The effect of different doses of the novel GR antagonist CORT125281 (6, 20 and 60 mg/kg/d) on the weight of (A) adrenal, (B) thymus and (C) spleen. Statistical significance was calculated using a one-way ANOVA with Tukey's multiple comparisons test.



Supplementary Figure 5: The effect CORT125281 on metabolic organ weight and hepatic TG of HFD-fed C57BL/6J mice. The effect of different doses of CORT125281 was examined on the weight of (A) iBAT, (B) gWAT and (C) liver. (D) The effect of different doses of CORT125281 was examined on liver triglycerides. Statistical significance was calculated using a one-way ANOVA with Tukey's multiple comparisons test. * $p<0.05$ vs. HFD, ** $p<0.01$ vs. HFD.