Weight loss after AT_1 receptor blockade in diet-induced rat obesity is at least partially related to an angiotensin(1-7)-dependent mechanism

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Supplementary Methods:

Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT):

Glucose and insulin levels were determined during an OGTT (1g glucose•kg_{bw}^{-1}) in rats that had been deprived of food for 18h. EDTA blood (80 µl) was withdrawn before and after glucose administration (Miesel *et al.*, 2012;Müller-Fielitz *et al.*, 2012;Müller-Fielitz *et al.*, 2014). For ITT, the glucose levels were monitored after insulin injections (0.6 IU•kg_{bw}^{-1}, s.c) in rats that had been deprived of food for 18h. Glucose was determined before and during a 360-min period in blood samples (Miesel *et al.*, 2012;Müller-Fielitz *et al.*, 2012;Müller-Fielitz *et al.*, 2014).

Leptin resistance test:

For the leptin resistance test, we injected leptin (R&D Systems, Minneapolis, USA) at 8 a.m., 11 a.m., 2 p.m., and 5 p.m. (100 µg•kg_{bw}^{-1} s.c. each time) and at 8 p.m. (200 µg•kg_{bw}^{-1} s.c.). The next day rats were treated with leptin again at 8 a.m. (100 µg•kg_{bw}^{-1} s.c.), 11 a.m. (100 µg•kg_{bw}^{-1} s.c.), and 2 p.m. (200 µg•kg_{bw}^{-1} s.c.). Body weight and energy intake were determined (Müller-Fielitz *et al.*, 2015). We recently showed that neither food intake nor body weight was influenced by leptin compared to saline-treated controls when rats were fed with the CD. In contrast, food intake and gain in body weight gain were lessened when control-fed rats received leptin instead of saline injections, which clearly indicated leptin sensitivity under normal

conditions (Müller-Fielitz *et al.*, 2015). Hence, we waived the requirement of additional saline injections in the present study.

RNA isolation and cDNA synthesis:

Hypothalami were dissected according to Paxinos and Watson (Paxinos G & Watson C, 1998). The brains were adjusted to -10°C, and coronal sections were made 0.26 mm (at the optic chiasm) and 4.8 mm posterior to the bregma. In order to cut the hypothalamus apart, the slice was turned on its posterior surface and cut sagittally 2.6 mm lateral to the midline directly before the amygdala and horizontally 7.4 mm under the cortical surface. Total RNA from the hypothalami, visceral fat, livers, or skeletal muscles was extracted on the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems, Darmstadt, Germany). The amount of total RNA was determined using a RiboGreen RNA quantitation assay (Invitrogen, Karlsruhe, Germany). Isolation of genomic DNA was avoided by thorough treatment with DNase I. First-strand cDNA was synthesized using oligo-(dT)15 primer and AMV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). cDNA was stored at -20°C until further analysis.

Quantitative real-time PCR (qPCR):

AT_{1A} and AT_{1B} receptors, ACE2, and Mas mRNA were determined in hypothalami and in visceral fat, skeletal muscle, and liver. mRNA steady-state levels of anorexigenic peptides [e.g., cocaine- and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH), and pro-opiomelanocortin (POMC)] and orexigenic peptides [e.g., prepro-orexin (PPO), neuropeptide Y (NPY), melanin-concentrating hormone (MCH), and agouti-related protein (AgRP)] were quantified in the hypothalami of the rats. Quantitative measurements of mRNA were performed by using SYBR green I as a fluorescent dye on the GeneAmp 7000 sequence detection system (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany). DNA-specific primer sequences for AgRP, CART, MCH, NPY PPO, and CRF (Miesel *et al.*, 2010), for AT_{1A}

and AT_{1B} receptors (Raasch *et al.*, 2004), and MAS (Santos *et al.*, 2010) have been published elsewhere. For ACE2 we used the following primers (forward 5`-ACT GTC GGG CGG TCA TCA TC-3`, reverse 5`-GGT GGA GAA AAG CAA GGA GA-3`). All primers were obtained from Invitrogen. Copy number calculations were based on the cycle threshold method by using serial dilutions of known amounts of specific cDNA fragments to generate standard curves. Expression values were normalized to the amount of total RNA/sample(Bustin, 2002).

Supplementary discussion on hypothalamic expression of (an-)orexigenic peptides:

Expression of hypothalamic (an-)orexigenic peptides did not reflect the lower food intake of TG rats or TEL-treated rats. Such an incoherence between food intake and hypothalamic mRNA levels of (an-)orexigenic peptides was already found previously (Müller-Fielitz *et al.*, 2012;Müller-Fielitz *et al.*, 2014). We only once demonstrated that hypothalamic levels of the orexigenic peptides MCH and PPO decreased after TEL in parallel to weight loss, reduction of energy intake, restoration of leptin sensitivity, and normalization of pSTAT3 signaling (Müller-Fielitz *et al.*, 2015). The latter study clearly differed in duration of treatment (3 weeks versus 3 months) and the fact that no functional phenotyping (e.g., indirect calorimetry, stress tests, glucose or insulin tolerance tests when rats were partially food-deprived) was performed prior to measuring peptide expression. Thus, we assume that caution should be exercised regarding the significance of (an-)orexigenic peptides as a biochemical surrogate parameter for food intake when functional tests influencing food intake have been performed before quantification.

Suppl. Tab. 1: Nutrition composition of chocolate and cookie bars.

	calorie value ($kJ \cdot g^{-1}$)	carbo-hydrates (%)	fat (%)	protein (%)	fiber (%)	
Bounty	19.7	58	25	4	2	Mars GmbH, Viersen, Germany
Knoppers	22.9	52	33	9	3	August Storck KG, Berlin Germany
Lion	20.6	66	23	5	1	Nestlé Deutschland AG, Frankfurt, Germany
Prinzen Rolle	20.6	68	21	6	3	Mars GmbH, Viersen, Germany
Snickers	20.2	60	23	9	2	Griesson - de Beukelaer GmbH & Co. KG, Polch, Germany
Twix	20.7	65	24	5	1	Mars GmbH, Viersen, Germany

Suppl. Tab. 2: mRNA levels of orexigenic (PPO, NPY, MCH, AgRP) and anorexigenic petides (POMC, CART, CRH) in hypothalami of Sprague Dawley (SD) rats or transgenic rats (TG) overexpressing Ang(1-7) that received control diet or cafeteria diet (CD).

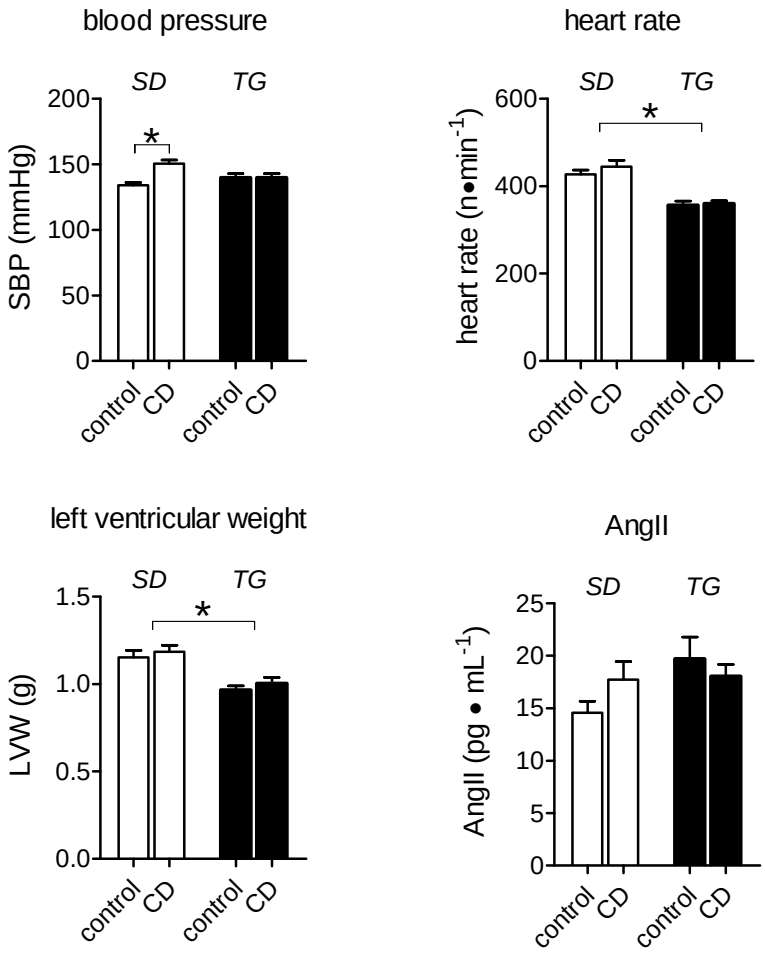
	$SD_{control}$	SD_{CD}	$TG_{control}$	TG_{CD}	
AgRP	4.2±0.3	3.6±0.3	5.0±0.5	5.6±0.2	†
MCH (x 10^4)	93.1±3.5	99.9±4.0	92.5±4.8	97.4±4.3	
NPY (x 10^4)	11.8±0.6	12.0±0.7	13.5±1.3	12.8±0.8	
PPO (x 10^4)	62.4±2.2	77.1±3.5*	69.1±4.9	70.6±2.5	
CART (x 10^4)	73.3±2.6	90.4±3.9*	76.3±4.1	82.6±2.9	
CRH (x 10^4)	1.1±0.1	1.3±0.1	1.2±0.1	1.3±0.1	
POMC (x 10^4)	18.2±1.8	20.6±1.6	18.3±2.6	21.6±1.0	

mRNA levels are expressed as copies•ng^{-1} RNA. Means+SEM, n=11-14. Statistical analysis was performed 2-ANOVA followed by Bonferroni post test.* $p<0.05$ vs. control of corresponding controls; † $p<0.05$ vs. strains

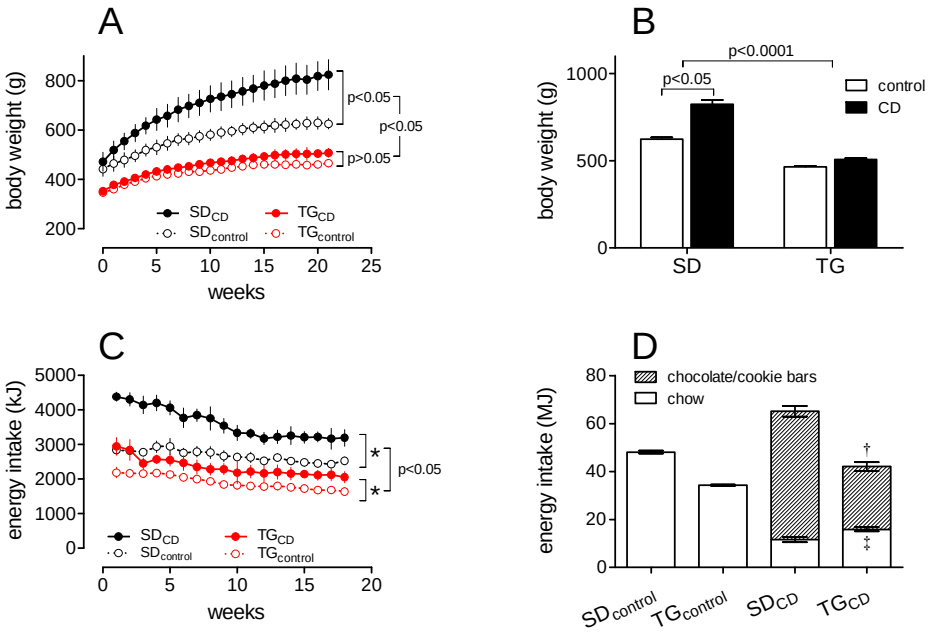
Suppl. Tab. 3: mRNA levels of orexigenic (PPO, NPY, MCH, AgRP) and anorexigenic petides (POMC, CART, CRH) in hypothalami of rats of CD-fed SD rats that were treated with TEL (8 $mg \cdot kg^{-1} \cdot d^{-1}$) or TEL plus A779 (24 or 72 $\mu g \cdot kg^{-1} \cdot d^{-1}$) while controls received vehicle+saline.

	vehicle +saline	TEL+saline	TEL+A779$_{24\mu g}$	TEL+A779$_{72\mu g}$
AgRP	100±4*	116±4	140±13	138±13
MCH	100±7	104±6	119±14	145±15*
NPY	100±5*	114±6	117±8	116±6
PPO	100±8	96±4	103±9	130±10*
CART	100±14	76±10	67±6	68±5
CRH	100±7*	137±12	122±6	135±7
POMC	100±4	93±6	107±12	105±7

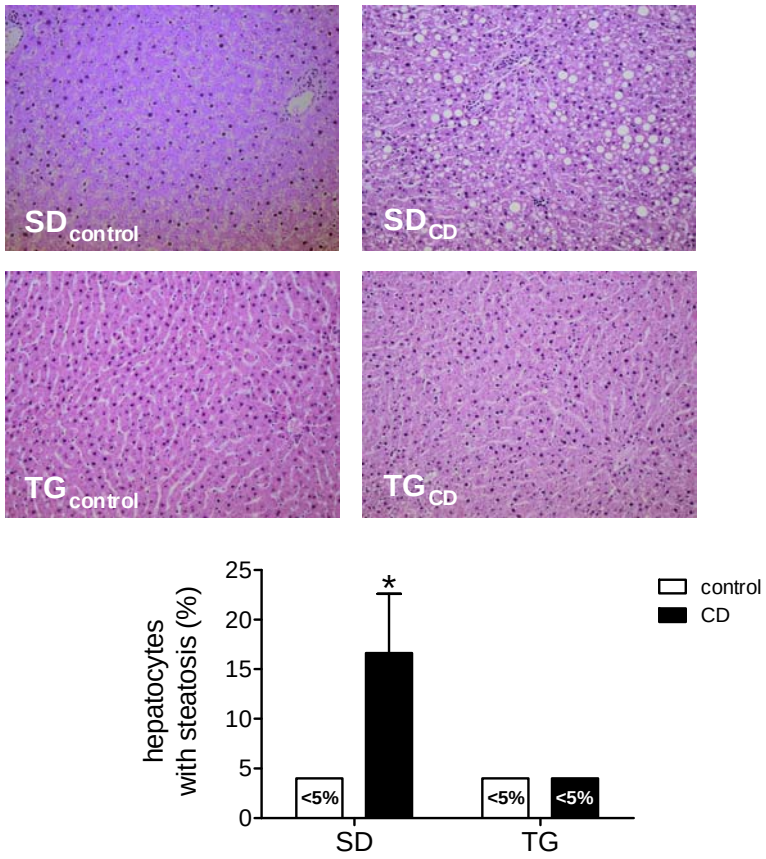
mRNA levels are expressed as % of $SD_{control}$. Means+SEM, n=11-12. Statistical analysis was performed by Wilcoxon Signed-Rank Test.* $p<0.05$ vs. TEL+saline



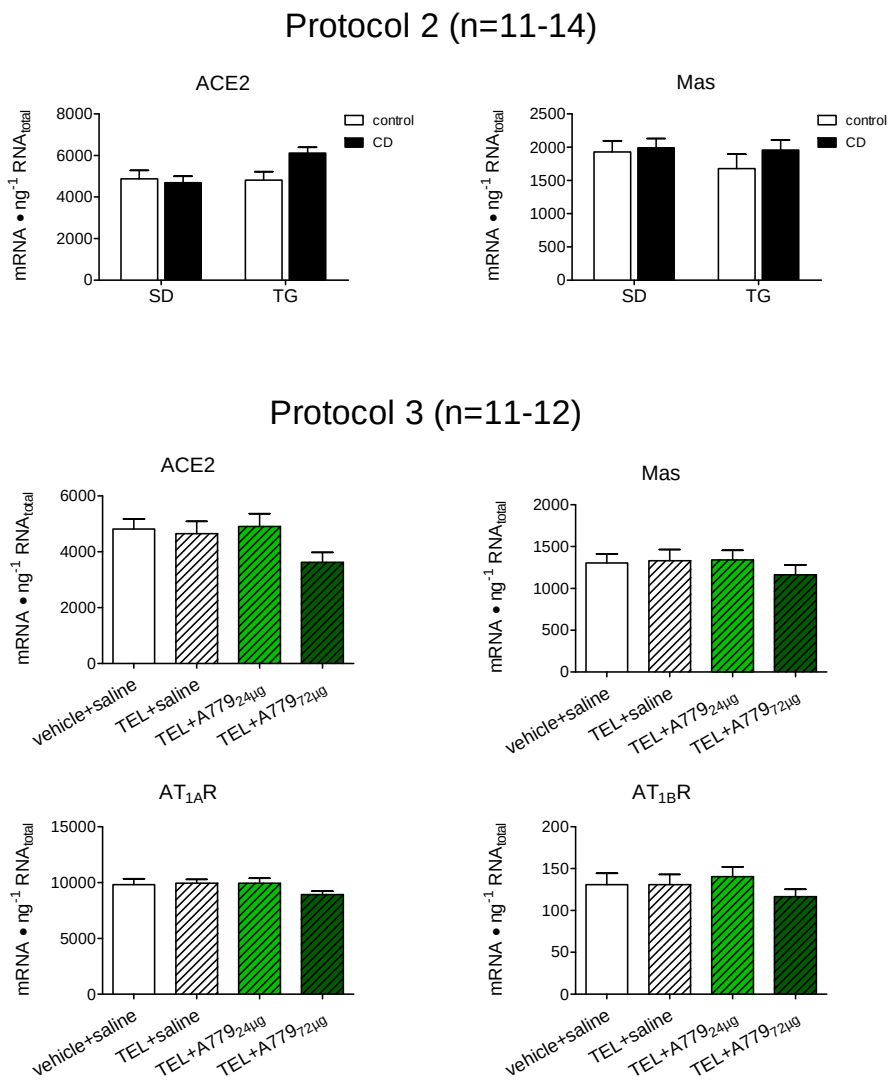
Suppl. Fig. 1: Systolic blood pressure (SBP), heart rate (HR), left ventricular weight, and AngII plasma concentration in SD (open bars) or TG rats (closed bars) depending on chow or CD feeding. Means+SEM, n=11-14. * $p<0.05$.



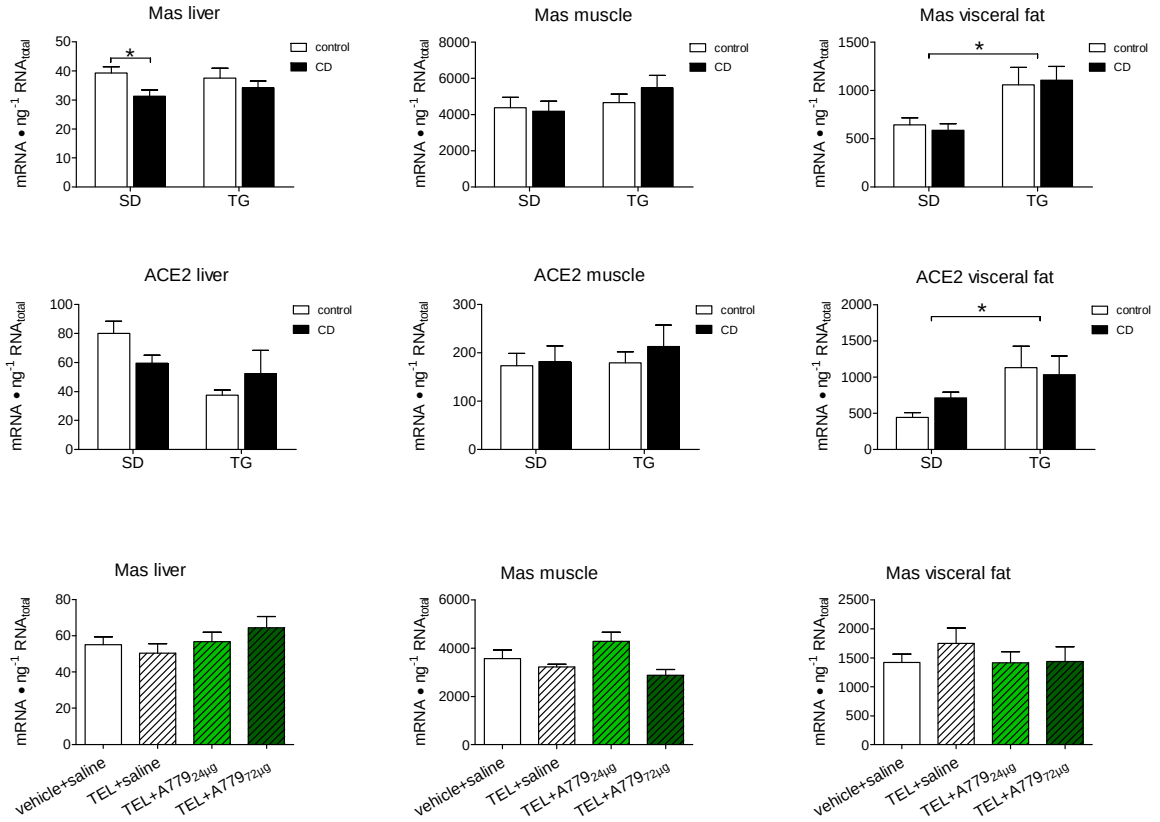
Suppl. Fig. 2: Body weight and energy intake are enhanced by CD feeding in older SD but not in TG rats (Protocol 2). **A**: Time-dependent increase in body weight. **B:** gain in body weight within the 12-week feeding period. **C:** Time-dependent energy intake. **D:** cumulative energy intake: open bars depict energy intake originating from chow intake whereas shaded bars represent energy intake from chocolate/cookie bars. Means±SEM, n=11-14. * $p<0.05$. † intake of chocolate/cookie bars: $p<0.05$ vs. SD_{CD}; ‡ chow-intake: $p<0.05$ vs. SD_{CD}.



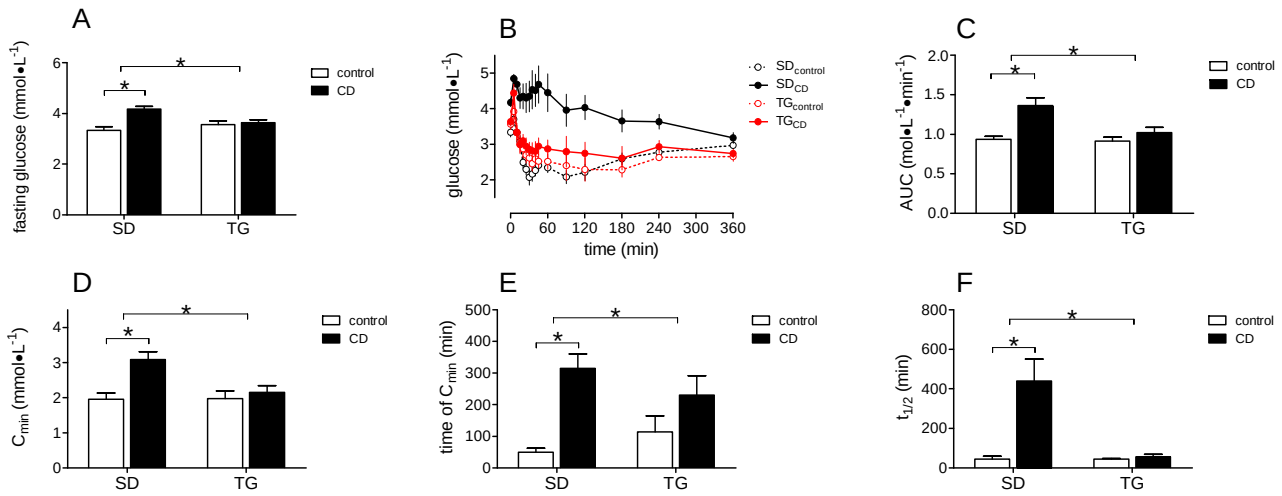
Suppl. Fig. 3: Histological findings of livers from control- or CD-fed SD and TG rats. Means±SEM, n=11-14. * $p<0.05$ vs. control. Tissue specimens were evaluated in a blinded manner based on and scored for hepatocytes with steatosis.



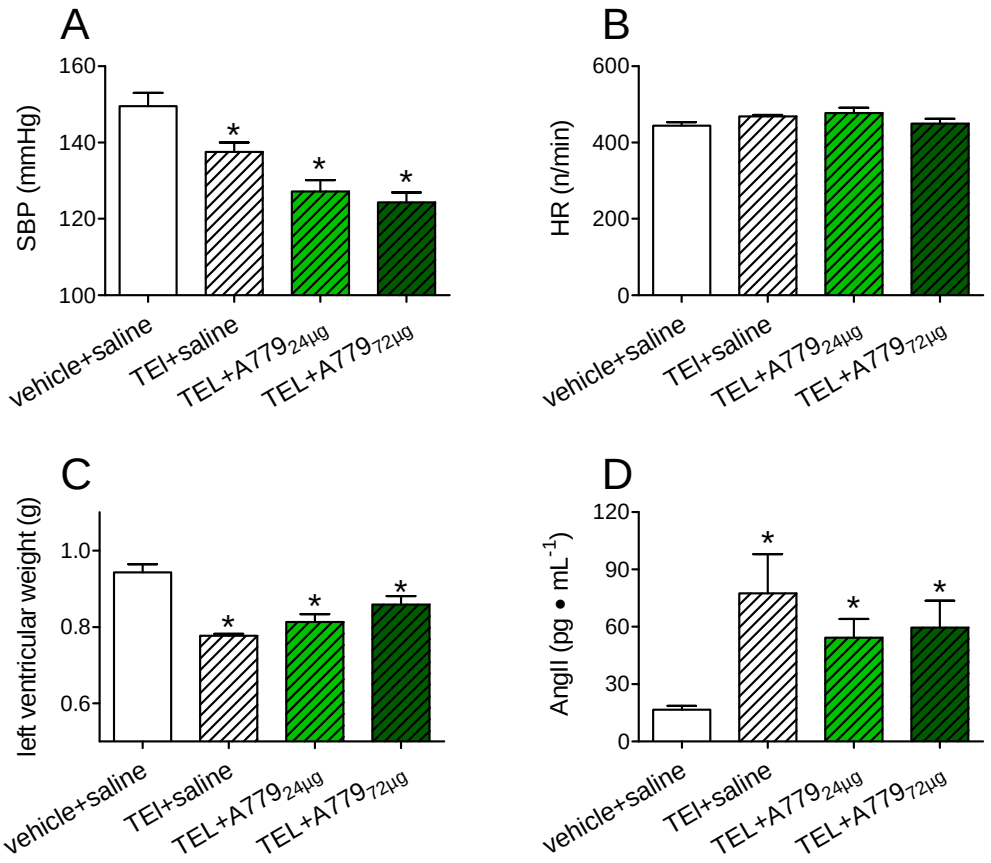
Suppl. Fig. 4: mRNA levels of components of the ACE2, Mas, and AT1A and AT1b receptors in hypothalami of rats of protocol 2 (control- or CD-fed SD and TG rats) and protocol 3, respectively (CD-fed SD rats that were treated with TEL [8 mg•kg^{-1}•d^{-1}] or TEL plus A779 [24 or 72 µg•kg^{-1}•d^{-1}] while controls received vehicle+saline). Means±SEM.



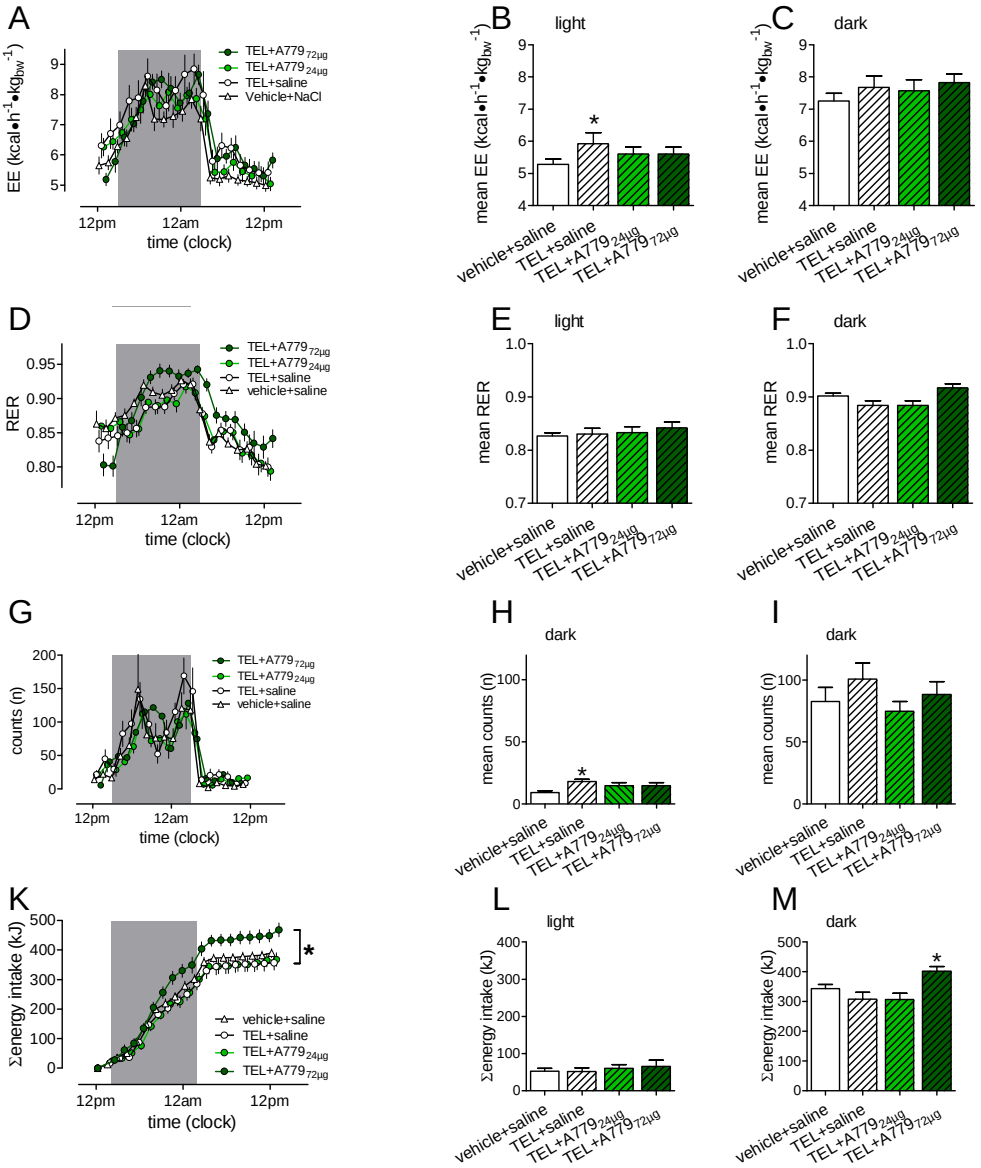
Suppl. Fig. 5: mRNA levels of MAS and ACE2 in different metabolic tissues of rats of protocol 2 (control- or CD-fed SD and TG rats) and protocol 3, respectively (CD-fed SD rats that were treated with TEL [8 mg•kg^{-1}•d^{-1}] or TEL plus A779 [24 or 72 µg•kg^{-1}•d^{-1}] while controls received vehicle+saline). Means±SEM.



Suppl. Fig. 6: Insulin response is impaired by CD feeding in SD but not in TG rats (Protocol 2). **A:** Fasting glucose levels; **B:** Glucose plasma concentrations after insulin injections (0.6 IU insulin•kg_{bw}^{-1}, s.c.). The AUC (**C**), the minimal glucose levels (**D**), the time points of minimal glucose levels (**E**) and the half-life of glucose decline (**F**) were higher in SD_{CD} than in $SD_{control}$, indicating impaired glucose control. A strain difference could be observed for all parameters. Means±SEM, n=11-14, * $p<0.05$.



Suppl. Fig. 7: Systolic blood pressure (SBP, A), heart rate (HR, B), left ventricular weight (C), and AngII plasma concentration (D) in CD-fed SD rats that were treated for 4 weeks with telmisartan (8 mg•kg^{-1}•d^{-1}) or telmisartan plus A779 (14 or 72 µg kg^{-1}•d^{-1}, by osmotic minipumps). Controls received vehicle and saline. Means±SEM, n=11-12. * $p<0.05$ vs. vehicle+saline.



Suppl. Fig. 8: Energy expenditure (EE; A-C) respiratory ratio (RER, D-F), locomotion (G-I) and energy intake (K-M) during indirect calorimetry measurements. Animals were housed for 3 days in calorimetry cages, but only the data of the 3rd day are depicted. Mean values during light and dark periods were calculated for EE (B, C), RER (E, F) and locomotion (H, I), whereas total energy intake was depicted specifically considering light (L) and dark periods (M). Means±SEM, n=12, * $p<0.05$ vs. vehicle+saline.

REFERENCES

Bustin SA. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* **29:** 23-39.

Miesel A, Müller H, Thermann, M Heidbreder, M Dominiak, P & Raasch W (2010). Overfeeding-induced obesity in spontaneously hypertensive rats: an animal model of the human metabolic syndrome. *Ann Nutr Metab* **56:** 127-142.

Miesel A, Müller-Fielitz H, Jöhren O, Vogt FM & Raasch W (2012). Double blockade of angiotensin II (AT(1))-receptors and ACE does not improve weight gain and glucose homeostasis better than single-drug treatments in obese rats. *Br J Pharmacol* **165:** 2721-2735.

Müller-Fielitz H, Hübel N, Mildner M, Vogt FM, Barkhausen J & Raasch W (2014). Chronic blockade of angiotensin AT(1) receptors improves cardinal symptoms of metabolic syndrome in diet-induced obesity in rats. *Br J Pharmacol* **171:** 746-760.

Müller-Fielitz H, Landolt J, Heidbreder M, Werth S, Vogt FM, Jöhren O et al. (2012). Improved insulin sensitivity after long-term treatment with AT1 blockers is not associated with PPARgamma target gene regulation. *Endocrinology* **153:** 1103-1115.

Müller-Fielitz H, Lau M, Geissler C, Werner L, Piehl M & Raasch W (2015). Preventing leptin resistance by blocking angiotensin II AT_1 receptors in diet-induced obese rats. *Br J Pharmacol* **172:** 857-868.

Raasch W, Jöhren O, Schwartz S, Gieselberg A & Dominiak P (2004). Combined blockade of AT1-receptors and ACE synergistically potentiates antihypertensive effects in SHR. *J Hypertens* **22:** 611-618.

Santos SH, Braga JF, Mario EG, Porto LC, Rodrigues-Machado MG, Murari A, et al. (2010). Improved lipid and glucose metabolism in transgenic rats with increased circulating angiotensin-(1-7). *Arterioscler Thromb Vasc Biol* **30:** 953-961.