Influence of AT\textsubscript{1} blockers

on obesity and stress-induced eating of cafeteria diet

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Running title: ARB and stress eating of cafeteria diet

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Based on findings that treatment with AT\textsubscript{1} receptor blocker (ARB) prevents diet-induced obesity and that the activity of the hypothalamic-pituitary-adrenal (HPA) axis is stimulated by AngII and blocked by ARBs, we aimed to investigate whether ARB treatment can reduce stress-induced eating of cafeteria diet (CD), thus contributing to alterations in eating behavior.

Sprague Dawley rats were fed with chow or CD and treated with telmisartan (TEL, 8mg/kg/d) or vehicle. At weeks 2 and 12, rats were stressed over 5 consecutive days by restraint stress (RS, 4h) and by additional shaking at d5. Tail blood was sampled during RS to determine hormone levels.

During the first period of RS, ACTH and corticosterone responses were diminished at d5 in CD-compared to chow-fed rats. Independently of feeding, TEL did not reduce stress hormones. Compared to food behavior before RS, the stress-induced CD eating increased in controls but remained unchanged in TEL-treated rats. After 12 weeks, TEL reduced weight gain and energy intake, particularly in CD-fed rats. Similar to the first RS period, corticosterone response was reduced in CD-fed rats at d5 during the second RS period. TEL did not further reduce stress hormones and did not lessen the CD eating upon RS.

We conclude that CD feeding compensates for stress reactions. However, stress-induced CD eating was only reduced by TEL after short-term, but not after long-term drug treatment. Thus, the potency of ARBs to lower HPA activity only plays a minor role in reducing energy intake to prevent obesity.
INTRODUCTION

In experimental studies, high-dosed AT1 receptor blockers (ARB) are established as a means to reduce body weight (Miesel et al., 2012, Muller-Fielitz et al., 2014, Muller-Fielitz et al., 2012, Muller-Fielitz et al., 2015, Muller-Fielitz et al., 2011). Weight loss has also been observed in patients during ARB therapy (Kintscher et al., 2007). Indeed, these anti-obese effects are known to be unrelated to the anti-hypertensive potency of ARBs (Muller-Fielitz et al., 2014, Muller-Fielitz et al., 2011) and are not a general feature of dampened renin-angiotensin system (RAS) activity as ACE inhibitors only marginally affected body weight (Miesel et al., 2012, Muller-Fielitz et al., 2014). Different mechanisms are currently thought to be involved, for example: 1.) A PPARγ-dependent mechanism might be involved as ARBs influenced adipocyte differentiation via PPARγ activation (Janke et al., 2006). However, such a mechanism is rather unlikely because typical PPARγ agonists such as thiazolidinediones increased body weight, food intake, fat quantity, and adipocyte size in rats and humans (de Souza et al., 2001, Larsen et al., 2003) and the expression of typical PPARγ target genes remained unaltered in visceral fat after ARB treatment in Western Blot microarray experiments (Muller-Fielitz et al., 2012, Schuster et al., 2018). 2.) A leptin-dependent mechanism might be involved as a) ARBs failed to reduce body weight in Zucker rats harboring a missense mutation in the leptin receptor (Muller-Fielitz et al., 2011); b) leptin sensitivity is restored by ARB treatment (Muller-Fielitz et al., 2014, Muller-Fielitz et al., 2015); and c) leptin transport across the blood-brain barrier was preserved by ARB treatment despite high-fat feeding (Schuster et al., 2018). 3) An AT1 receptor-independent mechanism might be involved as diet-induced obesity was not observed after ARB treatment in AT1 receptor-deficient mice (Rong et al., 2010). In this context, an angiotensin(1-7) [Ang(1-7)]-dependent mechanism was demonstrated to be relevant as diet-induced obesity was not observed in Ang(1-7) overexpressing rats and ARB-induced weight loss was partially antagonized by the Mas antagonist A779 (Schuchard et al., 2015).

In addition to these mechanisms, we speculated as to whether a hypothalamic-pituitary-adrenal (HPA) axis-dependent mechanism might play a role as the metabolic status and the activity in the HPA axis are tightly connected. Indeed, Dallman et al. reviewed this relationship in detail by particularly considering both physiological conditions and acute or chronic stress situations (Dallman et al., 2004): 1.)
Under physiological conditions, glucocorticoids stimulate caloric intake. 2.) High glucocorticoids after acute stress elevate blood glucose to ensure adequate substrate for brain and muscle for life-saving reasons and are related to increases in sweet, high-fat food ingestion. Following acute stress, the drive of the HPA axis is inhibited through a direct rapid-feedback mechanism. 3.) Following chronic stress, elevated glucocorticoids further activate the HPA axis. Thus, individuals did not become leaner because chronic stress inhibits food intake, probably through the anorexigenic effects of the corticotropin-releasing hormone (CRH). However, in the presence of sweet and high-fat foods, hedonic effects counteract stress-related weight loss, which is particularly influenced by insulin, and induce the development of obesity.

Since the availability of high-fat, high-carbohydrate foods and levels of perceived stress are high in western countries, Dallman accused the interaction between these two factors of being causally related to epidemic obesity in western societies (Dallman et al., 2004). Thus, it seems attractive to first ask whether dampening the drive of the HPA axis prevents diet-induced obesity from developing and, secondly, whether ARBs can do so. It has been established that the HPA axis is closely interrelated with the RAS as AT1 receptors are present in all organs of the HPA axis and are regulated during stress (Bali and Jaggi, 2013, Saavedra and Benicky, 2007). Vice versa, AngII stimulates the activity of the HPA axis via AT1 receptors (Abou-Samra et al., 1986, Armando et al., 2007, Jezova et al., 1998, Riviérer and Vale, 1983, Schoenenberg et al., 1987, Sumitomo et al., 1991). From the metabolic point of view, interaction between the RAS and the HPA axis was functionally shown to influence glucose control (Miesel et al., 2012, Muller et al., 2007, Muller-Fielitz and Raasch, 2013, Pavlatou et al., 2008).

Conflicting with Dallman’s aforementioned paradigm (Dallman et al., 2004), calorie intake was also found to be reduced upon chronic stress, independently of whether calorie intake originated from a high-carbohydrate (HC), high-fat (HF), or cafeteria diet (Zeeni et al., 2013). Thus, we first wanted to determine whether repeated stress alters the proportion of calories chosen from CD and/or whether HPA axis reactivity is affected by diet. Repeated restraint stress (RS) was applied over 5 days (d) in accordance with the protocol of Dallman’s group (Pecoraro et al., 2004). In contrast to that study using lard and sucrose (Pecoraro et al., 2004), we used sweet-tasting chocolate and cookie bars here, which
is more in line with our previous work concerning induced obesity, while controls only received chow diet (Miesel et al., 2010, Muller-Fielitz et al., 2014, Muller-Fielitz et al., 2012, Muller-Fielitz et al., 2015, Schuchard et al., 2015, Winkler et al., 2018, Winkler et al., 2016). As our previous findings showed that ARB treatment reduced both the intake of palatable food and HPA axis activity (Miesel et al., 2012, Raasch et al., 2006), we supposed that the HPA-RAS crosstalk functionally affects weight control. Therefore, we investigated whether eating sweet/fatty food would decrease in response to repeated (5d) RS if rats were treated short-term (2 weeks) or chronically (3 months) with TEL. As we did not include additional groups of nonstressed controls in this part of the study, we intraindividually compared stress-induced eating to conditions before applying stress, which admittedly could be considered a certain limitation of our study.

MATERIALS AND METHODS

Animals: All animal care and experimental procedures were conducted in accordance with the NIH guidelines for the care and use of laboratory animals and were approved by the animal ethics committee of the local regulatory authority (Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume des Bundeslandes Schleswig-Holstein). The results of all studies involving animals are reported in accordance with the ARRIVE guidelines. A total of 96 male Sprague Dawley (SD) rats (Charles River, Sulzfeld, Germany) were used in the experiments described here. One week before study begin, rats were placed in individual cages. According to our previous studies and also considering that female sex hormones affect the RAS at multiple levels (Fischer et al., 2002), we only used male rats, particularly to keep the number of rats low. The animals were kept at room temperature with a 12-h/12-h dark (2 p.m. - 2 a.m.)/light (2 a.m. - 2 p.m.) cycle. All animals were habituated to laboratory conditions for at least 10 days before experiments were started. All animals had free access to water.

Protocol 1 (Fig. S1): Four groups of SD rats (initial body weight 349±3g, not different between the groups) were included in protocol 1 (n=10 each group). Body weight was monitored daily. All groups were fed with chow (Maintenance 1320, Altromin, Lage, Germany) from d1 until d13. While two groups only received chow (designated as chow), two other groups additionally had free access at d5-7
and d9-d13 to one of six various commercial chocolate/cookie bars, these being switched daily in a regular manner [designated as CD throughout the text (Miesel et al., 2010); for the nutritional composition of chocolate and cookie bars, we refer the reader to (Schuchard et al., 2015)]. Individual food intake was monitored throughout the study by balancing the chow and chocolate/cookie bars. Due to the different calorie values of chow (11.7 kJ/g) and chocolate/cookie bars (20.8 kJ/g), we individually calculated the energy intake (in kJ) of each rat to correctly assess food intake on the basis of the consumed amounts of chow and chocolate/cookie bars. According to others (Pecoraro et al., 2004), one day intervened between pre-exposure of palatable food and onset of the repeated RS to avoid hogging eating of palatable diet when first offered. At d9-d13, one group of chow- or CD-fed rats was subjected to RS while unstressed rats served as controls. Feeding behavior was determined daily before and during stress. Blood was withdrawn before and during the stress period of 4h duration. To avoid any stress reactions due to blood withdrawal, blood was only taken at baseline conditions at d9 from non-stressed controls.

**Protocol 2 (Fig. S2):** Four groups of SD rats (initial body weight 207±1g, not different between the groups) were included in protocol 2 (n=12-14 each group). Two groups of SD rats were only fed with chow while two other groups received CD. One chow- and one CD-fed group of rats was treated with TEL (8 mg/kg bw, once per day by gavage, beginning at d8 until the end of the study). In our previous studies, 8 mg/kg TEL has been repeatedly shown to induce anti-obese efficacy in rats and mice (Miesel et al., 2012, Muller-Fielitz et al., 2014, Muller-Fielitz et al., 2012, Muller-Fielitz et al., 2015, Muller-Fielitz et al., 2011, Schuchard et al., 2015, Schuster et al., 2018, Winkler et al., 2016). For drug administration, TEL was suspended in distilled water by using gum arabic (10% w/v) to achieve final concentrations. A second group of chow- and CD-fed SD rats received an identical volume of vehicle (gum Arabic, 10% w/v, 1 ml per 1 kg bw/d) instead of TEL. Due to body weight-adapted drug/vehicle dosing, we monitored body weight continuously throughout the entire study. The daily energy intake of each rat was quantified by considering the difference between the initial and retained weights of chow and chocolate/cookie bars and their different caloric values (11.7 kJ/g or 20.8 kJ/g). All rats of each group were repeatedly subjected to RS at days 21-25 and for 5 consecutive days of week 14. As we abstained from including nonstressed animals in this part of the study, the prestress energy intake
served as appropriate controls. These prestress control values of the 1st or 2nd RS period were deter-
mined by averaging the food intake of d15-d18 and the last 4 days of week 13, respectively. Energy
expenditure was monitored at d25-28 and during week 12 by indirect gas calorimetry, measuring the
animal's oxygen consumption and carbon dioxide production to calculate the respiratory exchange rate
(RER) and energy expenditure (EE, PhenoMaster System™, TSE, Bad Homburg, Germany). Food
intake was simultaneously monitored by two precise weighing sensors equipped with food hoppers to
distinguish between the intake of chow and cookie/chocolate bars. This sensor technique was also
used to determine liquid consumption over time (PhenoMaster System™, TSE, Bad Homburg, Ger-
many) (Muller-Fielitz et al., 2014, Schuchard et al., 2015, Winkler et al., 2016). Locomotion was
monitored by sensing the body-heat image via infrared radiation (InfraMot System™, TSE, Bad
Homburg, Germany) (Muller-Fielitz et al., 2014, Schuchard et al., 2015, Winkler et al., 2016). Blood
was withdrawn before and during the 4h stress period. Rats were killed by decapitation after the final
RS and organs were removed for biochemical analysis. EDTA blood (2 ml) was collected for deter-
mining glucose and leptin levels. To determine AngII levels, blood (1 mL) was collected in an inhibi-
tor solution containing 12.1 mM EDTA and 20 µM bestatin (final concentration). After preparing the
femur, its length was measured using a vernier calliper.

Restraint stress (RS): On 5 consecutive days rats were restrained for 4h each day in a body weight-
adapted manner in hard plastic tubes (length/diameter ranging from 150/45, 160/60, and 220/80 mm
for animals with body weights <350g, 350-700g, or >700g). Tubes were closed at the top with perfo-
rated caps, thus allowing a sufficient air supply, and on the bottom with caps incorporating a small
hole to stick out the tail. The animals fit tightly into the restrainers and it was not possible for them to
move or turn around. RS started at 9 am each day. In addition to fixation, rats were shaken at d5 by
mounting tubes on a horizontal shaker (stroke distance 2 cm, frequency 30 Hz). Others have previous-
ly used this approach to avoid habituation to stress (Pecoraro et al., 2004). EDTA blood (80 µl) was
withdrawn from a tail nick immediately before applying stress as well as after 30, 60, 120, 180, and
240 min.
**Biochemical analysis**: Plasma concentrations of ACTH, corticosterone, leptin, and AngII were determined by RIAs using commercial kits (MP Biomedicals, Germany or Linco, St. Charles, Missouri, USA, or IBL, Hamburg, Germany) as recommended by the manufacturer, except that a reduced sample volume was employed (ACTH 50 µl, corticosterone 50 µl of a 1:200 dilution (Muller et al., 2007). Blood glucose was determined using sensors that detected glucose on the basis of amperometric measurements performed after enzymatic glucose oxidation (Ascensia® ELITE XL, Bayer) (Muller et al., 2007).

**Quantification of mRNA**: mRNA levels of the AT$_{1A}$ receptor or the ACTH receptor (melanocortin 2 receptor, MC2-receptor), CRH, and the orexigenic neuropeptide melanin-concentrating hormone (MCH) were quantified in organs of the HPA axis. The hypothalamus was prepared from whole brain and stored in a freezer, ensuring that the tissue did not defrost. Total RNA was isolated from organs. Isolation of genomic DNA was avoided by thorough treatment with DNase I. cDNA was synthesized using standard kits (Reverse Transcription System; Promega, Mannheim, Germany). Quantitative measurements of mRNA were performed by qPCR with the cycle threshold method, using SYBR green I as a fluorescent dye on the GeneAmp 7000 sequence detection system (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany). Primers for AT$_{1A}$, AT$_{1B}$, MC2 receptor, CRH, and MCH have been published elsewhere (Muller et al., 2007, Muller-Fielitz et al., 2014, Raasch et al., 2004, Schuchard et al., 2015, Winkler et al., 2016). All primers were obtained from Invitrogen (Karlsruhe, Germany). Product purity was confirmed by dissociation curve analysis and agarose gel electrophoresis in the presence of ethidium bromide. No-template controls served as negative controls (Jöhren et al., 2001). Expression values were normalized to total RNA content (Bustin, 2002).

**Calculations and statistics**: Means and standard errors are depicted as bars. In line graphs and tables, data are expressed as means±SEM. In order to quantify the total effect over the observation period in response to stress for changes in plasma concentrations of ACTH and corticosterone, the areas under the curves (AUC) were calculated for each individual animal on the basis of their delta values. All data were checked for outliers using the GraphPad® outlier calculator and tested for Gaussian distribution by performing D'Agostino&Pearson omnibus normality tests using Prism 5 for Windows (GraphPad Software).
Software, La Jolla, USA). One-way ANOVA for comparing more than 3 groups was only performed when Gaussian distribution and variance homogeneity were verified. A 2-way ANOVA was performed to examine the effects of two variables. When time was considered as one of the variables, we used matched 2-way ANOVA. Otherwise, unmatched tests were performed. In the figure and table legends, statistical testing is described precisely, also including the F- (or H-) and p-values of variance analyses. Bonferroni’s post-hoc test for multiple comparisons test was only performed if F reached P<0.05 and there was no significant variance inhomogeneity. Kruskal-Wallis test with Dunn's multiple comparison test was used when values were not distributed in a Gaussian fashion and variance inhomogeneity was detected. Differences were considered to be statistically significant at p<0.05. All correlation analyses were performed by two-tailed Pearson test.

RESULTS

Influence of CD feeding on stress reactions and stress-dependent energy intake

Baseline levels of stress hormones were not different between chow- or CD-fed rats. ACTH and corticosterone transiently increased upon RS and declined thereafter (Fig. 1). CmaxACTH did not differ between the groups at all 5 days while Cmaxcorticosterone was higher in chow-fed rats at d1 (749±35 vs. 529±44 ng/ml, p=0.0018) and d5 (762±50 vs. 557±55 ng/ml, p=0.012). Stress reactions time dependently abated within 4 days, but additional shaking during RS at d5 almost reconstituted them. This became obvious as AUCs of ACTH and corticosterone declined until d4 and recovered at d5 (Tab. 1). While ACTH responses did not differ between the two groups, corticosterone responses were lower at d1 and d5 in CD-fed rats (Fig. 1, Tab. 1). At all days except d4, a correlation was observed between the AUCs of the concentration time curves of ACTH and corticosterone, respectively. A decrease in stress reactivity may be attributed to an adrenal mechanism as the slopes of regression lines became time dependently less steep but also recovered at d5 (Fig 1, 3rd row). Energy intake and body weight were reduced in stressed rats (Fig. 2). Energy intake and corticosterone response tended to correlate negatively at d1 but positively at d5, while no correlations were observed at d2-d4 (Fig. 1, 4th row).
Influence of TEL on diet-induced obesity

The final body weight was higher in CD-fed rats (Fig. 3A). Although we did not quantify fat mass, we claim that CD feeding induced obesity as the ratio between body weight and femur was higher in these animals (Fig. 3B). TEL treatment prevented obesity from developing and also slightly lowered body weight in chow-fed rats (Fig. 3A/B). Furthermore, TEL lowered left ventricular weight, which is attributed to its antihypertensive action (Tab. 2). Food intake, plasma leptin, and blood glucose were increased in CD-treated animals (Figs. 3C-D). TEL lowered food intake in chow- and CD-fed rats (Fig. 3C). A 2-way ANOVA indicated a TEL group effect on glucose levels (p=0.014; Fig. 3D). TEL also normalized plasma leptin despite CD feeding (Fig. 3E). When leptin was correlated with energy intake, values of CD-fed rats were shifted rightwards compared to chow-fed controls when treated with vehicle, while TEL treatment induced a leftward shift, particularly in CD-fed animals (Fig. 3F). Thus, correlation analyses confirmed that rats became leptin resistant as a result of CD feeding and that TEL treatment prevented leptin resistance although we did not perform functional and/or immunohistochemical experiments as in previous studies (Müller-Fielitz et al., 2015, Schuster et al., 2018). The energy expenditure determined at w12 was higher in CD-fed rats and furthermore was enhanced selectively by TEL in CD-fed rats during the light and dark phase (Fig. 4A). In CD-fed rats, RER was lower during the light periods, but TEL did not affect either parameter (Fig. 4B). Locomotion of CD-fed rats was lower during the light and higher during the dark periods but was not affected by TEL during either phase (Fig. 4C).

Influence of TEL on stress reactions and stress-dependent energy intake

Baseline levels of stress hormones did not differ between groups before the 1st RS period at d21, while corticosterone, but not ACTH concentrations were selectively lower at w14 in CD-fed rats (Tab. S1). If any stress responses occurred, they were only slightly increased at d1 after CD feeding within the 1st RS period (Fig. 5A/C). In contrast, ACTH and corticosterone responses were clearly diminished at d5 in CD- as compared to chow-fed rats (Fig. 5B/D). TEL did not affect either stress hormone except for increasing corticosterone in chow-fed rats at d1 (Fig. 5C). Within the 1st RS period, the energy intake during stress declined time dependently in CD-fed rats and to a lesser extent in chow-fed animals (Fig. S3). To determine the influence of TEL on RS-induced
eating at d1, we compared energy intake with the prestress conditions during d15-d18. A 2-way ANOVA verified that acute stress lessened energy intake in chow- and CD-fed rats. Bonferroni post testing confirmed this for the VEH- and TEL-treated animals, but only for chow feeding and not for CD (Fig. 6A/B). As P-values of interactions were >0.05 in CD- and chow-fed animals, it seems likely that TEL had only minor effects on stress-induced eating at d1 of RS (Fig. 6A/B). However, stress-dependent intake of cookies at d1 was higher in VEH- but not in TEL-treated rats (Fig. 6C). This effect may contribute to the aforementioned observation that the stress-induced energy intake of VEH-treated rats was only reduced to a minor degree in CD-fed rats compared to chow-fed rats. To further investigate whether intake of cookies is enhanced by stress and whether TEL might reduce intake, we determined food intake at d5 of RS by using the phenomaster system (Fig. 6D). Energy intake was clearly higher in CD-fed rats during the first dark period following RS and TEL selectively normalized energy intake to chow levels (Fig 6E). This was attributed to an enhanced intake of cookies, which TEL reduced (Fig. 6F).

As the anti-obese efficacy of TEL is related to treatment duration (Schuster et al., 2018), we additionally performed stress tests after >3 months of treatment. Corticosterone responses during the 2nd RS period were lower in the CD-fed rats at d1 and d5, while ACTH responses were not affected (Fig. 7A-D, Fig. S4). In accordance with findings during the 1st RS period, TEL had no effect on stress hormones (Fig. 7A-D). In contrast to the results of the 1st RS, stress-induced energy intake was lower in both chow- and CD-fed rats (Fig. 7E-H). TEL reduced stress-induced chow and CD eating only at d5 but not d1 (Figs. 7F/H). Accordingly, TEL only diminished cookie intake at d5 of RS (Fig. 7I/J). Both baseline corticosterone levels and corticosterone responses upon RS (depicted as AUCs from corresponding concentration time curves, Fig. S4) correlated with plasma leptin (Fig. 8). Furthermore, we wanted to determine whether TEL treatment influences AT receptor expression in organs of the HPA axis. mRNA levels of AT1A/1B receptors were downregulated by TEL in adrenals but not in hypothalami and the pituitary glands. TEL markedly increased AngII levels but these levels were not affected by diet (Tab. S1). CD feeding did not influence AT1 receptor expression (Fig. 9). Additionally, adrenal MC2 expression was lower in TEL- than in VEH-treated animals (Tab. 2). mRNA levels of CRH were increased by TEL or by CD feeding, but there was no additive effect (Tab. 2).
DISCUSSION

Our investigation of whether a HPA axis-dependent mechanism might contribute to alterations in food behavior during ARB therapy gave the following key findings: 1.) ACTH and corticosterone responses upon chronic stress are decreased after short-term CD feeding, but only corticosterone responses declined after long-term CD feeding; and 2.) TEL diminishes energy intake, particularly intake of cookies, after stress but TEL did not diminish stress responses.

Effects of CD feeding on stress:

Due to repetitive RS, levels of ACTH and corticosterone declined until d4 but were reconstituted by additional shaking at d5 (Fig. 1), which was reported previously (Pecoraro et al., 2004). We used palatable chocolate cookies as comfort food, whereas Pecoraro offered sucrose solution plus lard. In line with Pecoraro’s stress protocols, we investigated stress-dependent feeding behavior 3 days after initiating the cafeteria diet and furthermore after 14w of diet. In accordance with Pecoraro, who found that stress-induced corticosterone response was lessened by comfort food at d5, we also observed this independent of feeding duration. In contrast to Pecoraro, who found no stress-induced corticosterone response at d1, we observed lower corticosterone responses upon CD feeding at d1, too, following long-term feeding and in the experiments of protocol 1 (Figs. 1, 5, 7). Findings on ACTH were inconsistent in the different experiments of our study (lower, unchanged higher, Figs. 1, 5, 7) and did not yield a clear pattern with respect to CD feeding (short- or long-term) or d1 or d5 of stress. In Pecoraro’s study the authors show that the ACTH response was reduced in the CD group at d1, but not d5 (Pecoraro et al., 2004). Authors attributed changes in stress hormones to a downregulation of hypothalamic CRH mRNA in animals that were fed with comfort food, thus supporting their metabolic feedback hypothesis (Pecoraro et al., 2004). However, they failed to confirm this finding in stressed animals and instead explained it by the time when brains were examined, either after applying heterotypic stress or habituation to repeated stress. In contrast, we found an increase in hypothalamic CRHmRNA, which more likely reflects dishabituation at d5 after additional shaking to overcome habituation to repeated stress as seen at d1-d4. The reduction in stress reactions is, however, not reflected by a down-regulation of adrenal AT_{1A/1B} receptors and MC2 receptor (Tab. 1), which are known to be the major...
regulators of ACTH-mediated corticosteroid biosynthesis in adrenal glands (Boston and Cone, 1996). As leptin certainly increased upon CD feeding (Fig. 1) and leptin reveals inhibitory effects on the HPA axis (Ahima et al., 1996, Bornstein et al., 1997, Heiman et al., 1997) via alpha-adrenergic- and/or orexin-mediated pathways (Bonnavion et al., 2015, Clark et al., 2008), leptin itself might cause the reduced stress responses. Our findings on the negative correlation between leptin and corticosterone support this notion (Fig. 8).

Effects of stress on energy intake:

In line with previous reports (Armario et al., 1984, Armario et al., 1990, Diane et al., 2008, Marti et al., 1994, Meerlo et al., 1996, Rybkin et al., 1997), we observed that energy intake was lower when animals were stressed (Fig. 2, 6B). This pattern is supported by the negative correlation between the corticosterone response and energy intake after acute stress at d1 (Fig. 1). However, the correlation became positive at d5 (Fig. 1) and, moreover, the RS-induced intake of CD and particularly sweet chocolate biscuits (Fig. 6F) was higher at d5 (Fig. 6E). These observations conflict with the “anhedonia hypothesis” of chronic stress, which claims that stress is more likely to reduce appetite activity (Willner et al., 1992). This inconsistency might be explained by the different diets, ranging from weak sucrose solutions with little taste or metabolic impact (Willner et al., 1992) to chocolate biscuits (as used in the present study) or lard and sucrose (Pecoraro et al., 2004) serving as calorically dense and palatable sweet food. Findings of Zeeni et al. (Zeeni et al., 2013) did not really support these assumptions, though, as they found that both eating and body weights were reduced in the rats upon chronic stress, independently of whether rats were fed with HC diet, HF diet, or CD. However, Zeeni’s group additionally showed that the sucrose preference was lower in stressed animals of the HC and HF but not of the CD groups compared with their corresponding non-stressed controls. This indeed indicated that the hedonism is still present despite chronic stress in rats when they were fed with peanut butter, chocolate, and biscuits, which, however, conflicts with their own findings showing that stress-induced CD-eating was reduced. Thus, our results are more in support of recent observations showing that palatable food intake is increased to compensate for chronic stress (Dallman et al., 2004), that chronic mild stress increases intake for sweets, especially as concentrations increase (Willner et al., 1998), and
that the increased intake of sucrose solution and lard as comfort food to compensate for stress is associated with diminished ACTH and corticosterone concentrations after RS (Pecoraro et al., 2004).

Effects of TEL on stress and energy intake:

In the present study, TEL clearly prevented obesity from developing via a reduction in energy intake and increase in energy expenditure, thus strengthening our experimental approach as a robust model to study the underlying mechanisms. The efficacy of TEL was verified by the marked increase in circulating AngII levels, which was attributed to a renin-dependent feedback mechanism and which was also observed previously (Ishiyama et al., 2004). Different mechanisms contributing to these anti-obese effects were briefly outlined in the introduction. Hence, we focus here on the effects of TEL on the HPA axis. Baseline levels of ACTH and corticosterone remained unchanged after short-term TEL treatment in chow- and CD-fed rats (Tab. S1). These findings are in line with others also showing unchanged plasma levels of stress hormones in rats after ARB treatment, irrespectively of the treatment duration, diet, or strain (Miesel et al., 2012, Muller-Fielitz et al., 2012, Raasch et al., 2006, Wincewicz et al., 2016). Nevertheless, these data conflict with other reports that baseline corticosterone decreased after ARB (Seltzer et al., 2004) or even increased (Sanchez-Lemus et al., 2012).

In our study, baseline corticosterone at least tended to be lower after long-term TEL treatment selectively in CD-fed rats (Tab. S1). Factors affecting this heterogeneity are unclear. Against our expectations and our findings that expression of adrenal MC2 and AT1A receptors were downregulated by TEL (Tab. 2, Fig. 9), TEL did not lower ACTH and corticosterone upon RS (Fig. 5/7). This is in contrast to previous studies showing that ARBs lessened HPA axis activity by applying different stress regimens such as CRH injections, forced swim tests, inflammation stress, and acute or long-term RS (Miesel et al., 2012, Raasch et al., 2006, Sanchez-Lemus et al., 2012, Wincewicz et al., 2016). It can be assumed that the lack of TEL efficacy on stress sensitivity is related to the normalization of leptin, thus dampening the suppressive potency of leptin on the HPA axis (for a discussion, see above). Moreover, lower HPA activity in SD rats per se may also contribute to the failure of TEL. This assumption is strengthened as AT1A receptor expression is higher in the pituitary glands of SHRs than in
normotensive WKY rats, leading to an exaggerated endocrine stress response of SHR to AngII (Johren et al., 2003). Whether Ang(1-7), which increases upon chronic TEL treatment (Igase et al., 2005, Ishiyama et al., 2004), contributes to the lower HPA axis regulation after TEL is unknown, but it is at least feasible as Ang(1-7) was shown to reduce AngII-induced corticosterone release in a Mas receptor-dependent mechanism (Zhu et al., 2014).

TEL also reduced chow and CD eating upon stress (Fig. 6A, S3A) without coincidentally affecting body weight (Fig. S3B). Wincewicz et al showed that TEL clearly lowered the corticosterone response upon chronic stress and that stress-induced loss of body weight was coincidently attenuated by TEL, which in contrast to our findings might indicate that TEL enhanced the stress-induced food intake (Wincewicz et al., 2016). As Wincewicz did not directly measure food intake and our trial and that of Wincewicz differed regarding the rat strain (SD vs. Wistar rats), duration of RS (5d vs. 21d), and TEL dose (8 vs. 1 mg/kg/d), we cannot say which parameter is responsible for the discrepant results. As discussed above, intake of chocolate/cookie bars increased at d1 and d5 during the 1st RS period. This stress-induced eating of palatable food was dampened by TEL (Fig. 6), as already found in previous experiments of our group (Miesel et al., 2012). TEL also lowered CD eating and particularly the intake of cookies within the second period of RS after 3 months although the effects are small (Fig. 7H/J).

In summary, we conclude that ARBs may lower stress-induced CD eating in an acute therapeutic approach but this effect becomes minor when ARBs are chronically administered. Considering the moderate effects on stressed-induced and CD eating and the lack thereof after chronic treatment, we must concede that a HPA axis-derived mechanism is thought to be involved to only a minor extent in ARB-dependent regulation of body weight and energy intake.
ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

VG, IS, and MW performed the research; WR and VG designed the research study; VG, MW, and WR analyzed the data; and WR and VG wrote the paper.
REFERENCES


FIGURE LEGENDS

Fig. 1: ACTH (1st row) and corticosterone (2nd row) profiles of chow (open circles) or CD-fed (filled circles) rats after restraint stress during 5 consecutive days of protocol 1. A matched 2-way ANOVA considering diet and time was calculated for ACTH and corticosterone plasma profiles. 2-way ANOVA highly indicated time differences for ACTH and corticosterone at d1-d5 (p<0.0001 for all days and both hormones). Pvalues in graphs specifically indicate diet differences in corticosterone responses. 3rd row: correlation and linear regression (±95% confidence interval) between ACTH and corticosterone responses; enlarged symbols represent means. 4th row: correlation and linear regression (±95% confidence interval) between corticosterone responses and energy intake; enlarged symbols represent means. Correlation analyses were performed by two-tailed Pearson test. Means±SEM; n=10 each group.

Fig. 2: Energy intake and development of body weight in chow- (A/C) and CD-fed rats (B/D) that were stressed between day 9 and 13 by restraint stress (RS). 2-way ANOVA was calculated considering time and RS (energy intake chow: F_{time}=3.7, p_{time}=0.009, F_{RS}=34.8, p_{RS}<0.0001; F_{interaction}=0.4, p_{interaction}=0.815; energy intake CD: F_{time}=65.4, p_{time}<0.0001, F_{RS}=34.6, p_{RS}<0.0001; F_{interaction}=1.2, p_{interaction}=0.311; body weight chow: F_{time}=475.5, p_{time}<0.0001, F_{RS}=4.5, p_{RS}=0.048; F_{interaction}=21.6, p_{interaction}<0.0001; body weight CD: F_{time}=410.0, p_{time}<0.0001, F_{RS}=1.6, p_{RS}=0.222; F_{interaction}=19.3, p_{interaction}<0.0001); * p<0.05 vs RS free conditions. Means±SEM, n=10 each group.

Fig. 3: Body weight (A, F_{TEL}=20.8, p_{TEL}<0.0001), obesity index (B, F_{TEL}=19.7, p_{TEL}<0.0001), cumulative energy intake (C, F_{TEL}=15.8, p_{TEL}=0.0002), blood glucose (D, F_{TEL}=6.5, p_{TEL}=0.0142.), and plasma leptin (E, F_{TEL}=7.5, p_{TEL}=0.009) of chow and chow+CD-fed rats that were simultaneously treated over the long term with vehicle or telmisartan. A 2-way ANOVA considering diet and TEL was calculated to determine differences. Panel (F, larger symbols represents means) depicts correlation (r=0.536, p<0.001) between leptin and energy intake. * p<0.05 vs VEH. Means±SEM.
Fig. 4: Energy expenditure (A;), RER (B), and locomotion (C) of chow- and CD-fed rats that were simultaneously treated with vehicle or telmisartan at week 12. Gray shading in figures on left represents dark periods. A 2-way ANOVA considering diet (exact p-values are shown in graphs) and TEL (EE<sub>light</sub>: F<sub>TEL</sub>=9.2, p<sub>TEL</sub>=0.004; EE<sub>dark</sub>: F<sub>TEL</sub>=5.6, p<sub>TEL</sub>=0.023; p>0.05 for RER and locomotion) was calculated to determine differences. * p<0.05 vs. VEH. Means±SEM.

Fig. 5: ACTH (A/B) and corticosterone (C/D) responses upon restraint stress during the first period at day 1 and 5 of RS application. A 2-way ANOVA considering diet (exact F- and p-values are shown in graphs) and TEL (ACTH d1 F<sub>TEL</sub>=0.6, p<sub>TEL</sub>=0.425; ACTH d5 F<sub>TEL</sub>=0.1, p<sub>TEL</sub>=0.983; corticosterone d1 F<sub>TEL</sub>=4.8, p<sub>TEL</sub>=0.032; corticosterone d5 F<sub>TEL</sub>=1.9, p<sub>TEL</sub>=0.173) was calculated to determine differences. * p<0.05 vs appropriate VEH. Means±SEM.

Fig. 6: Energy intake upon restraint stress (RS) during the first period at d1 (A-C) and d5 (E-F) of stress application. At d1, a 2-way ANOVA considering TEL (exact F- and p-values are shown in graphs) and stress (A: energy intake<sub>chow fed rats</sub> F<sub>RS</sub>=65.1, p<sub>RS</sub>&lt;0.0001; B: energy intake<sub>CD fed rats</sub> F<sub>RS</sub>=5.2, p<sub>RS</sub>&lt;0.032; C: chocolate/cookie intake F<sub>RS</sub>=4.8, p<sub>RS</sub>=0.038) was calculated to determine differences. * p<0.05 vs before RS. Fig D shows energy intake at d5 and the two following days, which was determined by the PhenoMaster System<sup>TM</sup> (TSE, Bad Homburg, Germany. A matched 2-way ANOVA considering time (p&lt;0.0001) and treatment (p=0.0006) was calculated to determine differences. † p&lt;0.05 vs initial value at the end of the dark period of the first post-stress day. To determine differences in total energy (E) and chocolate/cookie intake (F) at d5, a 2-way ANOVA considering diet (exact F- and p-values are shown in graphs) and TEL (E: F<sub>TEL</sub>=8.9, F: p<sub>TEL</sub>=0.005; F: F<sub>TEL</sub>=4.3, F: p<sub>TEL</sub>=0.045) was calculated; ‡ p&lt;0.05 vs VEH. Means±SEM.

Fig. 7: ACTH (A/B) and corticosterone responses (C/D) as well as chow (E/F), CD intake (G/H), and cookie eating (I/J) upon stress at d1 and d5 during the 2<sup>nd</sup> period of restraint stress (RS) at the end of
the study. A 2-way ANOVA was calculated considering the factors diet (F- and p-values are shown in graphs) and TEL for ACTH (panel A/B) and corticosterone (panel C/D) responses at d1 and d5 (all $p_{TEL}$-values > 0.05). A 2-way ANOVA was also calculated to determine differences in intake of energy or cookies considering RS (F- and p-values are shown in graphs) and TEL in chow-fed (d1 [panel E]: $F_{TEL}$=6.7, $p_{TEL}$=0.016; d5 [panel F]: $F_{TEL}$=17.5, $p_{TEL}$=0.0003) and CD-fed rats (energy intake at d1 [panel G]: $F_{TEL}$=0.1, $p_{TEL}$=0.851; energy intake at d5 [panel H]: $F_{TEL}$=21.8, $p_{TEL}$<0.0001; cookie intake at d1 [panel I]: $F_{TEL}$=4.6, $p_{TEL}$=0.043; cookie intake at d5 [panel J]: $F_{TEL}$=0.1, $p_{TEL}$=0.744).

Means±SEM, * $p<0.05$ vs. appropriate VEH controls; † $p<0.05$ vs. before RS.

Fig. 8. Correlation of plasma leptin with plasma corticosterone and corticosterone response to the 2nd restraint stress (RS) of chow- and CD-fed rats that were treated with TEL or vehicle. Correlation analyses were performed by two-tailed Pearson test.

Fig. 9: mRNA levels of AT$_{1A}$ and AT$_{1B}$ receptors in hypothalami, pituitary glands, and adrenals in telmisartan (TEL) or vehicle (VEH) treated rats. A 2-way ANOVA was calculated to determine differences considering diet (all p-values >0.05) and TEL-treatment (F- and p-values are shown in graphs). Means±SEM.

**LEGENDS SUPPLEMENTARY FIGURES**

Fig S1: Study protocol 1

Fig S2 Study protocol 2: all rats were fed with chow and group 3 and 4 additionally with various cookies, these being switched daily in a regular manner. Group 2 and 4 were treated with telmisartan (8 mg/kg/d), while group 1 and 3 received vehicle. All rats were stressed by restraint stress (RS) at days 21-25 and at week 14 and feeding behavior and energy expenditure were monitored at days 25-28 and during week 12.
Fig. S3: Energy intake during the 1st period (d1-d4) of RS (A) and changes in body weight after 5 days of RS (B). A: means±SEM (n=12-14). Using matched 2-way ANOVA considering the factors time and TEL we separately analyzed data for chow- ($F_{time}^{T_{TEL}}=2.8, p_{time}^{T_{TEL}}=0.048, F_{TEL}^{T_{TEL}}=10.0, p_{TEL}^{T_{TEL}}=0.004$) and CD-fed rats ($F_{time}^{T_{TEL}}=18.8, p_{time}^{T_{TEL}}<0.0001, F_{TEL}^{T_{TEL}}=19.8, p_{TEL}^{T_{TEL}}=0.0002$). Secondly, we performed a 2-way ANOVA for d1-d4 considering the two factors TEL and diet (d1: $F_{TEL}^{T_{TEL}}=23.2, p_{TEL}^{T_{TEL}}<0.0001, F_{diet}^{T_{TEL}}=205.5, p_{diet}^{T_{TEL}}<0.0001, F_{interaction}^{T_{TEL}}=4.7, p_{interaction}^{T_{TEL}}=0.034$; d2: $F_{TEL}^{T_{TEL}}=15.0, p_{TEL}^{T_{TEL}}=0.0003, F_{diet}^{T_{TEL}}=25.7, p_{diet}^{T_{TEL}}<0.0001, F_{interaction}^{T_{TEL}}=2.3, p_{interaction}^{T_{TEL}}=0.061$; d3: $F_{TEL}^{T_{TEL}}=22.9, p_{TEL}^{T_{TEL}}<0.0001; F_{diet}^{T_{TEL}}=2.0, p_{diet}^{T_{TEL}}=0.160, F_{interaction}^{T_{TEL}}=4.9, p_{interaction}^{T_{TEL}}=0.029$; $F_{TEL}^{T_{TEL}}=7.1, d4 p_{TEL}^{T_{TEL}}=0.010, F_{diet}^{T_{TEL}}=3.2, p_{diet}^{T_{TEL}}=0.081; F_{interaction}^{T_{TEL}}=2.3, p_{interaction}^{T_{TEL}}=0.135$). † p<0.05 CD+VEH vs. CD+TEL, ‡ p<0.05 chow+VEH vs. chow+TEL; B: means±SEM, A 2-way ANOVA considering diet (exact F- and p-values are shown in graphs) and TEL ($F_{TEL}^{T_{TEL}}=1.0, p_{TEL}^{T_{TEL}}=0.312$) was calculated to determine differences.

Fig. S4: ACTH and corticosterone plasma concentrations at day1 and 5 of the 2nd period of restraint stress at the end of the study. Means±SEM (n=12-14).
Tab 1: AUC of plasma concentration curves of ACTH and corticosterone at different days in rats upon restraint stress, Means±SEM (n=10);

<table>
<thead>
<tr>
<th>day</th>
<th>ACTH (ng/ml•min)</th>
<th>corticosterone (µg/ml•min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>chow</td>
<td>CD</td>
</tr>
<tr>
<td>1</td>
<td>75±13</td>
<td>72±9</td>
</tr>
<tr>
<td>2</td>
<td>49±7</td>
<td>48±7 *</td>
</tr>
<tr>
<td>3</td>
<td>40±6 *</td>
<td>42±6 *</td>
</tr>
<tr>
<td>4</td>
<td>27±4 *</td>
<td>28±3 *</td>
</tr>
<tr>
<td>5</td>
<td>54±6</td>
<td>42±3 *</td>
</tr>
</tbody>
</table>

Matched 2-way ANOVA was calculated to determine whether AUC differed in a time-dependent manner (ACTH AUC: $F_{time}=10.5$, $p_{time}<0.0001$, $F_{diet}=0.3$, $p_{diet}=0.581$, $F_{interaction}=0.3$, $p_{interaction}=0.853$; corticosterone AUC: $F_{time}=7.5$, $p_{time}<0.0001$, $F_{diet}=12.6$, $p_{diet}=0.001$, $F_{interaction}=3.9$, $p_{interaction}=0.006$; c); * p<0.05 vs. day 1; † p<0.05 vs chow.
Tab 2: Organ weights and mRNA levels (copies/ng total RNA) of adrenal melanocortin 2 receptors (MC2R) and of hypothalamic corticotropin-releasing hormone (CRH) and melanin-concentrating hormone (MCH) of chow- and CD-fed rats that were simultaneously treated with TEL. Means±SEM (n=11-14), 2-way ANOVA was calculated considering diet and TEL treatment, * p<0.05 VEH vs. TEL.

<table>
<thead>
<tr>
<th></th>
<th>chow</th>
<th>CD</th>
<th>F_{diet} (p_{diet})</th>
<th>F_{TEL} (p_{TEL})</th>
</tr>
</thead>
<tbody>
<tr>
<td>left ventricle (mg)</td>
<td>918±19</td>
<td>798±23*</td>
<td>6.7 (0.013)</td>
<td>24.9 (&lt;0.0001)</td>
</tr>
<tr>
<td>femur (mm)</td>
<td>40.6±0.2</td>
<td>40.3±0.2</td>
<td>0.02 (0.878)</td>
<td>1.81 (0.184)</td>
</tr>
<tr>
<td>kidney (mg)</td>
<td>1549±25</td>
<td>1435±26*</td>
<td>2.2 (0.140)</td>
<td>6.7 (0.012)</td>
</tr>
<tr>
<td>adrenal gland (mg)</td>
<td>30.8±1.1</td>
<td>30.7±1.0</td>
<td>1.7 (0.203)</td>
<td>0.3 (0.600)</td>
</tr>
<tr>
<td>MC2R (x10^7)</td>
<td>13.3±2.3</td>
<td>9.5±1.5</td>
<td>0.3 (0.235)</td>
<td>4.7 (0.035)</td>
</tr>
<tr>
<td>MCH (x10^5)</td>
<td>13.8±1.2</td>
<td>17.1±1.4</td>
<td>0.1 (0.839)</td>
<td>2.0 (0.165)</td>
</tr>
<tr>
<td>CRH (x10^5)</td>
<td>60.9±3.6</td>
<td>75.0±4.7*</td>
<td>6.7 (0.013)</td>
<td>1.9 (0.177)</td>
</tr>
</tbody>
</table>
Figure 1

173x195mm (300 x 300 DPI)
Figure 2

248x181mm (300 x 300 DPI)
Figure 3

268x145mm (300 x 300 DPI)
Figure 4

140x139mm (300 x 300 DPI)
Figure 5

260x171mm (300 x 300 DPI)
Figure 6

196x219mm (300 x 300 DPI)
Figure 7

158x243mm (300 x 300 DPI)
Figure 8

Leptin vs. baseline corticosterone

Leptin vs. stress-induced corticosterone response

Figure 8

103x138mm (300 x 300 DPI)
Figure 9

180x208mm (300 x 300 DPI)
Tab S1: Baseline levels of hormones in chow- and CD-fed rats at d21 and w14 before applying restraint stress or at the end of the study (=final). Means±SEM (n=11-14); 2-way ANOVA considering diet and TEL treatment.

<table>
<thead>
<tr>
<th></th>
<th>chow</th>
<th>CD</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VEH</td>
<td>TEL</td>
<td>VEH</td>
<td>TEL</td>
</tr>
<tr>
<td>ACTH (pg/ml) d21</td>
<td>55±7</td>
<td>44±6</td>
<td>54±6</td>
<td>50±4</td>
</tr>
<tr>
<td>ACTH (pg/ml) w14</td>
<td>60±6</td>
<td>74±9</td>
<td>59±6</td>
<td>57±3</td>
</tr>
<tr>
<td>corticosterone (ng/ml) d21</td>
<td>68±6</td>
<td>122±24</td>
<td>84±14</td>
<td>143±28</td>
</tr>
<tr>
<td>corticosterone (ng/ml) w14</td>
<td>161±14</td>
<td>168±19</td>
<td>110±21</td>
<td>67±9</td>
</tr>
<tr>
<td>AngII (pmol/L) final</td>
<td>16±4</td>
<td>148±22</td>
<td>11.9±2.9</td>
<td>124±20</td>
</tr>
</tbody>
</table>
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