Physiology & Behavior 105 (2012) 791–799

Contents lists available at SciVerse ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

The HPA axis modulates the CNS melanocortin control of liver triacylglyceride metabolism

Petra Wiedmer [a], Nilika Chaudhary [b], Michaela Rath [a], Chun-Xia Yi [b], Gayathri Ananthakrishnan [b], Rubén Nogueiras [c,d], Eva K. Wirth [e], Henriette Kirchner [f], Ulrich Schweizer [e], Wenke Jonas [a], Christelle Veyrat-Durebex [g], Francoise Rohner-Jeanrenaud [g], Annette Schürmann [a], Hans-Georg Joost [a], Matthias H. Tschöp [a,b], Diego Perez-Tilve [b,*]

[a] Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany
[b] Metabolic Disease Institute, Department of Internal Medicine, University of Cincinnati, Cincinnati, Ohio, United States
[c] Department of Physiology, School of Medicine, University of Santiago de Compostela, Instituto de Investigación Sanitaria (IDIS), Santiago de Compostela, Spain
[d] CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Santiago de Compostela, Spain
[e] Institute for Experimental Endocrinology, Charité, Universitätsmedizin Berlin, Berlin, Germany
[f] Integrative Physiology, Department of Molecular Medicine and Surgery, Karolinska Institute, Stockholm, Sweden
[g] Laboratory of Metabolism, Division of Endocrinology, Diabetology and Nutrition, Department of Internal Medicine, Faculty of Medicine, University of Geneva, Geneva, Switzerland

ARTICLE INFO

Article history:
Received 30 June 2011
Received in revised form 10 October 2011
Accepted 20 October 2011

Keywords:
Melanocortin receptor
Obesity
Adrenalectomy
Thyroidectomy
SHU9119
MTII

ABSTRACT

The central melanocortin system regulates lipid metabolism in peripheral tissues such as white adipose tissue. Alterations in the activity of sympathetic nerves connecting hypothalamic cells expressing melanocortin 3/4 receptors (MC3/4R) with white adipocytes have been shown to partly mediate these effects. Interestingly, hypothalamic neurons producing corticotropin-releasing hormone and thyrotropin-releasing hormone co-express MC4R. Therefore we hypothesized that regulation of hypothalamo-pituitary adrenal (HPA) and hypothalamo-pituitary thyroid (HPT) axes activity by the central melanocortin system could contribute to its control of peripheral lipid metabolism. To test this hypothesis, we chronically infused rats intracerebroventricularly (i.c.v.) either with an MC3/4R antagonist (SHU9119), an MC3/4R agonist (MTII) or saline. Rats had been previously adrenalectomized (ADX) and supplemented daily with 1 mg/kg corticosterone (s.c.), thyroidectomized (TDX) and supplemented daily with 10 μg/kg L-thyroxin (s.c.), or sham operated (SO). Blockade of MC3/4R signaling with SHU9119 increased food intake and body mass, irrespective of gland surgery. The increase in body mass was accompanied by higher epididymal white adipose tissue (eWAT) weight and higher mRNA content of lipogenic enzymes in eWAT. SHU9119 infusion increased triglyceride content in the liver of SO and TDX rats, but not in those of ADX rats. Concomitantly, mRNA expression of lipogenic enzymes in liver was increased in SO and TDX, but not in ADX rats. We conclude that the HPA and HPT axes do not play an essential role in mediating central melanocortinergic effects on white adipose tissue and liver lipid metabolism. However, while basal hepatic lipid metabolism does not depend on a functional HPA axis, the induction of hepatic lipogenesis due to central melanocortin system blockade does require a functional HPA axis.

1. Introduction

Obesity is recognized as an epidemic which is associated with multiple co-morbidities including diabetes, cardiovascular disease and cancer. Throughout industrialized nations, it poses one of the predominant health threats of our time [1,2]. Given that available pharmacologic agents have low efficiency and currently existing surgical approaches remain highly invasive and partially irreversible, safe and efficient pharmacologic approaches are urgently needed for curtailing further spread of obesity. The basis for appropriate anti-obesity drug development is the knowledge of how energy metabolism and body fat are regulated. In spite of significant progress in recent years, the specific pathways controlling body adiposity are not yet elucidated.

The current model suggests that the regulation of adiposity is the result of a coordinated interaction between peripheral and central pathways that measure and adjust body fat stores in the periphery. We and others have recently reported the existence of neuroendocrine circuits that directly control peripheral lipid metabolism in adipose tissue [3–6]. One of these circuits is mainly determined by the activity of the central nervous system (CNS) melanocortin system,

* Corresponding author at: Metabolic Disease Institute, University of Cincinnati, College of Medicine/Internal Medicine, 2180 E. Galbraith Road. A-125, Cincinnati, Ohio. 45220, United States. Tel.: +1 513 558 8658; fax: +1 513 558 0466.
E-mail address: pereztdo@ucmail.uc.edu (D. Perez-Tilve).

0031-9384/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.physbeh.2011.10.019

which consists of the receptor antagonist (inverse-agonist) agouti-related protein (AgRP), the neuropeptidergic agonist α-melanocyte stimulating hormone (α-MSH) and the melanocortin receptors 3 and 4 (MC3/4R). The CNS melanocortin system is one of the most essential circuitries for the CNS control of metabolic homeostasis. Endogenous signaling molecules such as insulin [7], leptin [8] and ghrelin [9] that signal the availability of peripheral energy stores directly target the CNS melanocortin system. Activation of MC3/4R by leptin induces proopiomelanocortin (POMC) secretion and processing to α-MSH, leading to negative energy balance and induction of lipolytic processes in white adipose tissue (WAT) [4,10]. In contrast, ghrelin stimulates AgRP levels and thereby blocks CNS melanocortin receptor activity, leading to positive energy balance and induction of lipogenic processes in the periphery [11].

We have recently shown that chronic blockade of the hypothalamic MC3/4R by a 7-day i.c.v. infusion of the synthetic MC3/4R antagonist SHU9119 considerably induces the expression of lipogenic enzymes in WAT [3]. These effects are associated with increased triacylglyceride (TG) content and increased TG synthesis in WAT. In addition, most of these effects are independent from SHU9119-induced hyperphagia, which points to an intrinsic and direct melanocortinergic effect on WAT.

Based on the available data, it appears most likely that sympathetic nerve fibers projecting from the CNS to adipocytes, hepatocytes and myocytes represent an important mechanism to mediate the peripheral effects of centrally administered melanocortin agonists and antagonists [3,12–14]. As an example, in the triple β-adrenoceptor knockout mouse, a model of impaired sympathetic nervous system (SNS) activity, the lipogenic effects of centrally administered SHU9119 in adipose tissue are blunted, indicating that a functional SNS is essential for the lipogenesis induced by blockade of the CNS melanocortin system [3].

Alternative mechanisms for transferring information from the central nervous system to the periphery are the classical hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-thyroid (HPT) axes, with circulating corticosterone and thyroid hormone levels as effector endpoints. Interestingly, populations of MC3/4R-expressing neurons in the periventricular hypothalamus co-express corticotropin-releasing hormone (CRH) [15]. In addition, neurons expressing thyrotropin-releasing hormone (TRH) co-localize with neurons expressing MC4R, α-MSH and AgRP in the paraventricular nucleus (PVN) of the hypothalamus [16–18]. Furthermore, AgRP deficient knockout mice (shift toward increased melanocortinergic activity) exhibit a lean phenotype that is associated with enhanced metabolic rate and increased plasma levels of thyroxin (T4) and triiodothyronine (T3) [19]. We therefore hypothesized that, in addition to the SNS, the HPA and HPT axes might mediate the effects of the CNS melanocortin system on peripheral lipid metabolism. Here we demonstrate that the integrity of the HPA and HPT axes is not essential for the regulation of food intake and body weight by CNS melanocortin signaling. However, our data indicate that the CNS MC3/4R-mediated control of the hepatic lipid metabolism is co-modulated by the HPA axis.

2. Materials and methods

All procedures were approved by the Animal Care and Use Committee at the state Ministry of rural development, environment and consumer protection of Brandenburg, Germany, in accordance with the German Animal Welfare Act.

2.1. Study design

To investigate the relevance of the HPA or the HPT axis in the central melanocortin effects on peripheral lipid metabolism, rats were either sham-operated (SO), adrenalectomized (ADX) or thyroidectomized (TDX) and chronically i.c.v. infused for 7 d with either saline, SHU9119 (melanocortin 3/4 receptor antagonist) or MTII (melanocortin 3/4 receptor agonist). Therefore, each group of pre-operated rats (either SO, ADX or TDX) included three treatment groups: a control group infused with i.c.v. saline; an i.c.v. SHU9119-infused group; and a MTII-infused group.

To prevent pre-operated rats from being metabolically affected by the insufficient production of either adrenal or thyroid hormones, rats received daily subcutaneous injections of either corticosterone (CORT) (to ADX rats) or L-thyroxin (T4) (to TDX rats) for the duration of the experiment (day 6 of i.c.v. treatment constitutes the day of last injection). The hormone supplementation is intended to provide sufficient CORT or T4 to maintain major basal metabolic functions influenced by either the HPA or the HPT axis. Rats received hormone supplementation daily between 9:00 and 10:30 am.

The morning of the seventh i.c.v. treatment day, rats were anesthetized with isoflurane and decapitated; trunk blood (collected in EDTA coated tubes with 150 µl of 1 mg/ml aprotinin solution) as well as several tissues were collected and frozen (−80 °C) for further analysis of gene expression and tissue triglyceride content.

2.2. Animals

Male Wistar rats (Crl:WI(Han)) that were previously operated with either sham or gland surgery were delivered by Charles River (L'Arbresle, France) with a weight of about 250 g. Three groups of previously operated rats were used: ADX rats, TDX rats (with reimplantation of the parathyroid) and SO rats (sham-operated for thyroidectomy, which represents the most deleterious surgery). Animals were maintained in individual cages under conditions of controlled temperature (22 °C) and illumination (12-hour light/12-hour dark cycle, onset of the dark cycle at 6 pm). They were allowed *ad libitum* access to standard laboratory chow (ssniff RM/H; ssniff Spezialdiäten GmbH, Soest, Germany). Since ADX rats suffer from decreased sodium retention, they needed sodium substitution which was provided by offering isotonic (0.9%) saline solution in addition to normal drinking water *ad libitum*. To achieve the same experimental conditions for all pre-operated groups, all rats were offered a bottle of isotonic saline in addition to drinking water. Food intake and body weight were measured daily during the experimental period.

2.3. Hormone substitution

Subcutaneous substitution of either CORT (1 mg/kg) or T4 (10 µg/kg) started on the third day after delivery (corresponding to the 12th day following ADX, TDX or SO). CORT (purity ≥ 92%, Sigma Aldrich) was dissolved in a small amount of ethanol (purity 99.8%) and then diluted in sesame oil (Sigma Aldrich) to yield a final concentration of 1 mg/ml (5% ethanol). T4 (Sigma Aldrich) was dissolved in sterile isotonic saline (Sigma Aldrich) under basic pH; this solution was further diluted with isotonic saline to yield a final concentration of 10 µg/ml. To control for the different vehicles for CORT and T4, all rats received equal volumes of oily and saline solutions. Vehicle solutions were made using the same procedure as the hormone solutions, except for the addition of the hormone. Thus, SO rats received 400 µl saline (T4 vehicle) and 400 µl sesame oil (CORT vehicle including 5% ethanol) without any hormone content. ADX rats received 400 µl of CORT solution and 400 µl of T4 vehicle. TDX rats received 400 µl of T4 solution and 400 µl of CORT vehicle.

2.4. I.c.v. infusions

Rats were equipped with a cannula (ALZET Brain Infusion Kit 2, DURECT Corporation, Cupertino, CA, USA) positioned in the right lateral cerebral ventricle (i.c.v.) fixed on the skull with Loctite 454 (Henkel AG & Co. KGaA, Düsseldorf, Germany). For chronic 7 d i.c.v. treatment, the cannula was connected via vinyl tubing to an osmotic

minipump (ALZET Model 2001, DURECT Corporation, Cupertino, CA, USA) delivering one of the following compounds at a rate of 1 µl/h: saline (Sigma-Aldrich), SHU9119 (24 nmol/d, Bachem) or MTII (1 nmol/d, Bachem). For the surgery, rats were anesthetized with intraperitoneal injection of ketamin hydrochloride (Ketamin Graeub, A. Albrecht GmbH, Aulendorf, Germany)/xylazine hydrochloride (Rompun®, Bayer Vital GmbH, Leverkusen, Germany) used at 100 mg/kg and 4 mg/kg, respectively. Following the surgery, rats received a single subcutaneous dose of 0.5 mg/kg carprofen (Rimadyl®, Pfizer GmbH, Karlsruhe, Germany).

2.5. Levels of plasma hormones and metabolites

Plasma T3 and T4 levels were determined using Milliplex™ MAP Rat Thyroid Hormone T3/T4 Panel - 2 Plex (Millipore, Billerica, MA, USA). Plasma CORT and ACTH levels were analyzed using Milliplex™ MAP Rat Stress Hormone Panel (Millipore, Billerica, MA, USA). Plasma leptin was determined using murine Leptin Quantikinine ELISA (R&D Systems). Plasma insulin levels were analyzed using rat insulin ELISA (DRG Instruments GmbH, Marburg, Germany). Plasma lipids were determined using commercially available kits: triglycerides (Sigma), free fatty acids (Wako).

2.6. Tissue triglyceride (TG) content

Liver samples (20 mg) were homogenized in ice-cold Chloroform/Methanol solution (2:1, vol/vol) for 2 min maximal speed using a tissue lyser (Qiagen). Triglycerides were extracted during 2-h shaking at room temperature. For phase separation, samples were centrifuged, and the organic bottom layer was collected. The organic solvent was dried using a SpeedVacPlus (SC210A, Savant Instruments, Farmingdale, NY) and the pellet was redissolved in chloroform. Triglyceride content of each sample was measured in triplicate using an enzymatic method (Randox Laboratories Ltd; Crumlin, UK).

2.7. Quantitative RT-PCR procedure

Total RNA was extracted from frozen epididymal adipose tissue and liver using TRIzol Reagent (Invitrogen) according to supplier's instructions. RNA integrity was assessed by performing 1% agarose gel electrophoresis in 1×MOPS and its concentration was determined by spectrophotometry (Nanodrop). cDNA templates for RT-PCR were synthesized using 2 µg of total RNA, primer random $p(dn)_6$ (Roche), dNTPs (Roche), 0,1 M DTT, 5x First-Strand-Buffer and Superscript III (Invitrogen). Quantitative real-time PCR was performed using *Power*SYBR® Green PCR master mix (Applied Biosystems), according to the standard protocol, using approximately 70 ng template cDNA. All primers were used at a final concentration of 0.5 µM. A standard curve was used to obtain the relative concentration of each experimental gene. Values were normalized to the concentration of hypoxanthine phosphoribosyltransferase (HPRT) in each sample. Primer sequences used are specified in Table 1. For the quantification of PVN CRH and TRH gene expression, frozen rat brains were sliced in coronal direction, slices between −1.3 mm and −2.1 mm from bregma containing the PVN were used. The bilateral areas containing the PVN were punched out by 20 gauge punch needle and kept in −80 °C. Total RNA from punched tissue was extracted using the RNeasy Micro Kit (Qiagen, CA), treated with DNAse I and eluted with RNAse free water according to the instructions provided in the kit. cDNA was synthesized by reverse transcription reaction from the RNA using Superscript III enzyme mix from Invitrogen. The qPCR gene expression assays were carried out using fluorescence labeled Taqman probes (Applied Bio Systems, CA). The cycle threshold (Ct) values obtained for TRH (22±0.3) and CRH (28±0.4) were normalized to the housekeeping gene L32 (27.5±0.2) and the fold change was calculated using the ΔΔCt method.

2.8. Statistical analysis

Data are shown as means±S.E.M. Data were analyzed for normal distribution (Shapiro–Wilk test) and for homogeneity of variances (Levene's Test for equality of Variances). Significant differences between the i.c.v. treatment groups on the different gland surgery background were tested using two-way analysis of variance (ANOVA) including the kind of gland surgery (SO, ADX or TDX), the i.c.v. treatment (saline, SHU9119 or MTII) and an interaction term between gland surgery and i.c.v. treatment. Bonferroni was used as a *post-hoc* test for multiple comparisons. Significance was assumed at $P<0.05$. Analysis was performed using GraphPad Prism 4 (GraphPad Software, Inc, La Jolla, CA 92037 USA).

3. Results

3.1. Validation of CORT or T4 clamping in ADX and TDX rats

Daily CORT replacement in saline infused ADX rats led to CORT plasma levels similar to saline infused SO and TDX rats indicating that CORT replacement was appropriate ($p=0.36$, Fig. 1A). Chronic i.c.v. infusion of SHU or MT-II affected neither the baseline of CORT (Fig. 1A) nor ACTH plasma levels (Fig. 1B) in sham or TDX rats. Furthermore, CORT levels in all ADX rats were clamped at the same level, irrespective if saline, SHU9119, or MTII was i.c.v. infused ($p_{treatment}=0.503$). ACTH levels were significantly increased in ADX rats in comparison with sham-operated rats, despite the appropriate CORT replacement (Fig. 1B). Regardless, CRH gene expression in the PVN was neither significantly affected by changes in MCR activation, nor by the CORT or T4 replacement (Fig. 1C).

T4 baseline levels were not significantly changed by the chronic i.c.v infusion of SHU or MT-II in sham or in ADX rats (Fig. 1D). Replacement with T4 in TDX rats led to increased T4 levels in saline- and SHU-treated TDX rats compared to SO and ADX rats (Fig. 1D), while MTII-treated TDX rats displayed normal T4 levels (2-way ANOVA: significant interaction term $p_{treatment\ x\ surgery}=0.049$, $p<0.01$ saline *vs.* MTII, Fig. 1D). Plasma levels of the active thyroid hormone, T3, were normal

Table 1
Primer sequences used for real-time PCR.

Gene	Accession no.	Forward sequence	Reverse sequence	PCR product	Annealing T (°C)
ACCα	J03808	5′-TCCGGCTTGCACCATGATAA-3′	5′-CCCCCAAAACGAGTAACAA-3′	104	54
FAS	M76767	5′-AGGATGTCAACAAGCCCAAG-3′	5′-ACAGAGGAGAAGGCCACAAA-3′	100	55
SCD-1	AF509569	5′-TGAAAGCTGAGAAGCTGGTG-3′	5′-CAGTGTGGGCAGGATGAAG-3′	83	57
LPL	L03294	5′-TCTCCTGATGATGCGGATTT-3′	5′-CAACATGCCCATCTGGTTTC-3′	97	54
CPT-1	L07736	5′-GGATGGCATGTGGGTAAAAG-3′	5′-TACTGACACAGGCAGCCAAA-3′	203	55
HPRT	NM_012583	5′-CAGTCCCAGCGTCGTGATTA-3′	5′-AGCAAGTCTTTCAGTCCTGTC-3′	139	60

Annealing T, annealing temperature

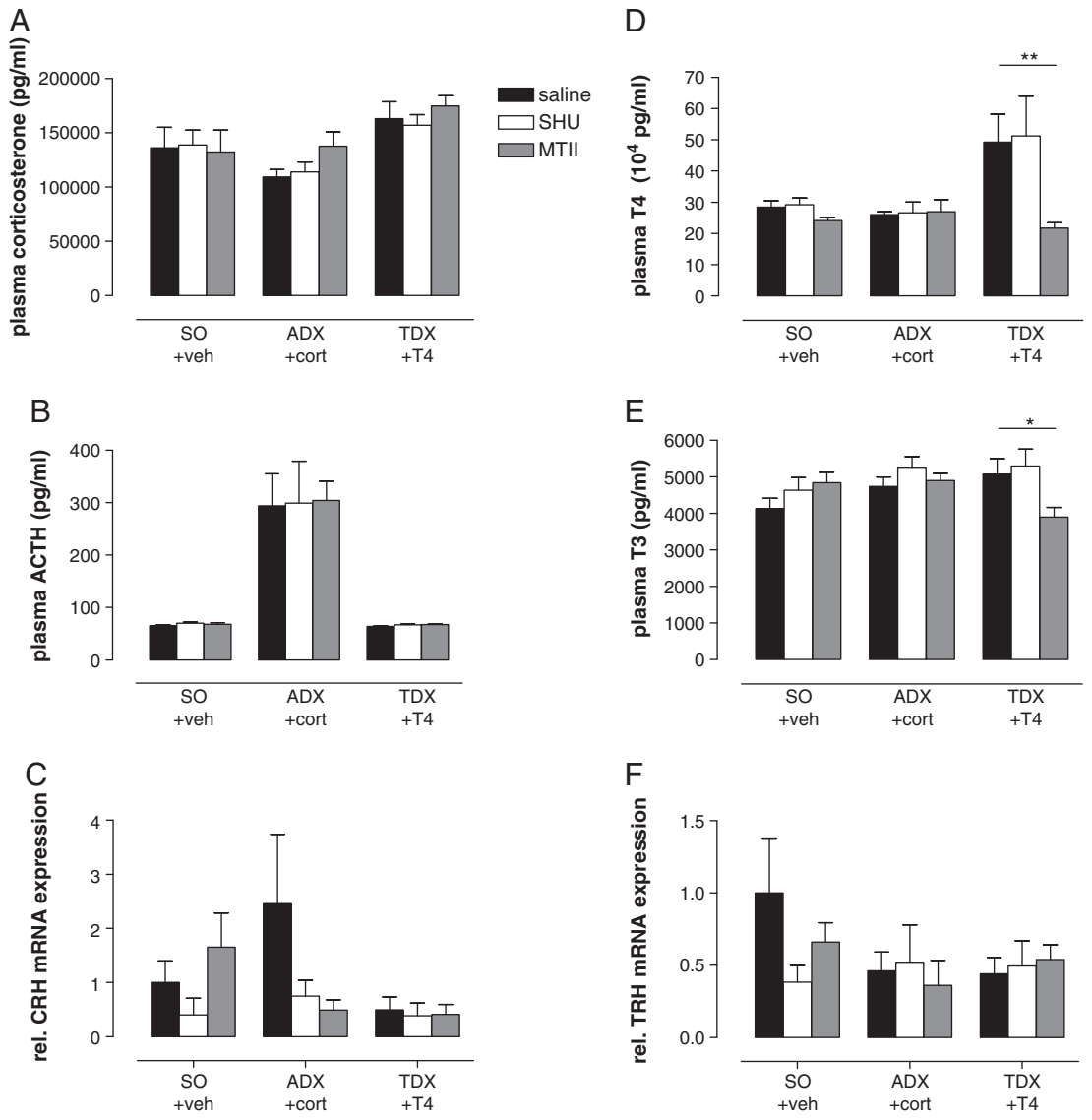

Fig. 1. Plasma levels of CORT (A) and ACTH (B), relative CRH mRNA expression in the PVN (C), plasma levels of thyroxin (T4) (D) and triiodothyronine (T3) (E) and relative TRH mRNA expression in the PVN (F) in vehicle-treated sham-operated (SO + veh), CORT-replaced adrenalectomized (ADX + cort) and T4-replaced thyroidectomized (TDX + T4) rats after a 7-day i.c.v. saline, SHU9119 (24 nmol/d) and MTII (1 nmol/d) infusion. Data are presented as mean ± SEM of 6–10 animals per group. $^{*}P<0.05$; $^{**}P<0.01$ *vs.* saline; Two-way ANOVA, Bonferroni *post hoc*-test.

in saline- and SHU-treated TDX rats, while MTII-infused TDX rats had decreased T3 levels (2-way ANOVA: significant interaction term $p_{\text{treatment x surgery}} = 0.046$, $p<0.05$ saline *vs.* MTII, Fig. 1E). TRH gene expression in the PVN was neither significantly affected by changes in MCR activation, nor by CORT or T4 replacement (Fig. 1F).

3.2. Effect of chronic i.c.v. SHU9119 or MTII infusion in SO, ADX and TDX rats on body weight gain and food intake

We have shown previously that a 7-day i.c.v. SHU9119 infusion increased body weight in male rats, and a 7-day i.c.v. MTII infusion decreased body weight [3]. These effects were confirmed here in SO rats and were also observed in ADX and TDX rats (Fig. 2A). Changes in body mass due to SHU9119-induced blockade or MTII-induced activation of the CNS melanocortin system in ADX and TDX male rats did not differ compared to SO rats. Increased body weight in SHU9119-infused rats was concordant with significantly increased food intake in SO, ADX and TDX rats ($P<0.001$) (Fig. 2B). Chronic i.c.v. MTII treatment significantly decreased food intake in SO and TDX rats ($P<0.001$), but not in ADX rats (Fig. 2B).

3.3. Effect of chronic i.c.v. SHU9119 or MTII infusion in SO, ADX and TDX rats on plasma levels of TG, FFA, leptin and insulin

We have reported earlier that the SHU9119-induced increase in body weight of normal rats was accompanied by increased TG synthesis and content in epididymal white adipose tissue (eWAT) [3]. Increased lipogenic activity in WAT may require increased transport of lipids from the liver to WAT and increased TG uptake for lipid accumulation. We therefore analyzed the levels of plasma lipid parameters (Fig. 3A–B). In keeping with our earlier observation in normal rats [3], chronic i.c.v. treatment of SO and ADX rats with either SHU9119 or MTII did not change plasma FFA levels, although MTII treatment increased FFA in TDX rats (Fig. 3A). Triglyceride levels were significantly decreased in SO ($p<0.01$) and ADX rats ($p<0.05$), but not in TDX rats treated with SHU (Fig. 3B). MTII did not

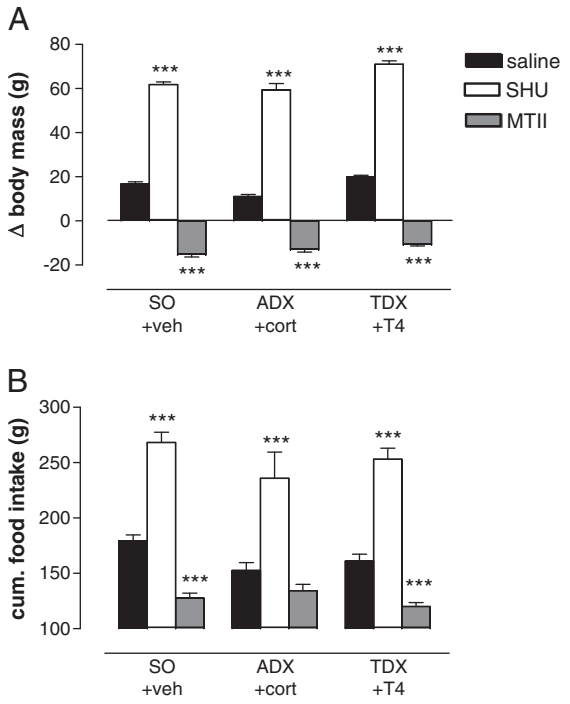

Fig. 2. Effect of a 7-day i.c.v. SHU9119 (24 nmol/d) and MTII (1 nmol/d) infusion in vehicle-treated sham-operated (SO + veh), CORT-replaced adrenalectomized (ADX + cort) and T4-replaced thyroidectomized (TDX + T4) rats on body weight gain (A) and cumulative food intake (B). Data are presented as mean ± SEM of 6–10 animals per group. ***$P<0.001$ *vs.* saline. Two-way ANOVA, Bonferroni *post hoc*-test.

significantly affect TG levels in any of the groups. Correlating with changes in body mass, leptin levels increased with chronic central MC3/4R blockade by i.c.v. SHU9119 treatment in all gland/sham-surgery groups (Fig. 3C). On the contrary, chronic central MC3/4R activation by i.c.v. MTII treatment decreased plasma leptin levels in all gland/sham-surgery groups, while the decrease was only significant for the SO and TDX rats. Consistent with earlier reports [3], plasma insulin levels were significantly increased by chronic CNS-MC3/4R blockade and not affected by CNS-MC3/4R activation in any of the gland/sham-surgery groups (Fig. 3D). Therefore, as compared to SO rats, the pattern of leptin and insulin levels was unaffected by thyroidectomy or adrenalectomy in the presence of basal hormone replacement.

3.4. Effect of chronic i.c.v. SHU9119 or MTII infusion in SO, ADX and TDX rats on epididymal fat pad mass and expression levels of lipogenic enzymes in WAT

To evaluate whether the lipogenic/lipolytic effects of MC3/4R inhibition/stimulation depend on physiologically variable CORT and T4 levels, we measured lipid accumulation in WAT. The mass of the epididymal fat pad significantly increased with the chronic blockade of central MC3/4R by i.c.v. SHU9119 treatment in SO, ADX and TDX rats (Fig. 4A). Therefore, neither adrenalectomy nor thyroidectomy with basal substitutive supplementation influenced the melanocortinergic effect on epididymal fat pad mass. To determine if changes in lipid accumulation in WAT correlated with changes in lipogenic enzyme expression, we quantified the mRNA expression of several enzymes involved in lipid metabolism. Two-Way ANOVA analysis indicates that chronic blockade of central MC3/4R by 7-day i.c.v. infusion of SHU9119 significantly increased the mRNA expression of acetyl-CoA carboxylase α (ACCα) in SO and TDX rats, but not in ADX animals (Fig. 4B), while it increased the mRNA expression of fatty acid synthase (FAS) in the SO and ADX rats, but not in the TDX rats (Fig. 4C). SHU9119 treatment increased stearoyl-CoA desaturase-1 (SCD-1) mRNA expression in all gland/sham-surgery groups (Fig. 4D). In addition, regardless of gland/sham-surgery type, lipoprotein lipase (LPL) mRNA expression was also increased by chronic SHU9119 treatment (Fig. 4E). I.c.v. melanocortin activation by MTII did not significantly affect the expression of the investigated enzymes involved in lipogenesis or lipid uptake (Fig. 4B–E).

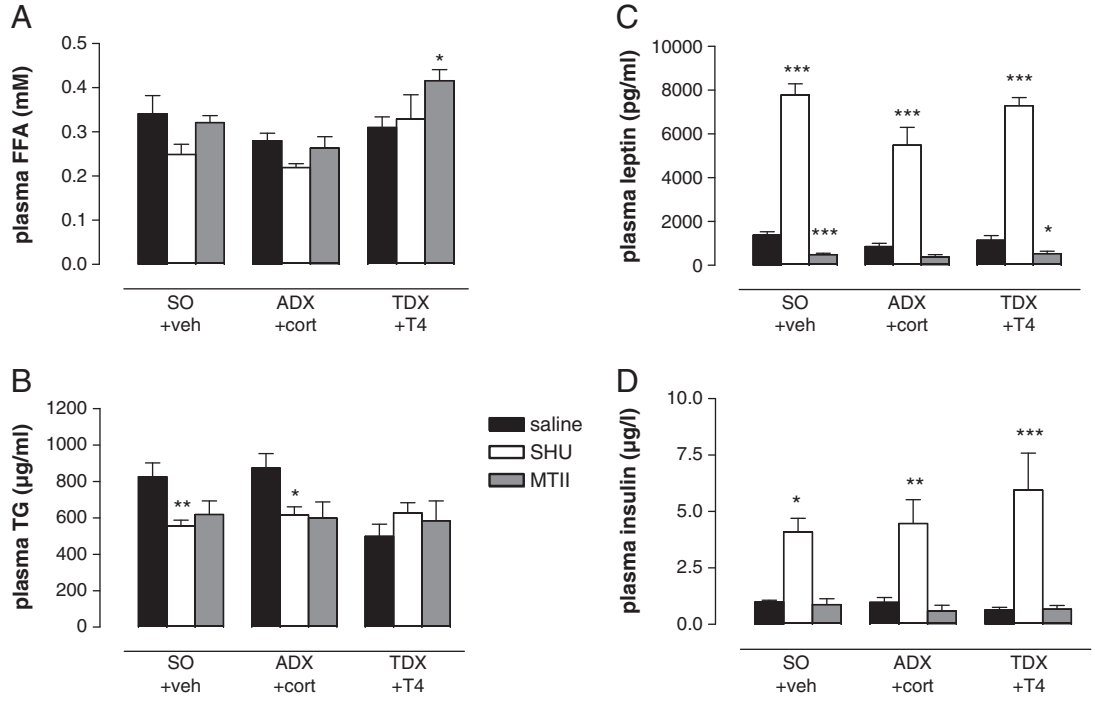

Fig. 3. Effect of a 7-day i.c.v. SHU9119 (24 nmol/d) and MTII (1 nmol/d) infusion in vehicle-treated sham-operated (SO + veh), CORT-replaced adrenalectomized (ADX + cort) and T4-replaced thyroidectomized (TDX + T4) rats on plasma levels of free fatty acids (FFA) (A), triglycerides (TG) (B), leptin (C), and insulin (D). Data are presented as mean ± SEM of 6–10 animals per group. *$P<0.05$; **$P<0.01$; ***$P<0.001$ *vs.* saline. Two-way ANOVA, Bonferroni *post hoc*-test.

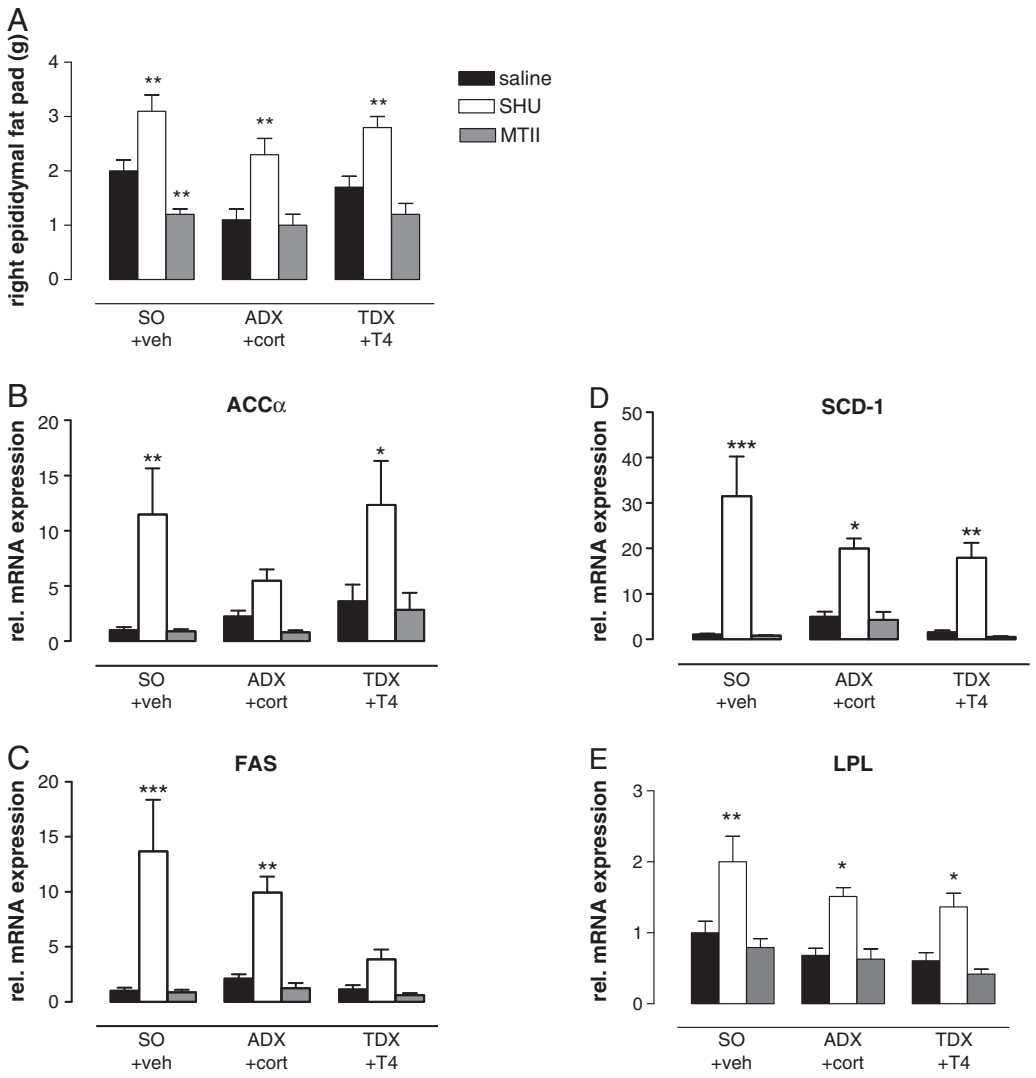

Fig. 4. Effect of a 7-day i.c.v. SHU9119 (24 nmol/d) and MTII (1 nmol/d) infusion in vehicle-treated sham-operated (SO + veh), CORT-replaced adrenalectomized (ADX + cort) and T4-replaced thyroidectomized (TDX + T4) rats on epididymal fat pad mass (A) and epididymal white adipose tissue mRNA expression of acetyl-CoA carboxylase α (ACCα) (B), fatty acid synthase (FAS) (C), stearoyl-CoA desaturase-1 (SCD-1) (D) and lipoprotein lipase (LPL) (E). Values were normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT). mRNA levels were calculated relative to saline-treated rats within the sham-operated group. Data are presented as mean ± SEM of 6–10 animals per group. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *vs.* saline. Two-way ANOVA, Bonferroni *post hoc*-test.

3.5. Effect of chronic i.c.v. SHU9119 or MTII infusion in SO, ADX and TDX rats on hepatic TG content and expression levels of lipid metabolizing enzymes in the liver

The liver is a major lipid-metabolizing organ in the body. We therefore speculated that liver lipid accumulation might be additionally affected by changes in central melanocortinergic activity similar to observed changes in WAT. Chronic blockade of central MC3/4R by i.c.v. SHU9119 increased hepatic TG content in SO and TDX rats, but not in ADX rats (Fig. 5A), MTII treatment, however, decreased liver TG content only in SO rats, and was ineffective in ADX and TDX rats. We next determined the hepatic mRNA expression of lipogenic enzymes and found that although chronic i.c.v. SHU9119 or MTII treatment did not modify the expression of ACCα mRNA (Fig. 5B), SHU9119 significantly increased the mRNA expression of FAS and SCD-1 in SO and TDX rats, but not in ADX rats (Fig. 5C–D). Taken together with the lack of increased hepatic steatosis in SHU9119 treated ADX rats (Fig. 5A), these gene expression data suggest that hepatic de-novo lipogenesis induced by blockade of the central melanocortin receptors requires a fully functional HPA axis. Carnitine-palmitoyl transferase-1 (CPT-1) mRNA expression was significantly altered by the i.c.v. infusion of MCR ligands ($P<0.001$). However, the *post-hoc* analysis only detected a statistically significant decrease of SHU9119 on CPT-1 gene expression in TDX rats (Fig. 5E).

4. Discussion

The autonomic nervous system plays a critical role in the transmission of the efferent information from the central melanocortin system to peripheral tissues [3]. However, the CNS melanocortin system also interacts with the HPA and HPT axes [15–18,20], two major endocrine systems with profound effects on the control of metabolic homeostasis. Here we tested whether the neuroendocrine hypothalamo-pituitary adrenal and thyroid axes mediate some of the effects of the melanocortin system on feeding and peripheral lipid metabolism. Our data indicate that the effect on feeding and adiposity induced by the pharmacological manipulation of the central melanocortin system in adult rats does not require neuroendocrine regulation of the HPA or HPT axes activity. However, the triglyceride accumulation and induction of lipogenic enzymes' gene expression in the liver observed upon blockade of the central melanocortin receptors requires a fully functional HPA axis. Importantly, these data

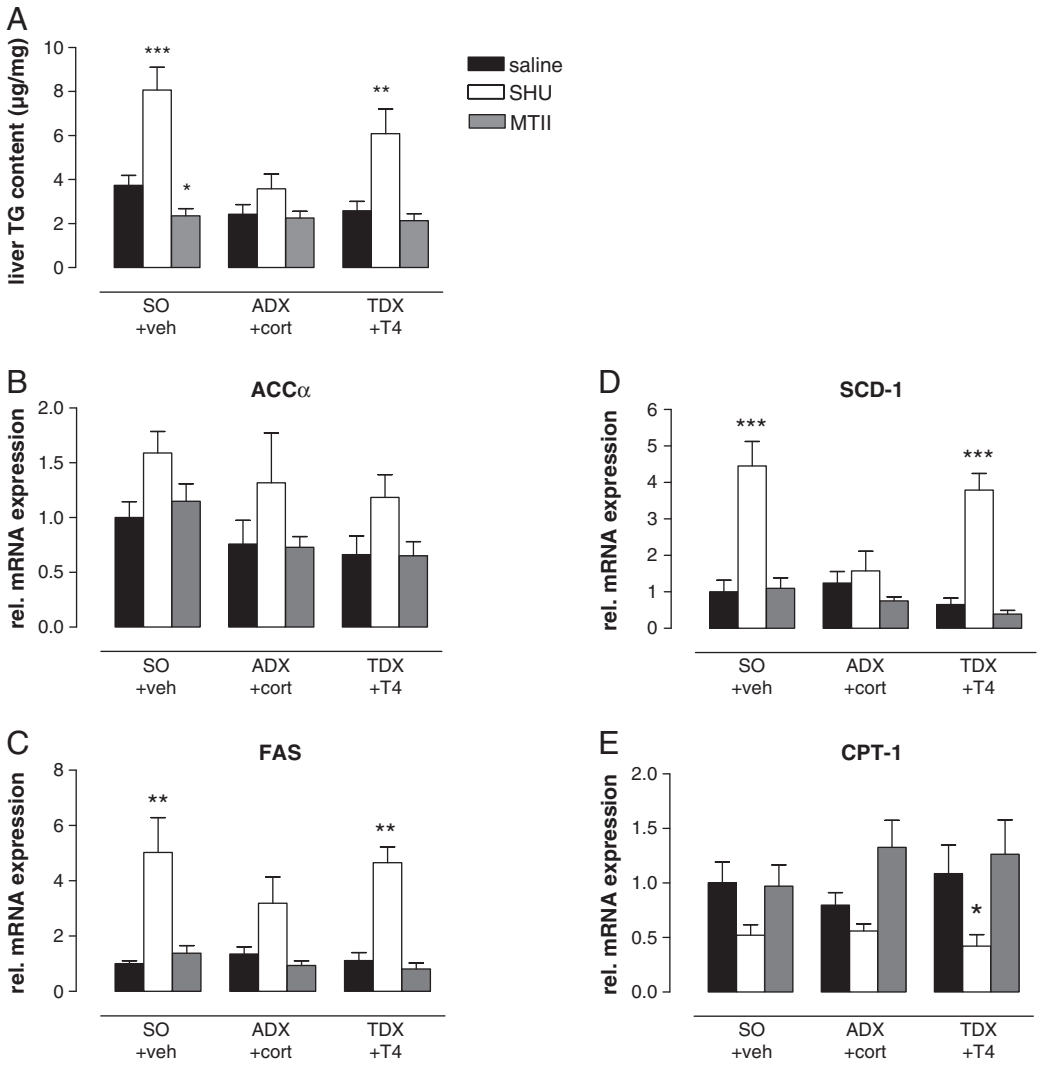

Fig. 5. Effect of a 7-day i.c.v. SHU9119 (24 nmol/d) and MTII (1 nmol/d) infusion in vehicle-treated sham-operated (SO + veh), CORT-replaced adrenalectomized (ADX + cort) and T4-replaced thyroidectomized (TDX + T4) rats on TG content in liver (A) and on liver mRNA expression of acetyl-CoA carboxylase α (ACCα) (B), fatty acid synthase (FAS) (C), stearoyl-CoA desaturase-1 (SCD-1) (D) and carnitine palmitoyltransferase 1 (CPT-1) (E). Values were normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT). mRNA levels were calculated relative to saline-treated rats within the sham-operated group. Data are presented as mean ± SEM of 6–10 animals per group. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *vs.* saline. Two-way ANOVA, Bonferroni *post hoc*-test.

illustrate how the CNS can control a specific metabolic process, such as *de novo* lipogenesis, simultaneously, but tissue-specifically in several peripheral tissues.

The CNS melanocortin system interacts with the HPA axis in the hypothalamic PVN and can modulate its activity. MC4R have been found in CRH neurons of the PVN [15]. Decreased activity of the CNS melanocortin system in leptin deficiency leads to massively increased systemic CORT levels, whereas i.c.v. infusion of specific melanocortin receptor agonists decreases CORT secretion in *ob*/*ob* mice [21]. In addition, in POMC$^{-/-}$ mice that exhibit increased food efficiency, decreased metabolic rate and increased body weight and fat pad weights, the pharmacological or the transgenic replacement of CORT exacerbates the obese phenotype. This implies that CORT can potentiate the obese phenotype originally induced by genetic (POMC$^{-/-}$ mice) [22] or pharmacologic (SHU treatment) inactivation of MC3/4R.

The melanocortin system also interacts with the HPT axis at the hypothalamic level. α-MSH and AgRP-containing nerve terminals innervate TRH neurons in the PVN [16,23] that co-express MC4R [20,24]. There is experimental evidence suggesting that the increase in melanocortin system activity leads to increased HPT axis activation. For instance, i.c.v. infusion of α-MSH increases plasma TSH in fasted rats, infusion of AgRP directly into the PVN decreases the circulating levels of TSH and T4 [25] and reduces TRH gene expression in the PVN [26]. Furthermore, T3 was reported to exert a negative feedback on both MC4R and TRH gene expression by binding on specific thyroid hormone responsive elements in the promoter region of these two genes [27].

Based on the experimental evidence listed above, we predicted that the pharmacological manipulation of the central melanocortin receptors would result in changes in the circulating levels of CORT and T4 when compared with the saline control group. SHU9119 or MT-II elicited the expected effect on feeding and body weight (Fig. 2A, B), although the i.c.v. treatments did not change significantly the circulating levels of CORT or T4 in the sham-operated rats (Fig. 1A, D) or the hypothalamic CRH or TRH gene expression (Fig. 1 C, F). In contrast with the somewhat stable T3 levels [28], corticosterone in rats circulates in a circadian pattern with highest levels at the end of the light phase [29,30]. Given that we only assessed the baseline hormonal levels at one time point at the end of the study, we cannot rule out the possibility that the manipulation of the central MC system could affect the daily circulating patterns of corticosterone and T3 by regulating the activity of the HPA and HPT axes, respectively.

An important limitation intrinsic to our experimental design relates to the combined removal of the endocrine gland plus exogenous

hormonal replacement. While this approach provides the desired lack of regulation of the HPA and HPT axes in the surgical groups, there is also a lack of the normal endogenous pattern of secretion of CORT or thyroid hormones [28], patterns that are likely preserved in the Sham-operated rats. Thus, the absence of a normal rhythm of glucocorticoid or thyroid hormone action in the ADX and TDX rats, in contrast to the control Sham groups, must be considered in the interpretation of these results.

The replacement of CORT in ADX rats resulted in similar levels of circulating CORT in comparison with SO and TDX rats. The increased ACTH levels found in ADX rats (Fig. 1B) suggest that the CORT replacement of only a single daily dose was insufficient to maintain baseline corticotropic activity in the pituitary and presumably also to maintain a physiological circadian profile. However, CRH mRNA levels in the ADX groups did not differ significantly from SO and TDX rats (Fig. 1C). Furthermore, we observed similar feeding between ADX and SO or TDX rats, as well as similar hyperphagia in response to the blockade of the central melanocortin receptors with SHU9119 (Fig. 2B). It was reported elsewhere that adrenalectomy in rats impaired both feeding and hyperphagic response to i.c.v. administration of the MCR antagonist AgRP which was prevented by CORT replacement [31]. Therefore our data suggest that the CORT replacement achieved in our experiments was sufficient to maintain the physiological regulation of food intake.

At the administered dose, T4 replacement of saline-infused TDX rats resulted in elevated T4 with normal T3 levels relative to the saline-infused SO group. T4-replaced TDX rats with i.c.v. SHU9119 infusion showed similar T4 and T3 levels compared to saline infused TDX rats, whereas MTII treatment decreased plasma T4 and T3 levels by 56 and 23%, respectively in T4-replaced TDX rats. This implies that the increased metabolic rate observed in centrally MTII-treated rodents [32] might be associated with increased T4 to T3 conversion and increased T3 action or degradation. The specific mechanism whereby the CNS–MC system might modify plasma thyroid hormone levels is currently unknown. A potential candidate would be the regulation of deiodinase (D1 to D3) activity in specific tissues such as the liver, kidney or brown adipose tissue. Characterization of the tissue-specific activity of these enzymes or changes in plasma levels of T3 and T4 in mice with specific deletion of deiodinase expression would provide evidence of the role of the CNS–MCR in the control circulating levels of thyroid hormones. Nevertheless, under the present experimental conditions, the fact that in T4-replaced TDX rats, SHU and MTII-related changes in body weight, food intake and gene expression in WAT or liver were comparable to the effects observed in SHU and MTII-treated SO and ADX rats implies that the SNS mediation of changes in melanocortinergic activity is sufficient to influence peripheral lipid metabolism in states of impaired HPT function.

The 7-day i.c.v. infusion of SHU9119 induced hyperphagia in ADX and TDX rats similar to SO rats. Accordingly, neither ADX nor TDX affected changes in body weight induced by manipulation of central melanocortin receptor activity when compared to SO rats.

The absence of fully functional HPA or HPT axis did not modify the effects exerted by the CNS melanocortin system on the regulation of insulin or leptin secretion. Spinedi and Gaillard have reported that circulating leptin levels decrease in ADX rats and are restored with CORT replacement [33]. In our experiments, leptin levels did not differ significantly between saline-infused SO and ADX rats, indicating that CORT substitution was sufficient to maintain normal leptin levels. The blockade of the melanocortin receptors with SHU9119 induced a similar increase in leptin levels in sham-operated, ADX and TDX rats, suggesting that the increase in leptin concentration is independent of the action of the melanocortin system on the HPA or HPT axis, but likely dependent on the changes in fat mass.

We have previously demonstrated that the blockade of the CNS melanocortin receptors with SHU9119 leads to adiposity by increasing the *de novo* lipogenesis in WAT, an increase that is independent of the changes in caloric intake [3]. Here we found that i.c.v. SHU increased epididymal fat pad weight to the same extent in SO, CORT-replaced ADX and T4-replaced TDX rats (Fig. 4A). Overall, this increase in fat pad weight correlated with the increase in the gene expression of lipogenic enzymes in eWAT in the three surgical groups. Altogether, these results suggest that increased adiposity induced by CNS melanocortin blockade does not essentially depend on functional HPA or HPT axes.

The similar hepatic triglyceride content and gene expression between the SO and ADX saline control groups suggests that the CORT replacement was sufficient to maintain hepatic lipid metabolism at baseline levels. These data are in agreement with previous reports, showing that CORT replacement is sufficient to maintain hepatic triglyceride levels [34]. Furthermore, ADX rats with CORT replacement develop hepatic steatosis when feeding a high sucrose/high fat diet [34]. Interestingly, the absence of a fully functional HPA axis prevented excessive triacylglyceride storage in the liver induced by i.c.v. SHU9119, despite the increased nutrient supply associated with the hyperphagia (Fig. 5A).

A deficient triacylglyceride storage in the liver of i.c.v. SHU9119-treated ADX rats is supported by the lack of significant up-regulation of the gene expression of lipogenic enzymes such as SCD-1 in comparison with the effect observed in SO and TDX rats (Fig. 5C, D). We have previously shown that similarly to what is observed in WAT, i.c.v. SHU increases hepatic SCD-1 gene expression independently of food intake [3]. Therefore, the lack of lipid accumulation and lack of induction of lipogenesis in the liver of i.c.v. SHU ADX rats, despite hyperphagia and activation of lipogenesis in WAT, suggests that the CNS melanocortin system control of hepatic lipogenesis requires a fully functional HPA axis.

The specific mechanisms by which the blockade of the CNS melanocortin system can regulate WAT but not hepatic lipogenesis in CORT-replaced ADX rats are currently unknown. It is possible that control of hepatic triglyceride metabolism requires the physiological daily fluctuations in CORT plasma levels [35], absent in our ADX rats. Alternatively, other adrenal hormonal signals may be required for the CNS–MC system control of hepatic lipogenesis, although such a possibility remains to be proven.

In summary, we have demonstrated that a lack of physiological regulation of circulating corticosterone and thyroid hormones is not sufficient to disrupt CNS melanocortin effects on food intake or body weight, while a fully functional HPA axis seems to play a distinct role for the regulation of peripheral lipid metabolism. Thus, lipid accumulation in the liver, but not in WAT, in the presence of increased nutrient supply due to MC3/4R blockade-induced hyperphagia, requires a functional HPA axis.

Acknowledgments

The skillful technical assistance of Kathrin Warnke is gratefully acknowledged.

Grants: This work was supported by the Sixth EU Research Framework Program (contract LSHM-CT-2003-503041, to M.H. Tschöp and F. Rohner-Jeanrenaud), by NIH grant 5R01DK077975 (to M.H. Tschöp) and by grant No. 310000-120147/1 of the Swiss National Science Foundation, Bern, Switzerland (to F. Rohner-Jeanrenaud).

References

[1] Flegal KM, Graubard BI, Williamson DF, Gail MH. Excess deaths associated with underweight, overweight, and obesity. JAMA 2005;293:1861–7.
[2] Pischon T, Boeing H, Hoffmann K, Bergmann M, Schulze MB, Overvad K, et al. General and abdominal adiposity and risk of death in Europe. N Engl J Med 2008;359:2105–20.
[3] Nogueiras R, Wiedmer P, Perez-Tilve D, Veyrat-Durebex C, Keogh JM, Sutton GM, et al. The central melanocortin system directly controls peripheral lipid metabolism. J Clin Invest 2007;117:3475–88.

[4] Buettner C, Muse ED, Cheng A, Chen L, Scherer T, Pocai A, et al. Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. Nat Med 2008;14:667–75.
[5] Koch L, Wunderlich FT, Seibler J, Konner AC, Hampel B, Irlenbusch S, et al. Central insulin action regulates peripheral glucose and fat metabolism in mice. J Clin Invest 2008;118:2132–47.
[6] Nogueiras R, Perez-Tilve D, Veyrat-Durebex C, Morgan DA, Varela L, Haynes WG, et al. Direct control of peripheral lipid deposition by CNS GLP-1 receptor signaling is mediated by the sympathetic nervous system and blunted in diet-induced obesity. J Neurosci 2009;29:5916–25.
[7] Benoit SC, Air EL, Coolen LM, Strauss R, Jackman A, Clegg DJ, et al. The catabolic action of insulin in the brain is mediated by melanocortins. J Neurosci 2002;22:9048–52.
[8] Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, et al. Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes 1997;46:2119–23.
[9] Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 2003;37:649–61.
[10] Gallardo N, Bonzon-Kulichenko E, Fernandez-Agullo T, Molto E, Gomez-Alonso S, Blanco P, et al. Tissue-specific effects of central leptin on the expression of genes involved in lipid metabolism in liver and white adipose tissue. Endocrinology 2007;148:5604–10.
[11] Theander-Carrillo C, Wiedmer P, Cettour-Rose P, Nogueiras R, Perez-Tilve D, Pfluger P, et al. Ghrelin action in the brain controls adipocyte metabolism. J Clin Invest 2006;116:1983–93.
[12] Nogueiras R, Wilson H, Rohner-Jeanrenaud F, Tschop MH. Central nervous system regulation of adipocyte metabolism. Regul Pept 2008;149:26–31.
[13] Bartness TJ, Song CK. Thematic review series: adipocyte biology. Sympathetic and sensory innervation of white adipose tissue. J Lipid Res 2007;48:1655–72.
[14] Brito MN, Brito NA, Baro DJ, Song CK, Bartness TJ. Differential activation of the sympathetic innervation of adipose tissues by melanocortin receptor stimulation. Endocrinology 2007;148:5339–47.
[15] Lu XY, Barsh GS, Akil H, Watson SJ. Interaction between alpha-melanocyte-stimulating hormone and corticotropin-releasing hormone in the regulation of feeding and hypothalamo-pituitary-adrenal responses. J Neurosci 2003;23:7863–72.
[16] Legradi G, Lechan RM. Agouti-related protein containing nerve terminals innervate thyrotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. Endocrinology 1999;140:3643–52.
[17] Mihaly E, Fekete C, Tatro JB, Liposits Z, Stopa EG, Lechan RM. Hypophysiotropic thyrotropin-releasing hormone-synthesizing neurons in the human hypothalamus are innervated by neuropeptide Y, agouti-related protein, and alpha-melanocyte-stimulating hormone. J Clin Endocrinol Metab 2000;85:2596–603.
[18] Lechan RM, Fekete C. Role of melanocortin signaling in the regulation of the hypothalamic-pituitary-thyroid (HPT) axis. Peptides 2006;27:310–25.
[19] Wortley KE, Anderson KD, Yasenchak J, Murphy A, Valenzuela D, Diano S, et al. Agouti-related protein-deficient mice display an age-related lean phenotype. Cell Metab 2005;2:421–7.
[20] Liu H, Kishi T, Roseberry AG, Cai X, Lee CE, Montez JM, et al. Transgenic mice expressing green fluorescent protein under the control of the melanocortin-4 receptor promoter. J Neurosci 2003;23:7143–54.
[21] Edwardson JA, Hough CAM. The pituitary-adrenal system of the genetically obese (ob/ob) mouse. J Endocrinol 1975;65:99–107.
[22] Smart JL, Tolle V, Low MJ. Glucocorticoids exacerbate obesity and insulin resistance in neuron-specific proopiomelanocortin-deficient mice. J Clin Invest 2006;116:495–505.
[23] Fekete C, Legradi G, Mihaly E, Huang Q-H, Tatro JB, Rand WM, et al. Alpha-melanocyte-stimulating hormone is contained in nerve terminals innervating thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and prevents fasting-induced suppression of prothyrotropin-releasing hormone gene expression. J Neurosci 2000;20:1550–8.
[24] Harris M, Aschkenasi C, Elias CF, Chandrankunnel A, Nillni EA, BjÃ¸rbÃ¦k C, et al. Transcriptional regulation of the thyrotropin-releasing hormone gene by leptin and melanocortin signaling. J Clin Invest 2001;107:111–20.
[25] Kim MS, Small CJ, Stanley SA, Morgan DG, Seal LJ, Kong WM, et al. The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. J Clin Invest 2000;105:1005–11.
[26] Fekete C, Sarkar S, Rand WM, Harney JW, Emerson CH, Bianco AC, et al. Agouti-related protein (AGRP) has a central inhibitory action on the hypothalamic-pituitary-thyroid (HPT) axis; comparisons between the effect of AGRP and neuropeptide Y on energy homeostasis and the HPT axis. Endocrinology 2002;143:3846–53.
[27] Decherf S, Seugnet I, Kouidhi S, Lopez-Juarez A, Clerget-Froidevaux MS, Demeneix BA. Thyroid hormone exerts negative feedback on hypothalamic type 4 melanocortin receptor expression. Proc Natl Acad Sci U S A 2010;107:4471–6.
[28] Kalsbeek A, Buijs RM, van Schaik R, Kaptein E, Visser TJ, Doulabi BZ, et al. Daily variations in type II iodothyronine deiodinase activity in the rat brain as controlled by the biological clock. Endocrinology 2005;146:1418–27.
[29] Atkinson HC, Waddell BJ. Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. Endocrinology 1997;138:3842–8.
[30] Leitch MM, Ingram CD, Young AH, McQuade R, Gartside SE. Flattening the corticosterone rhythm attenuates 5-HT1A autoreceptor function in the rat: relevance for depression. Neuropsychopharmacology 2002;28:119–25.
[31] Drazen DL, Wortman MD, Schwartz MW, Clegg DJ, van Dijk G, Woods SC, et al. Adrenalectomy alters the sensitivity of the central nervous system melanocortin system. Diabetes 2003;52:2928–34.
[32] Pierroz DD, Ziotopoulou M, Ungsunan L, Moschos S, Flier JS, Mantzoros CS. Effects of acute and chronic administration of the melanocortin agonist MTII in mice with diet-induced obesity. Diabetes 2002;51:1337–45.
[33] Spinedi E, Gaillard RC. A regulatory loop between the hypothalamo-pituitary-adrenal (HPA) axis and circulating leptin: a physiological role of ACTH. Endocrinology 1998;139:4016–20.
[34] Mantha L, Palacios E, Deshaies Y. Modulation of triglyceride metabolism by glucocorticoids in diet-induced obesity. Am J Physiol 1999;277:R455–64.
[35] De Boer SF, Van Der Gugten J. Daily variations in plasma noradrenaline, adrenaline and corticosterone concentrations in rats. Physiol Behav 1987;40:323–8.