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Treadmill training or caloric restriction delays aging-associated increase in urocortin 2-induced hyperthermia in middle-aged male Wistar rats

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Age-related obesity is a growing healthcare burden. Effectiveness of body weight reducing mediators changes with aging and these alterations may contribute to aging obesity. Our previous results suggested a potential contributions of urocortin 2 (UCN2) to aging obesity and age-related cachexia. Lifestyle interventions, such as caloric restriction or physical activity can improve body weight and body composition. Previous observations suggest that lifestyle interventions may also influence age-associated regulatory changes in energy balance. We aimed to study the effects of a 12-week treadmill training or caloric restriction on the central hypermetabolic responsiveness to UCN2 in middle-aged 6- and 12-month old male Wistar rats. Interventions started at age 3- or 9-months. Following the interventions, acute hypermetabolic/hyperthermic responses were tested upon intracerebroventricular injections of UCN2. Both the training and the caloric restriction lead to weight loss and training led to favorable body composition changes. UCN2-induced hypermetabolism/hyperthermia was diminished by both interventions in both age-groups. The *Ucn2* mRNA expression detected by RNAscope in situ hybridization in the hypothalamic paraventricular nucleus was also decreased by both interventions. UCN2-induced hypermetabolism/hyperthermia in the trained and caloric restricted middle-aged groups resembled those of young adult 3-month rats. Lifestyle interventions appear to delay age-related regulatory alterations of energy balance.

Keywords Urocortin 2, Obesity, Aging, Body temperature, Treadmill training, Caloric restriction

Obesity is a growing burden to the healthcare system worldwide. In 2022 16% of the population was obese and about 46% overweight[1]. Especially middle-aged and aging populations show high prevalence[2]. Obesity is a major risk factor for diabetes mellitus, fatty liver disease, cardiovascular diseases such as hypertension, myocardial infarction and stroke, cancer, osteoporosis, osteoarthrosis, Alzheimer's disease, depression, etc., and it decreases the quality of life[3]. Moreover, there are similarities between obesity and aging, such as higher glucose and insulin levels, decreased reproduction, shortening of telomers in the liver, higher mammalian target for rapamycin (mTOR) acitivity in both conditions[4]. Additionally, obesity accelerates aging[4]. Therefore, the prevention of obesity should be very important in the next few decades.

Previous studies indicate that age-related alterations in the central regulation of energy balance involving leptin, neuropeptide Y, melanocortins and corticotropins among other peptide mediators also contribute to the development of aging obesity[5–9]. The corticotropin family, via activation of its 2 types of receptors, can regulate the activation of the pituitary-adrenal axis, increase the heart rate and the body temperature. It also affects the anxiogenic and depressive behavior[10]. Anorexigenic, heart rate increasing effects are mediated by type 2 receptors of corticotropin releasing factor (CRF2R)[10]. A member of the corticotropin family, urocortin 2

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(UCN2) is known by its selective binding to the CRF2R. Our previous study demonstrated characteristic age-dependent changes in the effects of UCN2 that may have a role in the development of middle-aged obesity[9]. Our findings showed that the anorexigenic effects of UCN2 along with the hypothalamic *Ucn2* mRNA expression in the hypothalamic paraventricular nucleus (PVN) decreased in the middle-aged group[9].

Lifestyle factors, such as overeating, lack of physical activity and low energy expenditure also have an effect on the development of obesity[3]. Previous studies showed that physical activity, e.g. treadmill training can influence the release of different mediators. Treadmill training decreased the secretion of leptin[11]. Moreover, corticotropin releasing factor (CRF) another member of the corticotropin family might have a role in the development of training-induced anorexia[12]. Other studies reported that caloric restriction (CR) could influence the age-related changes in the anorexigenic and hypermetabolic effects of leptin[5]. Both physical training and caloric restriction may influence body composition in an advantageous way[4,13,14].

There is big body of data demonstrating that CR delays aging and even increases the life span[4,15]. Some evidence also indicate that treadmill training may improve the mitochondrial content thus delaying aging even if it did not prolong lifespan in rodents[16].

Based on previous research, it is known that various peptide mediators play an important role in the regulation of energy balance. It has been also demonstrated that age-related changes in the effects of such peptide mediators promote age-associated obesity[5,9]. Previously mentioned animal studies showed that long-term caloric restriction and treadmill training might delay aging[4,15,16]. We hypothesized that lifestyle interventions that reduce obesity such as treadmill training and caloric restriction may improve disadvantageous age-associated alteration in the central regulation of energy balance. They also might have a role on the effects of regulatory peptides, such as UCN2.

Our study is the first to test the effects of transient 12-week interventions of caloric restriction and treadmill training in middle-aged rats on the responsiveness to UCN2 with regard to energy balance.

Results

Effects of lifestyle interventions on food intake, body weight and body composition

In the CR groups daily food intake (FI) was 16 g standard chow per day. This limited FI was significantly lower than that of the *ad libitum* fed (NF) and treadmill training (TR) groups. In the younger 6-month rats one-way ANOVA with Tukey's *post hoc* test demonstrated these differences (CR6 vs. NF6 and TR6: $p < 0.001$). Among the older 12-month-old rats a similar statistical difference was shown (one-way ANOVA with Tukey's *post hoc* test, CR12 vs. NF12 and TR12: $p < 0.001$). Whereas the mean FI of the NF6 and TR6 groups remained similar, in the older age-group, chow consumption of TR12 rats became lower than that of NF12 during the course of the 12-week training (one-way ANOVA with Tukey's *post hoc* test, $p = 0.007$) (Fig. 1, Supplementary Table 8).

With regard to body weights (BW), the initial mean values did not differ either in the 3-month-old or the 9-month-old groups (Supplementary Table 1.). During the 12-week interventions, both the caloric restriction and the training induced weight loss in the CR6, CR12 and TR12 groups (one-way ANOVA with Tukey's *post hoc* test, $p < 0.001$) (Supplementary Table 1.).

The 12–week treadmill training significantly decreased the *post mortem* perirenal fat indicator in the TR12 group compared to that of the NF12 controls (NF12: 1.08 ± 0.07, TR12: 0.69 ± 0.06, one-way ANOVA with Tukey's *post hoc* test: $p = 0.019$). In addition, the training increased the value of the *post mortem* relative muscle indicator as compared with that of the NF12 group (NF12: 0.51 ± 0.04, TR12: 0.72 ± 0.0.4, one-way ANOVA with Tukey's *post hoc* test: $p < 0.001$). The 12-week caloric restriction (CR12) failed to change the perirenal fat indicator, but increased the relative muscle indicator significantly (NF12: 0.51 ± 0.04, CR12: 0.91 ± 0.02, one-way ANOVA with Tukey's *post hoc* test: for retroperitoneal fat $p = 0.23$, for muscles $p < 0.001$). By the end of the intervention there was a significant difference between the mean BW values of the TR12 and NF12 groups (NF12: 651.6 ± 31.99 g; TR12 504.2 ± 7.74 g; CR12 411.0 ± 7.48 g, one-way ANOVA with Tukey's *post hoc* test: $p < 0.001$) (Fig. 2).

Neither intervention changed the epididymal fat indicator.

The impact of lifestyle interventions on the thermoregulatory effects of central urocortin 2 injections in the younger middle-aged 6-month old male Wistar rats

Neither the training nor the caloric restriction changed the resting, baseline core temperature or oxygen consumption in the younger middle-aged groups. In the younger middle-aged 6-month old NF6 and in the treadmill-trained group (TR6), centrally applied UCN2 elicited a significant 420 min-long hyperthermic response as compared with the age- and physical activity-matched pyrogen free saline (PFS)-treated controls (repeated-measures ANOVA: NF6: $F(1,14) = 52.535$, $p < 0.001$; TR6: $F(1,10) = 73.69$, $p < 0.0001$). Similarly, the caloric-restricted younger middle-aged rats (CR6) showed a significant 420 min-long hyperthermic response upon the intracerebroventricular (ICV) injection of UCN2 compared with their PFS-treated controls ($F(1,21) = 12.97$, $p = 0.002$) (Fig. 3, Supplementary Table 4). With regard to the UCN2-induced increase in oxygen consumption, the rise was significant in all groups as compared with their respective PFS-injected controls (repeated-measures ANOVA NF6: $F(1,18) = 11.675$, $p = 0.003$ for 180 min TR6: $F(1,18) = 4.55$, $p = 0.047$ for 160 min, CR6: $F(1,14) = 16.92$, $p = 0.001$ for 180 min) (Fig. 3, Supplementary Table 5).

The UCN2-induced hyperthermia was significantly lower in the trained (TR6) than in the age-matched sedentary (NF6) younger adult group $F(1,15) = 42.59$, $p < 0.0001$). The UCN2-induced rise in oxygen consumption was similar in the NF6 and TR6 animals ($F(1,12) = 0.741$, $p = 0.406$). The UCN2-induced hyperthermia was significantly lower in the caloric-restricted (CR6) than in the age-matched sedentary (NF6) younger adult group $F(1,22) = 21.206$, $p < 0.001$) (Supplementary Table 4). The UCN2-induced rise in oxygen consumption did not differ in the NF6 and CR6 animals ($F(1,16) = 66,87$, $p = 0.084$). Neither UCN2-induced hyperthermia ($F(1,19) = 1.08$, $p = 0.311$ for 180 min, $F(1,19) = 0.049$, $p = 0.827$ for 420 min), nor UCN2-induced

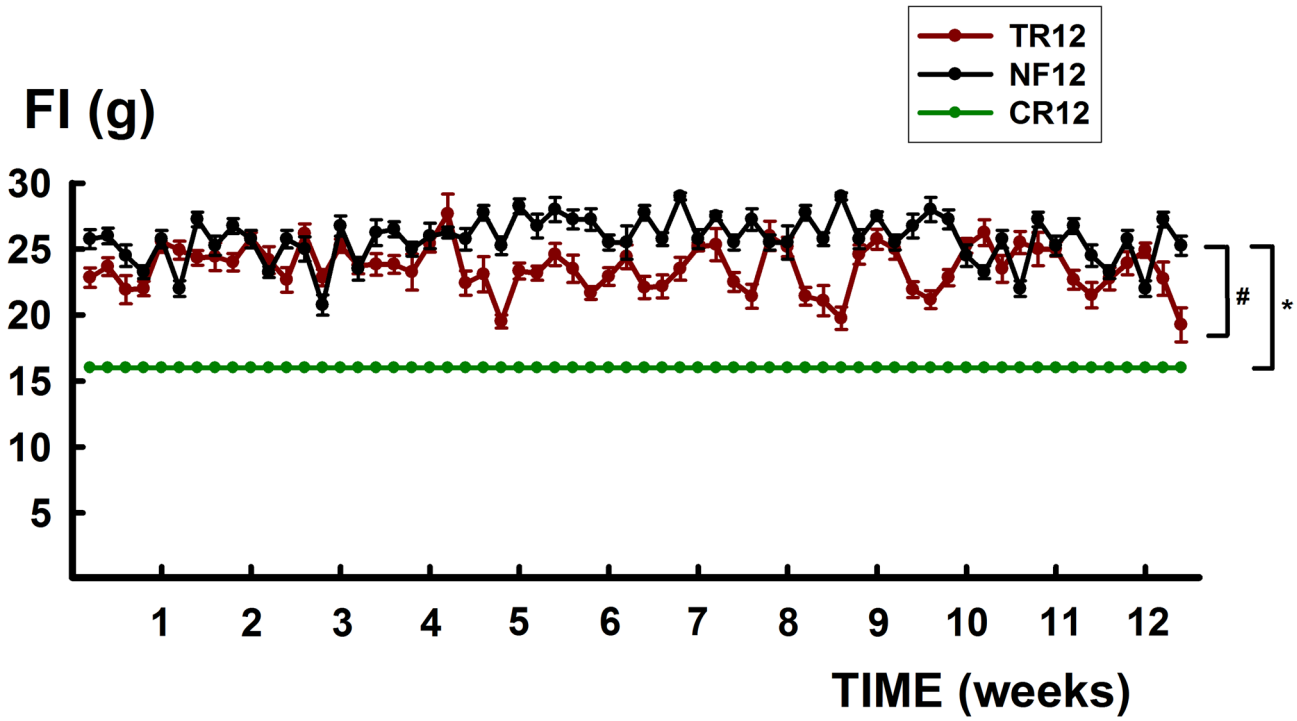

Fig. 1. Daily food intake (FI) values of *ad libitum* fed sedentary (NF), treadmill trained *ad libitum* fed (TR) and caloric restricted sedentary (CR) middle-aged male Wistar rats during a 12-week intervention or observation period starting at 9-months- and ending at 12-months of age. * indicates a significant difference between the CR12 and the NF12 or TR12 animals, # indicates a significant difference between the TR12 and NF12 groups, shown by repeated-measures ANOVA with Tukey's *post hoc* test. Number of animals per group: CR12 $n = 18$, NF12 $n = 16$, TR12 $n = 20$.

hypermetabolism differ ($F(1,16) = 0.277$, $p = 0.606$ for 180 min, $F(1,16) = 0.110$, $p = 0.745$ for 420 min) in the CR6 and TR6 groups (Supplementary Table 5).

Heat loss, indicated by the changes in tail skin temperature increased moderately by the end of the observation period in the NF6 and CR6 groups (repeated-measures ANOVA: NF6: $F(1,15) = 12.053$, $p = 0.003$, CR6: $F(1,17) = 9.907$, $p = 0.006$). Tail skin temperature did not increase upon UCN2 administration in the TR6 animals (repeated-measures ANOVA: TR6: $F(1,12) = 0.21$, $p = 0.886$)(Supplementary Table 2).

The impact of lifestyle interventions on the thermoregulatory effects of central urocortin 2 injections in the older middle-aged 12-month old male Wistar rats

Neither the training nor the caloric restriction changed the resting, baseline core temperature or oxygen consumption in the older middle-aged groups. All older middle-aged 12-month groups showed significant hyperthermic responses following central administration of UCN2. In the NF12 and the treadmill-trained TR12 rats, centrally applied UCN2 induced a significant 420 min-long hyperthermic response as compared with their age- and physical activity-matched PFS-treated controls (repeated-measures ANOVA: NF12: $F(1,10) = 25.807$, $p < 0.001$; TR12: $F(1,29) = 15.37$, $p < 0.001$ for 180 min, $F(1,25) = 10.47$ $p = 0.003$ for 420 min). Similarly, the caloric-restricted older middle-aged rats (CR12) showed also a significant 420 min-long hyperthermic response upon the ICV injection of UCN2 compared with their PFS-treated controls ($F(1,21) = 9.44$, $p = 0.006$ for 180 min, $F(1,14) = 46.62$, $p < 0.001$) (Supplementary Table 6). With regard to UCN2-induced hypermetabolism, in the NF12 group the UCN2-induced increase in the VO_2 remained higher compared to controls for 420 min (repeated-measures ANOVA: $F(1,10) = 7.767$, $p = 0.019$) (Supplementary Table 7). In the TR12 animals VO_2 increased during the first 80 min upon the central UCN2 injection (repeated-measures ANOVA: $F(1,22) = 4.55$, $p = 0.044$). In the CR12 rats, UCN2 induced a 90-min initial rise in VO_2 (repeated-measures ANOVA: $F(1,14) = 4.742$, $p = 0.047$), followed by another rise later from 290 min to 360 min ($F(1,10) = 8.035$, $p = 0.018$) (Fig. 4, Supplementary Table 7).

The UCN2-induced hyperthermia was significantly lower in the trained (TR12) than in the age-matched sedentary (NF12) group ($F(1,24) = 22.00$, $p < 0.0001$). The UCN2-induced rise in oxygen consumption was similar in the NF12 and TR12 animals ($F(1,17) = 3.94$, $p = 0.063$). The UCN2-induced hyperthermia was significantly lower in the caloric restricted (CR12) than in the age-matched sedentary (NF12) group ($F(1,23) = 26.59$, $p < 0.001$). The UCN2-induced rise in oxygen consumption did not differ in the NF12 and CR12 animals ($F(1,17) = 1.42$, $p = 0.254$). Neither UCN2-induced hyperthermia ($F(1,29) = 2.263$, $p = 0.143$ for 180 min, $F(1,22) = 0.0489$, $p = 0.829$ for 420 min), nor UCN2-induced hypermetabolism differ ($F(1,13) = 0.609$, $p = 0.449$ for 180 min, $F(1,13) = 1.47$, $p = 0.247$ for 300 min) in the CR12 and TR12 groups.

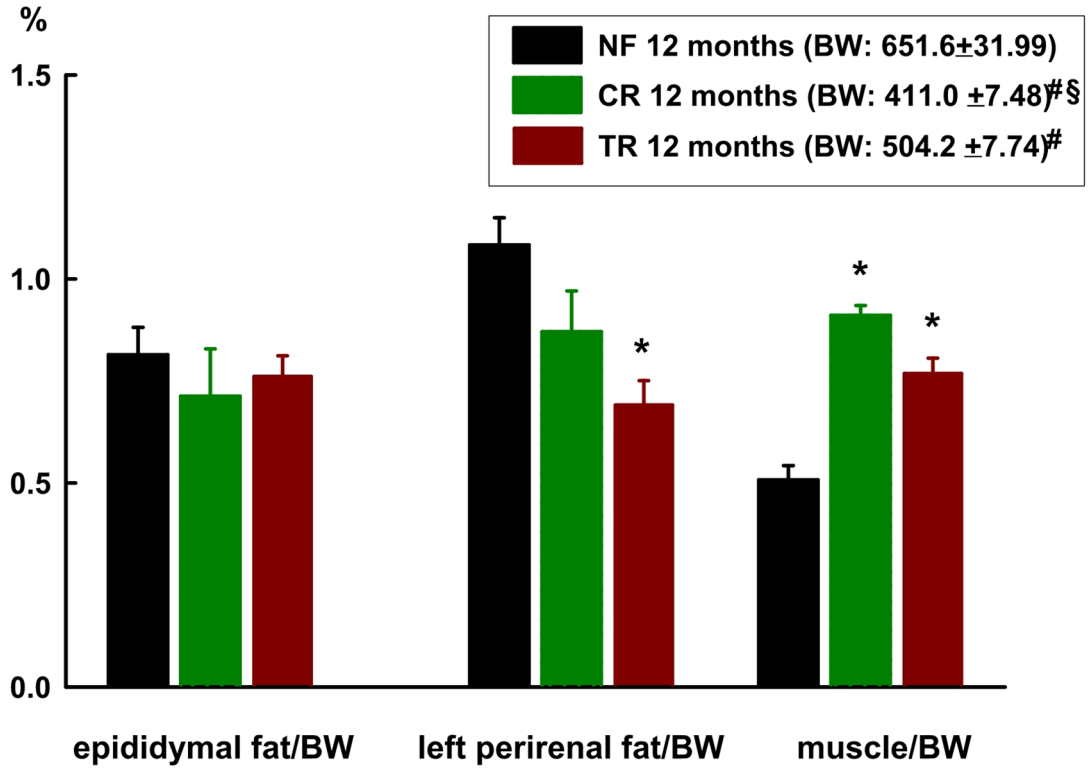

Fig. 2. Relative *post mortem* body composition indicators of intact *ad libitum* fed sedentary (NF12), treadmill trained *ad libitum* fed (TR12) and caloric restricted sedentary (CR12) 12 months old male Wistar rats ($n = 5$/group). Wet weights of epididymal fat pads, left sided perirenal fat mass and – as muscle indicator – the sum of the wet weights of the tibialis anterior muscle, the m. extensor digitorum longus, the soleus muscle and the m. tibialis anterior were measured. Wet weights of the fat and muscle indicators were divided by the body weight (BW) and multiplied by 100. # indicates body weight difference between groups TR12 or CR12 versus NF12, § indicates difference in body weight between groups CR12 *versus* TR12 or NF12 shown by one-way ANOVA with Tukey's *post hoc* test. * indicates differences between TR12 or CR12 *versus* NF12 *post mortem* indicators of perirenal fat and muscle mass.

Heat loss, indicated by the rise in tail skin temperature increased moderately by the end of the observation period in the NF12 and CR12 groups (repeated-measures ANOVA: NF12: $F(1,14) = 22.0583$, $p < 0.001$, CR12: $F(1,13) = 21.0717$, $p < 0.001$). Tail skin temperature did not increase upon UCN2 administration in the TR12 rats (repeated-measures ANOVA: TR12: $F(1,14) = 0.33$, $p = 0.575$) (Supplementary Table 3).

The effects of lifestyle interventions on the hypothalamic expression of urocortin 2 mRNA in the paraventricular nucleus of older middle-aged 12-month old male Wistar rats

Both the 12-week training and the 12-week caloric restriction decreased the expression of *Ucn2* mRNA significantly in the PVN as compared to the control NF12 group (NF12: 23.0 ± 5.13, TR12: 6.9 ± 2.37, CR12: 8.0 ± 0.89 – independent samples Kruskall-Wallis test for non-parametric data: $p = 0.011$ for TR12 vs. NF12, $p = 0.034$ for CR12 vs. NF12). There was no difference between the TR12 and CR12 groups ($p = 0.671$) (Fig. 5).

Discussion

In our study we have tested the effects of lifestyle interventions, i.e. 12-week caloric restriction and 12-week treadmill training in middle-aged male Wistar rats. The 12-month-old older middle-aged rats provide an animal model of age-related obesity. We have measured the FI, body weight (BW), body composition indicators of these animals followed by thermoregulatory tests on the acute central effects of UCN2, a member of the central corticotropin family involved in a wide variety of fields from stress- to food intake regulation and thermogenesis[17]. In addition, we have also tested the *Ucn2* mRNA expression in the paraventricular nucleus of the hypothalamus to identify the changes in the intrinsic activity of this neuropeptide elicited by our lifestyle interventions. The tests of UCN2-induced thermoregulatory responses were also carried out in younger middle-aged, 6-month-old groups, as well.

The 12-week treadmill training surprisingly decreased food intake to a small extent but statistically significantly in the TR12 group (Fig. 1). It is opposed to the expected increase that we predicted because of the increased energy consumption. Our results are in accord with such human observations that described the smallest daily food intake in individuals engaged in light to low moderate physical activity as compared with both sedentary and very active groups[13,18]. As underlying mechanisms it was suggested that exercise decreases the

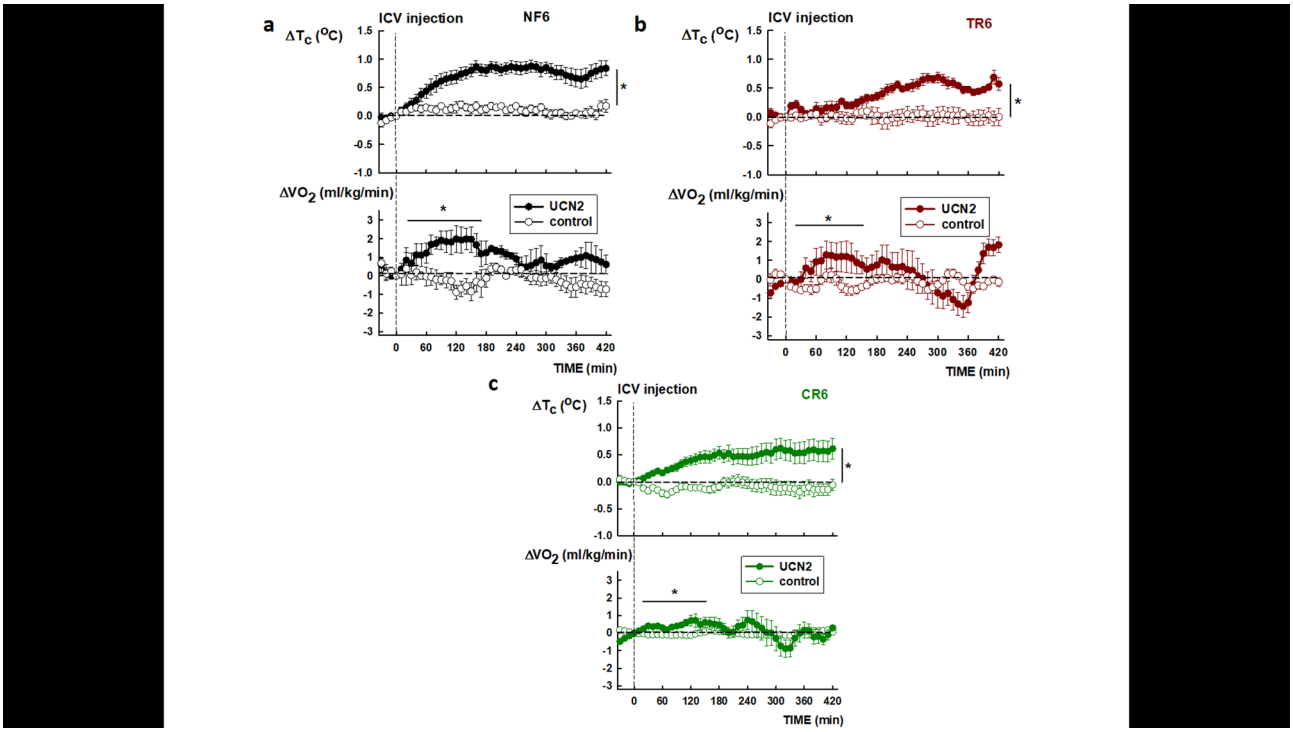

Fig. 3. Changes in core temperature (ΔTc) and oxygen consumption (ΔVO_2) of 6 months old male Wistar rats of different intervention groups following an intracerebroventricular (ICV) injection of urocortin 2 (UCN2). Dark symbols indicate UCN2-treated, empty symbols indicate results of age-matched controls. (Panels a, b, and c show the results obtained in *ad libitum* fed sedentary NF6, *ad libitum* fed treadmill trained TR6 and caloric restricted sedentary CR6 rats, respectively.) Control animals received pyrogen-free saline (PFS). Asterisks indicate significant differences between the UCN2-treated and control animals shown by repeated-measures ANOVA. Number of animals per group: TR6 treated $n = 7$, TR6 control $n = 8$, NF6 treated $n = 8$, NF6 control $n = 8$, CR6 treated $n = 10$, CR6 control $n = 9$.

concentrations of ghrelin and also decreases blood leptin concentration. Exercise also increases the basal blood insulin level and also the postprandial sensitivity to insulin and leptin. Therefore, the energy intake matches the energy expenditure better[13,19,20]. However, long-term the energy intake should increase as a compensation. Previous observations also showed an increase in early morning appetite showing great variations among individuals. On the other hand, another study of the literature on physical activity and food intake reported that very intensive physical training decreased the appetite even more strongly than moderate exercise[21]. In this latter study those participants engaged in very intensive training tended to consume smaller meals[20].

Our findings are in accord with the report of Zhang and Bi[22] that describes decreased FI and weight loss following voluntary wheel-running in male OLETF rats. Their review describes a short-term increase in CRF[12,23] and a training-induced increase in thyrotropin-releasing hormone (TRH) release in the dorsomedial hypothalamus of Sprague-Dawley rats and male OLETF rats along with a suppression of neuropeptide Y release[22,23]. All these changes may contribute to a decrease in FI.

Previous research raised the hypothesis that treadmill training may induce stress in rats that involves activation of the CRF neurons of the PVN resulting in the activation of the hypothalamus-pituitary-adrenocortical (HPA) axis[24,25]. Another study described that treadmill training increased the blood level of norepinephrine and consequently affected the production of adrenocorticotropic hormone (ACTH)[26]. Contrary to these findings, Lalanza and coworkers reported that long-term, 32-week treadmill-training decreased the ACTH level[27].

With regard to corticotropins, previously treadmill training has been reported to induce activation of the corticotropin system of the PVN[24,25]. However, when rats become habituated to chronic, repeated treadmill training, the sensitivity of PVN neurons appear to be adjusted, the serotonin release of the dorsal raphe nucleus rises and the stress-associated depressive-like behavior of the rats improve[28]. Thus, in the long run, the stress-reducing anxiolytic, beneficial effects of the treadmill training emerge.

In our study the 12-week treadmill training decreased the body weight slowly. By the end of the intervention there was a significant difference between the mean BW values of the TR12 and NF12 groups (Fig. 2). The weight loss was much faster and stronger in the CR12 rats.

With regard to *post mortem* body composition indicators, the 12-week training reduced the relative perirenal fat and increased the relative muscle indicator value significantly as compared with the NF12 animals. On the other hand, the 12-week CR did not induce any significant change in the relative value of either fat type (epididymal or perirenal), despite those observations that showed a reduction in the body fat following caloric restriction[4]. The relative value of the muscle indicator increased in the CR12 group as compared with the NF12

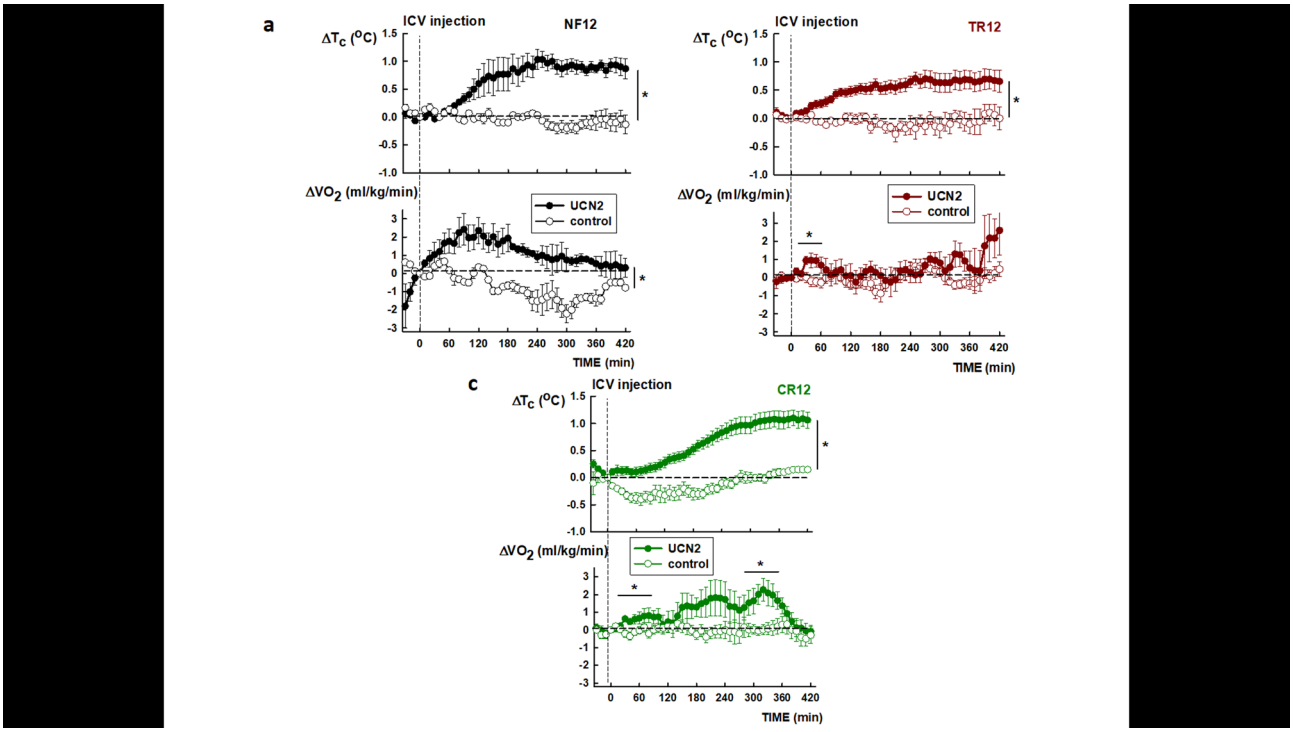

Fig. 4. Changes in core temperature (ΔTc) and oxygen consumption (ΔVO_2) of 12 months old male Wistar rats of different intervention groups following an intracerebroventricular (ICV) injection of urocortin 2 (UCN2). Dark symbols indicate UCN2-treated, empty symbols indicate results of age-matched controls. (Panels a, b, and c show the results obtained in *ad libitum* fed sedentary NF12, *ad libitum* fed treadmilltrained TR12 and caloric restricted sedentary CR12 rats, respectively.) Control animals received pyrogen-free saline (PFS). Asterisks indicate significant differences between the UCN2-treated and control animals shown by repeated-measures ANOVA. Number of animals per group: TR12 treated $n = 10$, TR12 control $n = 10$, NF12 treated $n = 8$, NF12 control $n = 8$, CR12 treated $n = 10$, CR12 control $n = 8$.

(Fig. 2). Thus, we did not expect any reduction in the capacity for heat production in either the TR12 or the CR12 groups.

Our results confirm literature data showing that at least medium length training (lasting for 3 months), increases the energy expenditure and leads to loss of body fat resulting in the increase of lean mass[13]. However, in our model in middle-aged male Wistar rats the 12-week caloric restriction did not decrease either the fat mass indicator or the relative muscle mass. Our results differ from the findings of the Comprehensive Assessment of the Long-term Effects of Reducing Energy Intake (CALERIE) Study, showing a marked loss of visceral adipose tissue with a modest loss of muscle in young non-obese individuals following long-term mild caloric restriction[29].

Centrally applied UCN2 induced a significant increase in the core temperature (Tc) of all groups. These responses were smaller in the NF6 than in the NF12 rats that support our previous observations. Earlier, we have demonstrated a consistent age-related increase in the UCN2-induced hyperthermic/hypermetabolic responses in male Wistar rats from 3- to 18 months of age[9]. The hyperthermic reactions of trained and caloric-restricted rats were lower than those of the respective NF animals. Although all groups showed some rise in oxygen consumption compared with their corresponding control groups, the hypermetabolic responses of the CR6, TR6, and CR12 and TR12 groups were shorter than those of the corresponding NF groups (Figs. 3 and 4). Only the NF12 rats maintained a significant UCN2-induced hypermetabolism throughout the long observation period (Figs, 3,4).

With regard to the effects of treadmill training, TR6 and TR12 groups showed lasting but moderate UCN2-induced hyperthermia with only a modest increase in oxygen consumption. Our findings suggest that the treadmill training may have rejuvenated the metabolic/thermogenetic reactions of the middle-aged rats. Observations of Garcia-Valles and her coworkers reported, that treadmill training may not have improved the lifespan in rodents, but it could improve the mitochondrial content thus delaying aging[16].

As an alternative explanation, exercise-induced increase in uncoupling (*via* UCP-1) may be suggested in the trained animals. Earlier studies reported controversial results about the treadmill training-induced upregulation of thermogenesis genes such as Prdm16 and UCP1. Certain studies supported the idea of treadmill training increasing the level of Prdm16 and UCP1 expression in white adipose tissue[30,31]. However, other research groups reported that training could not affect the mRNA level of *Ucp1*[32] or just weakly stimulated the brown adipose tissue (BAT) thermogenesis without a rise in the level of UCP1[33].

With regard to heat loss, we have observed a gradual rise in Ts in the NF and CR groups that reached statistical significance. This moderate increase in the heat loss has not prevented the development of UCN2-induced

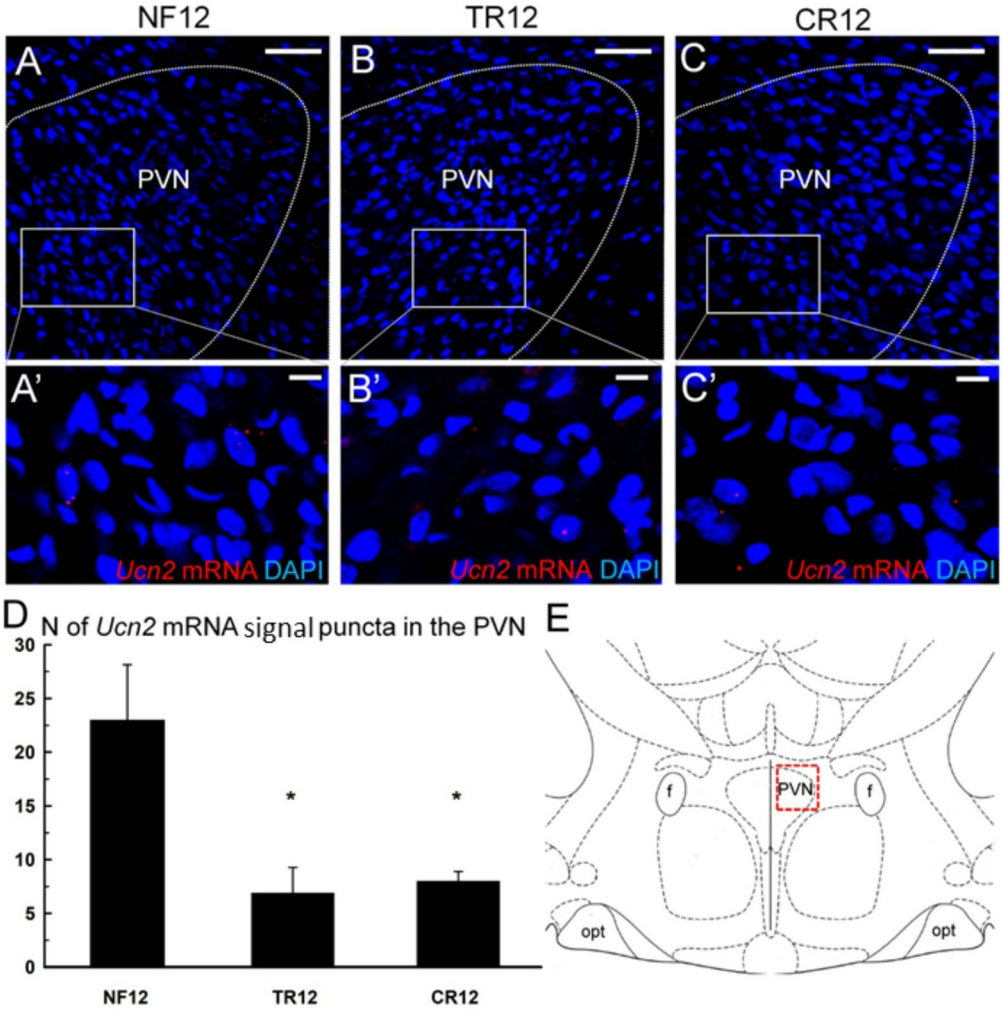

Fig. 5. Urocortin 2 (*Ucn2*) mRNA expression in the paraventricular nucleus of the hypothalamus (PVN). Panels A-C show representative confocal images in the PVN of 12-month-old rats that were normally fed (NF12), subjected to physical training (TR12) or caloric restriction (CR12). Red signal dots correspond to *Ucn2* mRNA transcripts. The blue color represents nuclear counter staining with 4′,6-diamidino-2-phenylindole (DAPI). Due to the relatively low *Ucn2* expression level, higher magnification images of the boxed areas in the bottom panels (A'-C') illustrate the individual *Ucn2* signal puncta more efficiently. Histogram D provides a quantitative comparison of *Ucn2* expression in the PVN across experimental groups. * $p < 0.05$, compared to the NF12 group, according to Tukey's *post hoc* test upon one-way analysis of variance ($F_{2, 12}$=8.98, $p = 0.004$, $n = 5$/group). Cartoon E illustrates the neuroanatomical localization of the imaged areas shown in A-C. f: fornix, opt: optic tract. Bars: 50 µm in A-C, 10 µm in A'-C'.

hyperthermia, however it may have contributed to the development of the plateau phase of the hyperthermic response. Trained rats with the most moderate hyperthermic responses in each age-group did not show any significant rise in Ts.

Considering the effects of caloric restriction, the hyperthermic/hypermetabolic effects of the central UCN2 injection were similar in the caloric-restricted and trained groups. Caloric restriction may also have rejuvenated the metabolic/thermogenetic reactions of the middle-aged rats to UCN2. Numerous previous studies indicated that caloric restriction shows tendencies to delay aging[4] even the lifespan of rodents could increase[15]. On the other hand, it has been suggested that there are similarities between obesity and aging[4]. Thus, obesity may promote aging, caloric restriction may delay it.

However, experimental data suggest other potential mechanisms as well. Some studies emphasize that caloric restriction decreases the basal metabolic rate and the body temperature[34]. It also affects the plasma level of various hormones[34]. The CR-induced decrease of leptin and melanocortins may also suppress core temperature and metabolic rate[34]. Other observations suggest that uncoupling may be suppressed in the muscles and in the fat tissue due to caloric restriction[35]. However, our 12-week 30% caloric restriction programme has not influenced resting body temperature or oxygen consumption significantly. The *post mortem* body composition indicators did not show any sarcopenia either (Fig. 2), that could decrease the heat production capacity of the animals. Nevertheless, the mechanisms through which caloric restriction influences body temperature and metabolic rate

still remain controversial, since some studies also reported that CR can induce fat browning in white adipose tissue, and suggest that the thermogenesis increases with these changes[36,37].

When assessing the *Ucn2* mRNA expression, we have to state a limitation of this study: we were unable to provide data on the dynamics of UCN2 at peptide level. This is on one hand because of the lack of a reliable antibody. On the other hand, the reliable immunohistochemical detection of neuropeptides requires often ICV colchicine pre-treatment. Because of the serious side effects of this procedure would have compromised the outcome of this experiment, we have decided to focus on the changes of *Ucn2* expression at mRNA level. Both training and caloric restriction appeared to suppress the *Ucn2* mRNA expression in the PVN of the hypothalamus as compared with data of the NF12 group (Fig. 5). These results are in accord with the observed changes in the hyperthermic/hypermetabolic responsiveness to central UCN2. It may raise the possibility that the effects of transient, late-onset lifestyle interventions may influence central regulatory mechanisms.

Conclusions

Both treadmill training and caloric restriction show tendencies in delaying aging in middle-aged rats indicated by the altered hyperthermic/hypermetabolic responsiveness to centrally-applied UCN2. There are similarities with aging and obesity, thus we hypothesized that interventions reducing obesity may improve disadvantageous age-associated alteration in central regulation of energy balance. Based on our results, we can confirm that the consequences of lifestyle interventions such as treadmill training and caloric restriction may delay aging.

Methods

Animals

Male Wistar rats were used (6–10 rats/group except for the immunohistochemistry where n = 5/group) from the colony of the Institute for Translational Medicine of the Medical School, University of Pécs, Hungary. We established two age groups, 6-month-old (younger middle-aged) and 12-month-old (older middle-aged) animals for our investigations. Rats were placed individually into (37.5 cm x 21.5 cm x 18 cm) plastic cages, covered with steel grids. The rodents were housed at an ambient temperature of 22–24 °C with a 12 h/12 h dark/light cycle. In our study we formed 3 lifestyle groups, the *ad libitum* fed NF sedentary, the CR sedentary and the *ad libitum* fed TR groups. All three groups were fed with a standard laboratory rat chow (11 kJ/g; CRLT/N rodent chow, Szindbád Kft., Gödöllő, Hungary). For the NF and TR groups food and water were available *ad libitum*. For the CR animals, daily 16 g of rat chow was available representing 70% of the *ad libitum* food consumption with *ad libitum* water access[5]. The rats in the TR group undertook a daily 45-min treadmill training 5 days/week [modified after[38]. For these training sessions, we used a special treadmill device, which was designed for rats (Harvard Apparatus Panlab, Treadmill Control LE8710). Following a week of gradual habituation to the treadmill device, our training protocol included a 5-min rest followed by a 45-min training at 17.5 m/min speed using a 10% slope each day. The lifestyle interventions in the CR and TR groups started at the age of 3-months or 9-months and lasted for 12 weeks. Our protocols and procedures were approved by the Animal Welfare Committee of the University of Pécs and by the National Scientific Ethical Committee on Animal Experimentation of Hungary. The license was granted by the Government Office of Baranya County (BA02/2000-9/2020). They were also in accordance with the directives of the European Union (86/609/EEC, Directive2010/63/EU) and the rules of the Hungarian Government (40/2013.II.14.) on the protection of animals used for scientific purposes. This study was reported in accordance with the ARRIVE guidelines.

Surgical intervention

Following the end of the interventions, at the age of 6- or 12-months, a 22 gauge stainless-steel leading cannula was implanted into the right lateral cerebral ventricle of each Wistar rat for the ICV injections. For the surgeries general anesthesia was performed. We administered a combination of ketamine [78 mg/kg (Calypsol, Richter) and xylazine (13 mg/kg Sedaxylan, Eurovet)] intraperitoneally. To prevent infections, 2 mg Gentamycin was also given to the rats. When the anaesthesia was complete, we fixed the head of the animals into a stereotaxic apparatus. Then, the skin was incised over the skull and the bone was cleaned. For the leading cannula and the miniature fixing screws, altogether three holes were drilled into the bone. The cannula was placed A: −1.0 mm to the bregma, L: 1.5 mm right lateral to the bregma and V: 3.5 mm ventral to the dura[39]. The guide cannula and the screws were secured on the skull with dental cement. The lumen was closed with a stylet, which could be replaced with an injection cannula during the experiments[40].

To check the appropriate location of the guide cannula, ICV injected prostaglandin E_2 (Sigma-Aldrich, Budapest, Hungary, P5515, 500 ng/5 µL) was administered to the rats. If there was an at least 1.0 °C rise in the core temperature within an hour, then the appropriate location of the cannula was confirmed. After the end of the experiments, the rats were euthanized by an intraperitoneal overdose injection of urethane (2.8 g/kg, Reanal, Budapest, Hungary)[9,40]. The injection sites were checked macroscopically *post mortem*, by coronal sections of the removed and fixed brains. Only those results were included in the statistical analysis where the location of the cannula was appropriate[40].

Substances applied

UCN2 (Bachem, Switzerland, Product-No. 4040984) dissolved in PFS or PFS were administered slowly in 5 µL volumes. For the thermoregulatory tests we administered 5 µg UCN2 also in 5 µL volume. In the control group, rats received ICV injections of 5 µL PFS.

Assessment of body weight and food intake

The daily FI and the BW of the rats were measured manually 5 days a week.

Assessment of thermoregulatory functions

Following the end of the interventions, at the age of 6- or 12-months, when the rats recovered from the implantation of the ICV leading cannula, thermoregulatory responsiveness to UCN2 was tested in an indirect calorimeter system (OxyletPro-Physiocage, Harvard Apparatus, MA, USA). The tests were carried out during the daytime period, between 08:00 h and 17:00 h. The partially restrained rats were confined in cylindrical wire-mesh cages in metabolic chambers (size: 20 × 30 × 18.5 cm). Before the experiments the rats were carefully, gradually habituated to the confiners to minimize the restraint stress during the experiments. Oxygen consumption was measured every 10 min, which represents the metabolic rate. The Tc, tail-skin temperature (Ts) and the ambient temperature (Ta) were detected with thermocouples attached to a Benchtop thermometer (Cole-Parmer). The data were recorded on the computer. The heat loss index (HLI) was calculated as follows: (Ts – Ta)/(Tc – Ta). The value of the HLI ranges from 0 to 1. The value 0 indicates maximal vasoconstriction (Ts = Ta), the value of 1 (Ts = Tc) shows maximal vasodilation and heat-loss[9,40].

RNAscope in situ hybridization for Ucn2 mRNA

Animals from each 12 months old treatment group were anesthetized intraperitoneally with an overdose of urethane (D = 2.8 g/kg, Merck KGaA, Darmstadt, Germany) and transcardially perfused with 50 ml of 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 250 ml 4% paraformaldehyde in Millonig's buffer. Brains were dissected and post-fixed. Thirty µm coronal sections were made by a vibratome (Leica Biosystems, Wetzlar, Germany) between the optic chiasm and middle cerebellar peduncle. The success of surgery was verified during sectioning by observing the path of the guide cannula from the cortical surface to the lateral ventricle. Free floating sections were first collected in RNAase free PBS containing 0.01% sodium azide and then transferred to anti-freeze solution for storage at −20°C. Per animal, two coronal sections between bregma – 1.56 mm and – 1.92 mm interspaced by 150 µm were manually selected. These sections bilaterally contained the PVN and were identified based on the rat brain atlas by Paxinos and Watson[39]. After a modified pretreatment procedure for RNAscope, as we recently published[41,42], the staining protocol was applied according to the supplier's suggestions. The *Ucn2* mRNA was visualized by Cyanine 3 (Cy3; 1:3000) with the help of a probe for rat *Ucn2* (Cat. No: 829641-C2, Advanced Cell Diagnostics, Newark, CA, USA). The nuclear counterstaining was carried out with 4',6-diamidino-2-phenylindole (DAPI) and the sections were finally covered with glycerol-PBS (1:1) solution.

In order to confirm the sensitivity of our test, triplex positive (Cat. No: 320891, Advanced Cell Diagnostics, Newark, CA, USA) and negative control (Cat No: 320871, Advanced Cell Diagnostics, Newark, CA, USA) probes were used on randomly selected PVN sections. The positive controls showed well recognizable fluorescence while the negative controls showed no signal (images not shown).

For digitalization of the sections, an Olympus FluoView1000 confocal microscope with 40x (NA: 0.8) objectives was used in analog mode. The excitation and emission of fluorophores were set according to the built-in settings of the FluoView software (Fv10-ASW; Version 0102). With regard to the dyes, blue (DAPI) and red (Cy3) virtual colors were assigned. Four PVN cross-section areas per animal were digitalized. The signal dots indicating the presence of mRNA were manually counted by two independent researchers using the ImageJ software (version 1.52a, NIH). From each animal, four non-edited digital pictures were included, and the average of the counting results (per animal) were used in the statistical analysis. For publication, the Adobe Photoshop software was used for cropping, contrasting and editing the selected representative images.

Determination of post mortem body composition indicators

During the autopsy of those intact animals used for RNAscope measurements also relative *post mortem* body composition indicators were determined. Wet weights of epididymal fat pads, left sided perirenal fat mass and as a muscle indicator the sum of the wet weights of the tibialis anterior muscle, the m. extensor digitorum longus, the soleus muscle and the m. tibialis anterior were measured and then divided by the body weight of the animals and multiplied by 100.

Statistical analysis

Experimental groups contained 6–10 animals. For immunohistochemistry each group contained 5 rats. The normal distribution of data and the homogeneity of variance was examined and confirmed for all datasets except for data describing the number of *Ucn2* mRNA signal puncta. Therefore, this latter dataset was evaluated by the nonparametric Kruskal-Wallis test, followed by Bonferroni correction. All results are shown as mean ± SEM. For the statistical analysis, repeated-measures ANOVA and one-way ANOVA with Tukey's *post hoc* test were applied using SPSS for Windows 25.0 software. The significance was set at the level of $p < 0.05$. With regard to the figures, they were made with the SigmaPlot 11.0 software.

Data availability

The data used to support the findings of this paper are available from the corresponding author upon reasonable request.

Received: 12 August 2025; Accepted: 10 December 2025
Published online: 17 December 2025

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Acknowledgements
The authors are grateful for the expert technical assistance of Ms. M. Koncsecskó-Gáspár, Ms. A. Bóka-Kiss, Ms. A. Jech-Mihálffy, Ms. É. Sós and Ms. I. Orbán.

Author contributions
MB and DKK designed the study, DKK carried out the *in vivo* tests, MB, PE and DKK carried out the statistical analysis. RNAscope methodology and imaging was carried out by BG, VK and GB, evaluation of non-edited digital images, DKK, EP and BG. DKK and MB wrote the original draft, they were responsible for the manuscript preparation and final editing, EP was responsible for funding acquisition. All authors agreed with regard to the interpretation of the data, all authors contributed to the final manuscript and gave approval to the submitted final version.

Funding
This study was supported by the National Research, Development and Innovation Fund of Hungary (K138452 to E.P.); the European Union's Recovery and Resilience Facility (RRF, RRF-2.3.1-21-2022-00011 to M.B.); B.G. was supported by the Thematic Excellence Program 2021 Health Sub-program of the Ministry for Innovation and Technology in Hungary, within the framework of the EGA-16 project of Pécs University (TKP2021-EGA-16); and by the grant NKFI-146117. János Bolyai Research Scholarship of the Hungarian Academy of Sciences (BO/00750/22/5 to V.K.); and Hungarian Research Network (HUN-REN-TKI14016 to V.K.).

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-32585-8.

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