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Effect of Intracerebroventricular Administration of Apelin-13 on the Hypothalamus–Pituitary–Thyroid Axis and Peripheral Uncoupling Proteins

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Abstract Apelin, a ligand for G protein-coupled APJ receptor, is a peptide hormone. Although apelin and APJ receptors are determined in hypothalamus and thyroid gland its role in the hypothalamus–pituitary–thyroid (HPT) axis and mechanism of action on energy metabolism is not clear. This suggests that apelin may play a role in the HPT axis and energy metabolism. This study was designed to determine possible effects of centrally administered apelin-13 on the HPT axis and energy metabolism. A total of 40 adult male Sprague Dawley rats were divided into four groups (n = 10 each group). Intact rats served as control group while the sham group received vehicle of apelin. Apelin-13 was injected intracerebroventricularly at the doses of 1 and 10 nmol, for 7 days in the rats in the experimental group. At the end of the experimental protocol, animals were decapitated and brain, blood, white and brown adipose tissues samples were collected. There was no significant difference between the groups in terms of hypothalamic TRH mRNA levels. Serum TSH levels were significantly higher in all groups compared to the control group ($p < 0.05$). Serum fT3 and fT4 levels were significantly lower in apelin-13 administered groups ($p < 0.05$). Moreover, apelin-13 administered groups had lower levels of UCP1 mRNA in white and brown adipose tissues. UCP3 mRNA expression in muscle tissue was also lower in apelin-13 treated groups ($p < 0.05$). These results indicates that apelin-13 exhibits a decreasing effect on energy consumption through a mechanism involving the peripheral rather than central arms of the HPT axis.

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Introduction

Energy homeostasis relies on a concerted response of the nervous and endocrine systems to signals evoked by food intake, energy storage, and expenditure of energy. Thyroid hormones (THs) are included in meeting immediate energy demands, thus placing the hypothalamus–pituitary–thyroid (HPT) axis at a central interface (Joseph-Bravo et al. 2015). Several hypothalamic nuclei synthesize TRH (e.g. arcuated nucleus and paraventricular nucleus; ARC and PVN, respectively), but the TRH neurons that control thyrotrophin (TSH) release are the hypophysiotropic neurons present in the PVN (Fekete and Lechan 2014; Joseph-Bravo et al. 2015). TSH, which is synthesized and released by the anterior pituitary, is the primary regulator of the synthesis and release of THs at the thyroid gland. The THs thyroxine (T4) and triiodothyronine (T3) control the production and secretion of TRH and TSH by negative feedback to protect physiological levels of the major hormones of the HPT axis. Different other factors, which are neural, humoral, and local factors, may regulate THs levels by affecting this axis directly or indirectly (Ortiga-Carvalho et al. 2016). The roles of THs are central and peripheral nervous system development, growth, energy metabolism, and thermogenesis. These hormones also regulate the hepatic metabolism of nutrients, fluid homeostasis and the cardiovascular system.

Apelin is a peptide hormone originally isolated from the bovine stomach and is the endogenous ligand of the APJ receptor (Tatemoto et al. 1998). There are the apelin forms with many biological activities such as apelin-13, apelin-17, and apelin-36 which derived from a 77-amino-acid precursor molecule. Apelin-13 has a stronger biological activity than other apelin forms (Sandal et al. 2015; Tatemoto et al. 1998). Apelin is defined as a significant neuroprotective peptide in the central nervous system (Khaksari et al. 2012). High expression levels of APJ and apelin in the hypothalamic-neurohypophyseal (especially the hypothalamic PVN and SON) suggest that the control of the circadian rhythm of this peptide has an effect on nutrition, fluid balance and pituitary hormone release (Bao et al. 2016; Hosoya et al. 2000). Moreover, the increased levels of apelin/APJ in adipose tissue and plasma with obesity (Boucher et al. 2005; Dray et al. 2010) and the effect of apelin on the expression of uncoupling protein (UCPs, markers of peripheral energy consumption) in fat and muscle tissue indicate that this peptide may have peripheral effects on energy metabolism.

This study was performed to determine possible effects of apelin that is considered to play a role or roles in energy metabolism on UCP1 mRNA expression levels in fat tissue and UCP3 mRNA expression levels in muscle tissue which are accepted as an indicator of the hormones and energy use in the HPT axis.

Materials and Methods

The Number of Animals and the Formation of the Groups

40 male Sprague Dawley rats (mean weight: 250–270 g) were used in the study. If the highest difference between the mean weights of the animals was 4.6 g and the standard deviation of the mean weights of the animals was 3.2 g and type 1 error (α) was 0.05 and type 2 error (β) was 0.81, power analysis showed that there should be at least ten animals in each group. The animals were divided into four groups including the control group, the sham (vehicle) group, the low dose apelin-13 group (APLN-1 nmol) and the high dose apelin-13 group (APLN-10 nmol) such that their mean body weights are close together (n = 10). The rats were kept at 21 ± 1 °C temperature and under 12 h light/dark conditions during the experiment. Moreover, they were fed ad libitum with standard rat chow and they drank tap water. This study was approved by the Animal Experimentation Ethics Committee of Inonu University Faculty of Medicine (Protocol number: 2013/A-57), and also all applications in the study were performed as stated in the protocol of ethics committee.

Experimental Preparation

Apelin-13 used in the experiment was provided by Sigma-Aldrich Inc. (Catalog no: A6469, USA). Apelin-13 was dissolved within artificial cerebrospinal fluid (vehicle, aCSF; 124 mM NaCl, 5.0 mM KCl, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, and 30 mM glucose, pH: 7.2) and then two different concentrations of Apelin-13 were prepared to be used in the experimental study (low dose; 1 nmol and high dose; 10 nmol). Osmotic mini-pumps (Alzet 2ML1, USA) were filled with the apelin concentrations (aCFS for the rats in the sham group). The pumps were connected to the brain infusion kits which are designed as infused into the lateral ventricle of the rats (Alzet Brain Infusion Kit-1, USA).

Experimental Protocol

Other rats except for the rats in the control group were anesthetized with a combination of 70 mg/kg ketamine (Richter Pharma AG, Australia) and 8 mg/kg xylazine (Alfazyne, Holland). After anesthesia, the animals with shaved head

were fixed in the stereotaxic device (Small Animal Stereotaxic System, ASI Instruments, USA) and bone structure was reached by cutting the skin with a scalpel. The location of the lateral ventricle was determined by going 1.40 mm medial and 0.8 mm posterior from the bregma according to the Paxinos and Watson rat brain atlas and then the marked segment was drilled by using a drill. The brain infusion kit (Alzet) was placed in the right lateral ventricle of the animal using the azlet holder for intracerebroventricular (icv) infusions and then the head of the kit was fixed to the skull with a dental adhesive. Osmotic mini-pumps that had been previously connected to the cannula of the kit was implanted under the neck skin of the animals (Sandal et al. 2015). After the incision was sutured, the postoperative care of the animals was performed. Apelin-13 was infused into the lateral ventricle of the rat in a volume of 10 µl/h for 7 days by osmotic mini-pumps (aCFS for the rats in the sham group).

The animals were decapitated after infusion and then blood, hypothalamus, muscle (biceps muscle), interscapular white and brown adipose tissues were collected. The hypothalamus, muscle, and adipose tissue samples were placed in DNase/RNase-free eppendorf tubes containing RNAlater solution and also the tissues were stored at − 80 °C until analyzes were performed. Moreover, the amount of liquid in them was calculated after osmotic mini-pumps were collected and also the pumps were confirmed to run.

ELISA Analysis

The blood samples were centrifuged for 10 min at 4500 rpm and the serum was obtained from blood. Moreover, the levels of serum TSH (USCN Life, China; Catalog number: E0463r) and free T3 (fT3) and free T4 (fT4) (Elabscience, China; Catalog number: E-EL-R1097 and E-EL-R0390) were determined using ELISA kits.

Quantitative Real-Time RT-PCR Analysis

Muscle or Adipose Tissue UCP mRNA Levels

Total RNA was isolated from white and brown adipose, and muscle tissues using TRIzol reagent (Invitrogen, USA). cDNAs were created by reverse-transcription of total RNA samples with a High Capacity RNA to cDNA Synthesis kit (Invitrogen, USA). PCR reactions were prepared, in triplicate, and heated to 50 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. Cycle threshold values versus template concentration were prepared for each target gene, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Rn-01775763-g1, Applied Biosystems, USA) as the endogenous reference in each sample. The real-time PCR analysis was performed with the ABI Prism 7500 Fast Real-Time PCR Instrument (Applied Biosystems, USA) using Tag Man Master Mix (Applied Biosystems, USA). All results were standardized to the levels of GAPDH. The samples were quantified for UCP-1 (Rn-00562126-m1, Applied Biosystems, USA), UCP-3 (Rn00565874_m1, Applied Biosystems, USA), using the comparative *Ct* ($^{\Delta\Delta}Ct$) method, as described in the Assays-on-Demand User's Manual (Applied Biosystems) (Etem et al. 2014).

Hypothalamic TRH mRNA Levels

Total RNA in the hypothalamus was extracted with Pure RNA Tissue kit (Roche Diagnostics GmbH, Germany). cDNA synthesis was performed by Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany). Real-Time PCR was performed in a Light Cycler Instrument (Roche Applied Science) using Start Essential DNA Probes Master Kit (Roche Diagnostics GmbH, Germany) and Real-Time Ready Assay (β-Actin Lot no: 90,015,222; TRH Lot no: 90,015,384, Roche Diagnostics GmbH, Germany). Reaction volumes were set at 10 µl. 5 µl master mix containing 0.5 µl real-time ready mix, 2 µl PCR grade water, and 2.5 µl cDNA was prepared. Samples were run in triplicate. The cycling protocol was set as the follows; an initial 10 min. denaturation step at 95 °C, followed by 55 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 s. To determine the change in TRH gene expression among the groups, the β-Actin gene was selected as housekeeping gene and relative mRNA expression levels were calculated according to housekeeping genes using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The statistical analysis of the data was performed using IBM SPSS statistics version 22.0. The suitability of data with the normal distribution was assessed by the Kolmogorov–Smirnov test. The Kruskal–Wallis H test was used to compare changes in gene expression and serum hormone levels in the brain tissue. The Dunn's test was used for multiple comparisons. The Student's *t* test for independent samples was used to determine fold changes in mRNA levels in fat and muscle tissue samples. Gene expression levels in the brain tissue and TSH, T3 and T4 levels in the blood tissue were given as the median (min–max) and also fold changes in mRNA levels in fat and muscle tissues were given as mean ± standard deviation (SD). Statistical significance was accepted at $p < 0.05$.

Results

Serum TSH, fT3 and fT4 Levels

The serum TSH, fT3, and fT4 levels of the groups after icv administration of apelin-13 were shown in Fig. 1. Serum TSH level was significantly higher in the other groups compared to the control group after the infusion ($p < 0.05$). However, there was no statistically significant difference between the apelin-13-injected groups and the sham group in terms of serum TSH level (Fig. 1a). Although serum fT3 and fT4 levels decreased significantly in both the low dose apelin-13 group and the high dose apelin-13 group compared to the control group ($p < 0.05$), serum fT3 and fT4 levels decreased significantly in only the high dose apelin-13 group compared to the sham group (Fig. 1b, c, $p < 0.05$).

TRH, UCP1, and UCP3 mRNA Levels

The changes in TRH mRNA expression after icv administration of apelin-13 were shown in Fig. 2. There was no statistically significant difference between the apelin-13-injected groups and the control group and the sham group in terms of TRH mRNA level. Moreover, there was no statistically significant difference between the sham group (aCSF-injected group) and the control group in terms of TRH mRNA level.

UCP1 RNA levels in white and brown adipose tissue were significantly lower in both the low dose apelin-13 group and the high dose apelin-13 group (Fig. 3a, b, $p < 0.05$). This decrease occurred as a dose-dependent especially in white adipose tissue (Fig. 3a). Moreover, UCP3 mRNA expression in muscle tissue was significantly suppressed in both the low dose apelin-13 group and the high dose apelin-13 group compared to the control group and the sham group (Fig. 3c, $p < 0.05$).

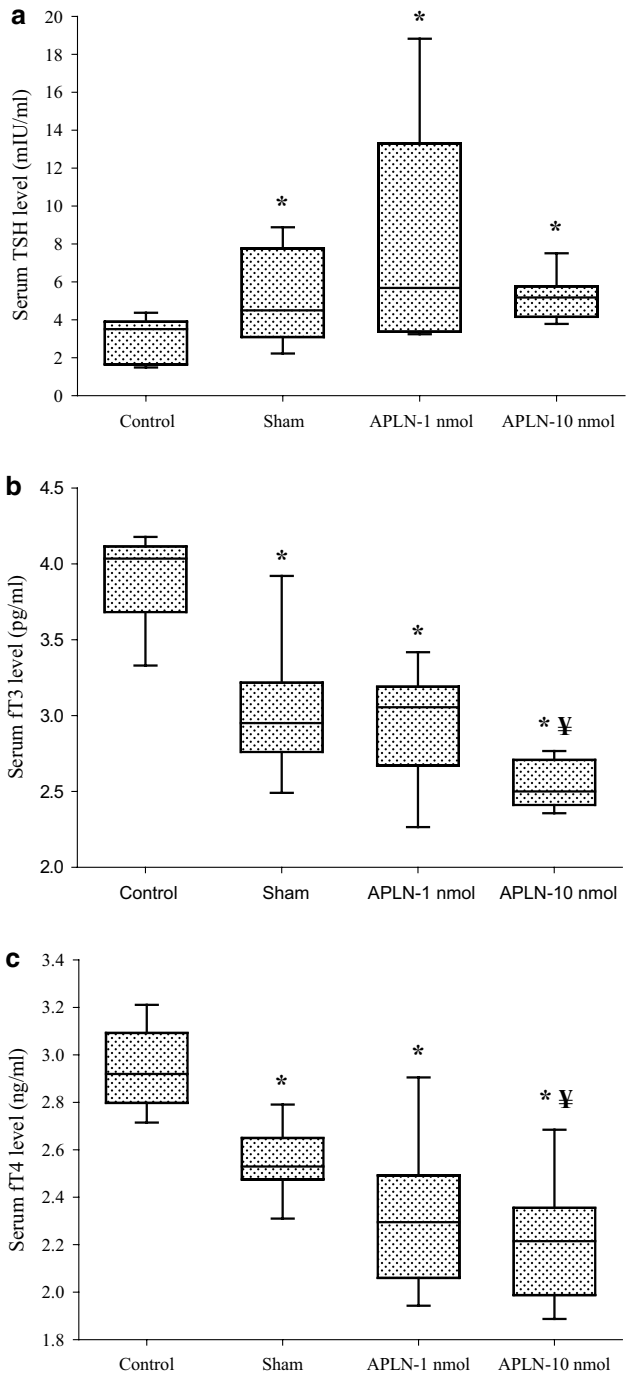

Fig. 1 Serum levels of TSH (**a**), fT3 (**b**) and fT4 (**c**) of the groups. Icv irisin infusion, for 7 days, significantly reduced serum fT3 and fT4 levels, while increasing serum TSH level. Values were expressed as median (min–max). *$p < 0.05$ compared to the control group, and ¥$p < 0.05$ compared to the sham group (Kruskal–Wallis H test and Dunn's test, where appropriate)

Discussion and Conclusions

Apelin/APJ has been shown to be expressed in the hypothalamic nuclei such as the PVN and SON and arcuate nucleus and in the hypothalamus–pituitary axis (O'Carroll et al. 2013; Pope et al. 2012). These areas are known as the control center in the energy metabolism and neuroendocrine functions (Joly-Amado et al. 2014; Ortiga-Carvalho et al. 2016). The presence of apelin/APJ in these brain areas shows that the peptide may have several roles in these physiological processes. Moreover, the expression of apelin in the peripheral areas such as thyroid gland, fat and muscle tissues (Lee et al. 2000; O'Carroll et al. 2013) supports that this peptide may have an effect on the energy metabolism and the HPT axis.

Limited number of studies is insufficient to reveal the possible relationship between apelin and THs. Serum apelin

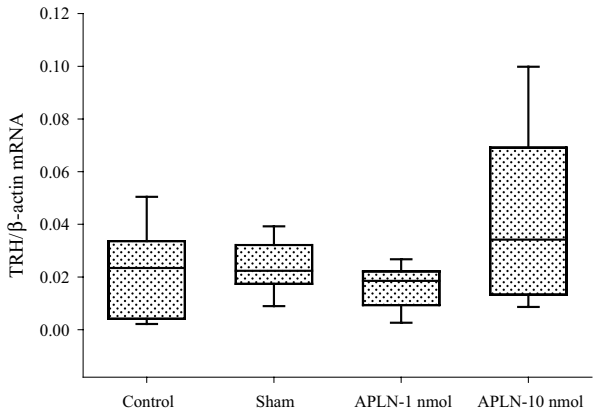

Fig. 2 Effects of icv apelin-13 infusion on hypothalamic TRH mRNA expression levels. No significant was determined between the control group and apelin-13 groups. Values were expressed as median (min–max). In the comparison of quantitative variables among groups, Kruskal–Wallis H test was used The Dunn's test was used for multiple comparisons

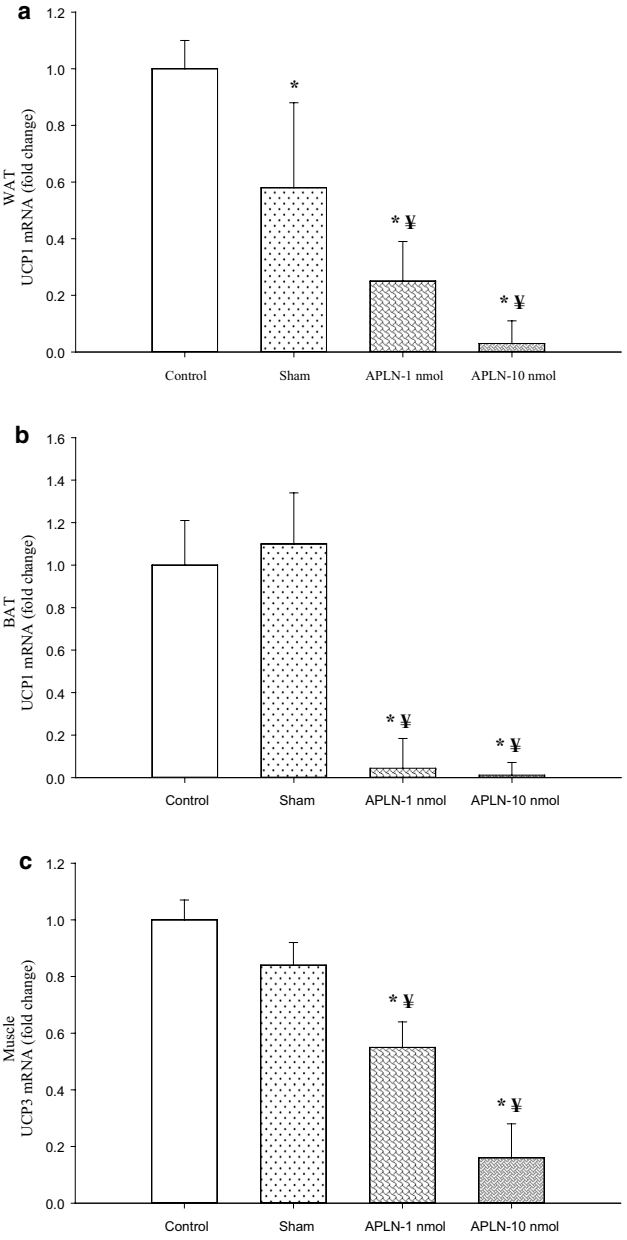

Fig. 3 Effects of apelin-13 on UCP1 mRNA levels in the adipose and UCP3 levels in the muscle tissue. Infusion of 1 and 10 nmol apelin-13 significantly reduced UCP1 mRNA levels in the WAT (**a**) and BAT (**b**), and UCP3 mRNA level in the muscle tissue (**c**). In the comparison of quantitative variables among groups, Kruskal–Wallis H test was used The Dunn's test was used for multiple comparisons. Values were expressed as median (min–max). *$p < 0.05$ compared to the control group, and $^{¥}p < 0.05$ compared to the sham group

levels were found to be high in patients with obese and insulin resistance in the present studies (Castan-Laurell et al. 2011; Krist et al. 2013). However, suppressed apelin synthesis and low serum apelin concentration have been shown to be associated with weight loss and improved insulin sensitivity (Krist et al. 2013). In our previous studies it has been shown that central and peripheral injections of apelin-13 in rats increased feed intake and body weight (Sandal et al. 2014; Tekin et al. 2014). In contrast to these results, there are the studies showing that apelin did not affect food intake (Higuchi et al. 2007; Taheri et al. 2002) and also the administration of apelin caused weight loss (Higuchi et al. 2007; Yamamoto et al. 2011). On the other hand, it is suggested that apelin may be effective in the treatment of obesity and other metabolic disorders resulting from impairment of energy metabolism (Boucher et al. 2005; Castan-Laurell et al. 2011). Zorlu et al. showed that there was no significant difference between healthy individuals and people with subclinical hypothyroidism in terms of serum apelin levels. Moreover, the researchers reported that serum apelin level was positively correlated with body mass index (Zorlu et al. 2014). Gurel et al. showed that serum apelin level was higher in patients with thyroid dysfunction compared to healthy individuals but this level was not statistically significant (Gurel et al. 2015).

The different results were found in the studies conducted on the hormones such as leptin, ghrelin and adiponectin having the similar effects with apelin. Cusin et al. reported that icv administration of leptin reduced serum TSH and T4 levels and also there was no any significant change in T3 level (Cusin et al. 2000). Mahmoudi et al. showed that plasma TSH, T3 and T4 levels were dose-dependently after chronic central administration of ghrelin (Mahmoudi et al. 2011). In another study, although icv administration of ghrelin reduced in T3 and T4 levels, icv administration of leptin increased significantly in the levels of these hormone (Amoo-Rajabi et al. 2012). In this study, we found that administration of apelin-13 increased

TRH mRNA and serum TSH levels but this increase did not show the difference in the apelin-13-injected group compared to the sham group. Moreover, it was shown that administration of apelin-13 has a suppressive effect on THs (T3 and T4) and also this effect occurred significantly in the high-dose apelin-13 group. Our results show that icv administration of apelin-13 has no effect on the HPT axis at the central level and also it exhibits its peripheral effect depending on the administered dose.

UCPs which are among mitochondrial transport protein family are expressed in the mitochondrial inner membrane (Rousset et al. 2004). These play a role in mitochondrial biogenesis, regulation of cellular energy and non-shivering thermogenesis (Erden et al. 2015). Attane et al. reported that chronic administration of apelin-13 increased mitochondrial biogenesis and oxidative capacity in muscle tissue (Attane et al. 2012). It was shown that UCP3 mRNA expression in skeletal muscle was suppressed significantly in rats with apelin gene transfer compared to normal rats (Yamamoto et al. 2011). In contrast to these results, it was reported that administration of apelin increased the expressions of UCPs (Higuchi et al. 2007; Masaki et al. 2012). We found that it reduced UCP1 mRNA expression in white and brown adipose tissue and UCP3 mRNA expression in muscle tissue in the apelin-13-injected groups. We think that it performs this effect by reducing T3 and T4 levels after administration of apelin. The present studies seem to support our opinion. The researchers consider that UCPs expressed by skeletal muscle and adipose tissue might have important roles on the metabolic effects of the hormones of the HPT axis (Gurel et al. 2015; Lanni et al. 2003). Lee et al. reported that the injection of T3 into the culture of human adipocytes increased dose-dependently UCP1 mRNA level (Lee et al. 2012). It was shown that the injection of T3 in rats with thyroidectomy increased UCP1 mRNA expression in brown fat (Bianco and Silva 1987). It was revealed that THs can regulate UCP3 expression in muscle tissue as its function in adipose tissue (Queiroz et al. 2004). In another study, it was shown that the injection of T3 in rats increased UCP3 mRNA level in muscle tissue (de Lange et al. 2007).

Consequently, it was shown that central administration of apelin has no significant effect on TRH mRNA and TSH levels but it reduced serum T3 and T4 levels. This situation shows that apelin-13 has an effect on peripheral areas on the HPT axis. Moreover, the suppression of UCP1 mRNA expression in adipose tissue and of UCP3 mRNA expression in muscle tissue by apelin-13 supports that apelin can reduce energy use.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable institutional and national guidelines for the care and use of animals were followed. In this study, protocols for animal experiment were approved by the institutional animal ethical committee.

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