Age- and nutritional state-related changes in central leptin effects on energy balance

Doctoral (PhD) Thesis

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List of abbreviations

ACT	locomotor activity
AgRP	agouti-related peptide
alpha-MSH	alpha-melanocyte stimulating hormone
AMPK	5’-adenosine monophosphate-activated protein kinase
ARC	arcuate nucleus of the hypothalamus
BAT	brown adipose tissue
BDNF	brain-derived neurotrophic factor
BPM	beats per minute
BW	body weight
CART	cocaine-amphetamine-regulated transcript
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
CO_2	carbon-dioxide
CR	calorie-restricted
CRH	corticotropin-releasing hormone
DMH	dorsomedial nucleus of the hypothalamus
ERK	extracellular signal-regulated kinase
FI	food intake
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT4	glucose transporter type 4
GnRH	gonadotropin-releasing hormone
HF	high-fat diet-induced obese
HLI	heat loss index
HR	heart rate
ICV	intracerebroventricular
IL	interleukin
IP	intraperitoneal
IRS	insulin receptor substrate
JAK2	Janus kinase 2
LHA	lateral hypothalamic area
MCH	melanin-concentrating hormone

mRNA	messenger ribonucleic acid
NF	normally fed
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
Ob-R	leptin receptor
Ob-Rb	long isoform of leptin receptor
PFS	pyrogen-free saline
PI3K	phosphatidylinositol-3-kinase
PO/AH	preoptic area/anterior hypothalamus
POMC	proopiomelanocortin
PTP-1B	protein tyrosine phosphatase-1B
PVN	paraventricular nucleus of the hypothalamus
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SHP2	SH2 domain containing protein tyrosine phosphatase 2
SOCS3	suppressor of cytokine signaling 3
STAT3	signal transducer and activator of transcription 3
STAT5	signal transducer and activator of transcription 5
Ta	ambient temperature
Tc	core body temperature
TRH	thyrotropin-releasing hormone
Ts	tail skin temperature
UCP	uncoupling protein
VMN	ventromedial nucleus of the hypothalamus
VO_2	oxygen consumption
VTA	ventral tegmental area
WHO	World Health Organization

1. Introduction

Abnormalities of energy balance present major public health problems with rapidly increasing prevalence [World Health Organization (WHO) 2012, 2014, 2015a]. They include changes of body weight (BW) and/or body composition. Obesity is defined as excessive accumulation of body fat, while wasting disorders are characterized by progressive loss of BW, especially that of muscle mass. During aging two common tendencies are observed: middle-aged people tend to gain weight and develop obesity (Scarpace et al., 2000b), while at old age, anorexia (loss of appetite) with a consequent cachexia and progressive muscle atrophy (sarcopenia) develops. Mean BW increases gradually until 45-55 years of age (predominantly fat mass), then after a stagnation until the age of 65-75, a decline in active tissues (especially muscle mass) is seen without any apparent disease (Steen et al., 1979; Steen, 1988).

In 2014 39 % of adults (> 18 years of age) were overweight, 13 % were obese worldwide (WHO 2014). According to a survey in Hungary, 54 % of adults were overweight or obese in the same year. The ratio was highest in the middle-aged population (71 % of men and 53 % of women were either overweight or obese between 35 and 64 years of age) (Központi Statisztikai Hivatal, 2015).

Another global phenomenon is population aging. By WHO estimation, the proportion of the world's population over 60 years of age will nearly double to 22 % until 2050 (WHO 2015b). In Hungary, the ratio of people over 65 years was 17 % in 2013 and it is predicted to reach 30 % by 2060 (Központi Statisztikai Hivatal, 2013). On the other hand, obesity appears to accelerate aging and age-related degenerative processes (e.g. muscle atrophy, neurodegeneration) (Balaskó et al., 2013; Carter et al., 2013; Balaskó et al., 2014).

Age-related changes can be observed not only in humans, but also in other mammals suggesting the contribution of altered basic regulatory mechanisms, in addition to environmental factors (e.g. increasingly sedentary lifestyle and imbalanced dietary choices) in the background. Thus, one of the common features of aging and obesity is a dysregulation of energy homeostasis. Such dysregulation involves resistance to different regulatory peptide hormones, e.g. leptin or insulin, leading to abnormalities of BW and/or body composition (Ahima, 2009; Carter et al., 2013).

Obesity is strongly associated with cardiometabolic disorders including type 2 diabetes mellitus (Colditz et al., 1995), hypertension and coronary heart disease (Hong et al., 2003), atherosclerosis and ischemic stroke (Rexrode et al., 2001), and also with certain malignancies (Carrol, 1998; Morimoto et al., 2002). The consequences of (senile) cachexia are also severe: muscle weakness leading to frailty, falls and bone fractures (Fried et al., 2001), a higher risk for decubitus (pressure ulcer) (Thomas, 2001), impaired quality of life (Crogan and Pasvogel, 2003), loss of independence (Roubenoff and Houghes, 2000). As both types of body composition changes increase the risk of morbidity and mortality, investigation of regulatory alterations that develop in obesity or during the course of aging is of outstanding importance.

1.1. Regulation of energy balance

Energy balance involves the regulation of two interconnected circles. One is responsible for the maintenance of BW. This long-term regulation is based on the balance between food intake (FI) and metabolic rate (MR). The other short-term balance refers to thermoregulation (core body temperature, Tc), it defends the stability between MR (heat production) and heat loss (Fig. 1).

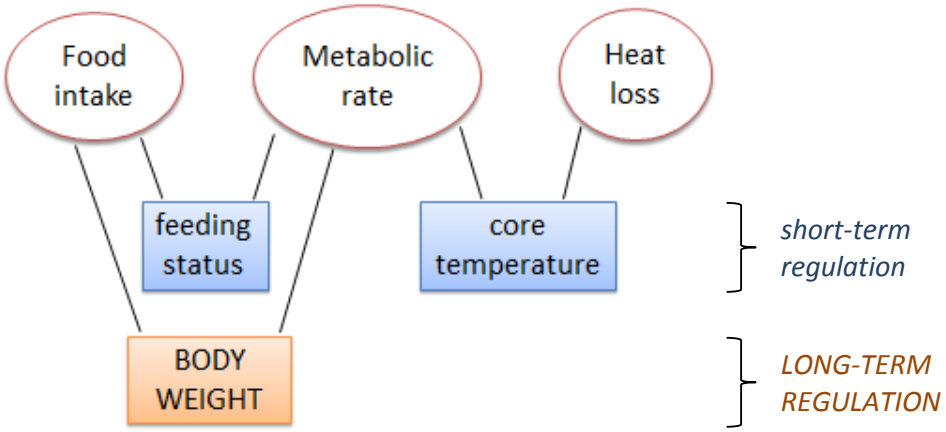

Fig. 1 *Components of energy balance*

Acute changes in FI and MR determine the feeding status (hunger, satiety), chronic dysregulation leads to abnormal nutritional states (obesity, cachexia). These changes also have an influence on the thermoregulatory status. Fasting or starvation induces

hypometabolism and a tendency for hypothermia, while a postprandial state is characterized by hypermetabolism and hyperthermia. On the other hand, temperature changes also modify feeding behaviour. In a hot environment, anorexia develops. Cold exposure leads to an increase in MR to maintain homeothermy (stable Tc independent of external influences), that is followed by hyperphagia in order to preserve a stable BW (Székely and Szelényi, 2005).

Neuroendocrine regulation of energy balance involves a cross-talk between the periphery and the central nervous system, consisting of sensory inputs, central integrative circuits, and autonomic outputs (sympathetic and parasympathetic) (Carrascosa et al, 2009; Münzberg et al., 2016). Sensory input to the brain is accomplished by neural or humoral route; signals may act on chemo- and mechanosensitive endings of the abdominal vagus (Székely, 2000), humoral substances in the circulation (secreted from peripheral organs) can act directly at the brain (Janig, 1996).

Peripheral signals can be assigned into the following types: (1) hunger and satiety signals regulating the short-term eating behavior, such as gastrointestinal hormones [e.g. FI increasing (orexigenic) ghrelin or FI decreasing (anorexigenic) cholecystokinin (CCK)], or changes of gastrointestinal tension (stretch) (Stanley et al., 2005; Székely and Szelényi, 2005); (2) nutrient signals, such as glucose or fatty acids, which reflect the whole-body nutrient status and can modulate appetite (Hu et al., 2003) and (3) long-term adiposity signals, such as leptin and insulin, indicating the amount of fat stored in the body (Schwartz et al., 2000; Székely and Szelényi, 2005; Carrascosa et al., 2009).

These factors interact with specific brain areas such as hypothalamic or brainstem [e.g. the nucleus of the solitary tract (NTS)] nuclei. These brain areas play a key role in integrating peripheral signals which modify the activity of central regulatory peptides, and generate homeostatic responses, transmitted by the autonomic nervous system to regulate FI and energy expenditure (Badman and Flier, 2005).

The arcuate nucleus of the hypothalamus (ARC) has a principal role in the control of FI. It is a region with a „leaky" part of the blood-brain barrier allowing the access of peripheral hormones. The ARC contains orexigenic [expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP)] and anorexigenic [producing proopiomelanocortin (POMC) and its derivative alpha-melanocyte stimulating hormone (alpha-MSH), and also cocaine-amphetamine-regulated transcript (CART)] „first-order" neurons, that send projections to the paraventricular nucleus of the hypothalamus (PVN) and to the lateral hypothalamic area (LHA). They modulate the secretion of other regulatory peptides [anorexigenic

corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) in the PVN and orexigenic melanin-concentrating hormone (MCH), orexins in the LHA] of „second-order" neurons (Valassi et al., 2008; Ramamoorthy et al., 2015) (Fig. 2).

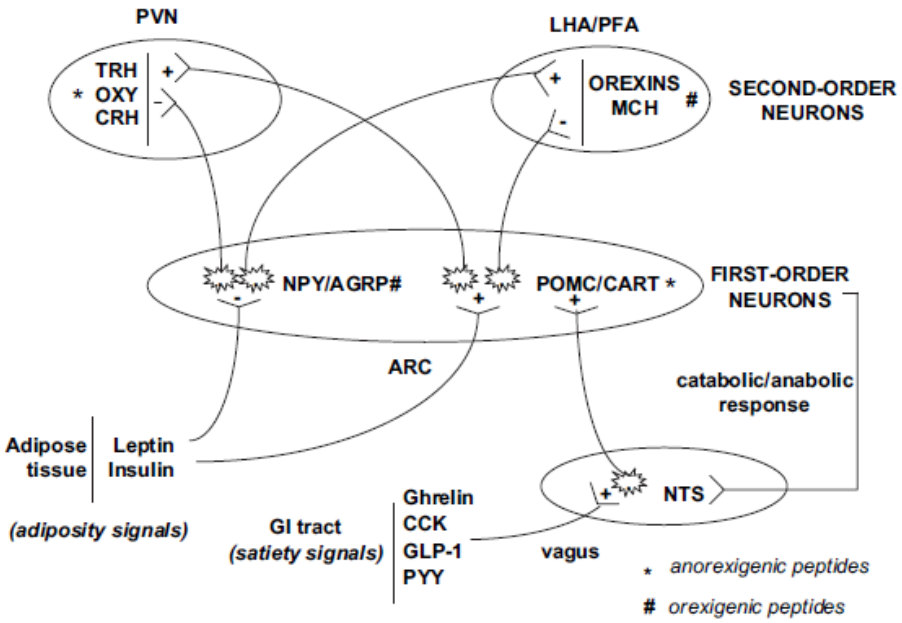

Fig. 2 *Brain pathways involved in the regulation of food intake. ARC: arcuate nucleus of the hypothalamus, NTS: nucleus of the solitary tract, CCK: cholecystokinin, GLP-1: glucagon-like peptide 1, PYY: peptide YY, PVN: paraventricular nucleus of the hypothalamus, LHA: lateral hypothalamic area, PFA: perifornical area, NPY: neuropeptide Y, AGRP: Agouti-related peptide, POMC: proopiomelanocortin, CART: cocaine-amphetamine-regulated transcript, CRH: corticotropin-releasing hormone, TRH: thyrotropin-releasing hormone, OXY: oxytocin, MCH: melanin-concentrating hormone, GI tract: gastrointestinal tract. (Valassi et al., 2008)*

In the regulation of MR, the ventromedial nucleus of the hypothalamus (VMN) and the PVN have fundamental roles. The VMN controls the influence of sympathetic tone on brown adipose tissue (BAT) heat-production. PVN has an influence on the parasympathetic dorsal vagal nucleus and spinal sympathetic preganglionic neurons (Székely and Szelényi, 2005; Shabalina and Nedergaard, 2011).

The preoptic area/anterior hypothalamus (PO/AH) region of the hypothalamus has outstanding role in temperature regulation. It is connected to nuclei involved in the regulation of MR and in the control of heat loss mechanisms (e.g. vasomotor functions).

Local thermosensitive neurons, incoming neural and humoral signals are responsible for the thermoregulatory effector function (heat conservation or heat loss) (Cannon and Nedergaard, 2004; Székely and Szelényi, 2005).

Components of energy balance can be influenced separately, but key modulators induce coordinated regulatory changes. Anabolic substances increase FI (orexigenic agents) with a decrease in MR in order to increase the energy content of the body, catabolic modulators suppress FI (anorexigenic factors) while inducing hypermetabolism, in a coordinated way (Székely and Szelényi, 2005).

1.2. Leptin

1.2.1. History of leptin

In 1959, Hervey performed a parabiosis study (surgical union of two animals with a common blood supply allowing the investigation of circulating substances) with rats made obese by electrical hypothalamic (VMN) lesions (Hervey, 1959). The non-obese parabiotic partners (with intact hypothalamus) of obese rats consumed less food and lost weight, suggesting the presence of a circulating factor that functioned as a feedback signal in the regulation of energy balance. This hypothesis was supported by subsequent similar observations (Parameswaran et al., 1977; Coleman, 1978; Harris et al., 1987).

The ob/ob mouse is homozygous for an autosomal recessive mutation of the ob gene which arose spontaneously in 1949 (Ingalls et al., 1950). Observations that obese ob/ob mice did not produce the hypothesized satiety feedback signal led Friedman's group to identify leptin. The protein was cloned from rodent adipose tissue in 1994, as the product of the ob gene (Zhang et al., 1994). The name "leptin" was taken from the Greek word "leptos", which means thin, as mice lacking the ob gene are hyperphagic and obese (Tartaglia et al., 1995). These mice gain weight rapidly throughout their lives, they develop decreased basal MR, diminished thermogenic capacity as well as hyperinsulinemia, accompanied by type 2 diabetes (Bray and York, 1979; Friedman and Halaas, 1998). Leptin treatment in such cases dramatically reduces FI, increases MR and Tc, and reverses hyperglycemia and hyperinsulinemia (Pelleymounter et al., 1995; Harris et al., 1998).

1.2.2. Leptin synthesis and secretion

Leptin is a 16-kDa protein consisting of 167 amino acids and it belongs to the long-chain helical cytokine family (Zhang et al., 1997). The protein is one of the most important adipokines that is produced predominantly in the subcutaneous and to a lesser extent in the visceral white adipose tissue (Friedman and Halaas, 1998). Numerous other sites of leptin production have been identified in the gastric epithelium, in placental trophoblasts (Masuzaki et al., 1997; Badd et al., 1998), in skeletal muscles (Wang et al., 1998), in the heart (Purdham et al., 2004), and also in the brain of rats and humans (Morash et al., 1999; Wiesner et al., 1999). Circulating concentrations of the hormone refer to the amount of fat mass, but leptin release shows circadian fluctuations with a nadir in the morning and a maximum in the middle of the night (Licinio et al., 1997).

Leptin expression and secretion are regulated by several other factors. Food consumption and the postprandial rise in insulin increase the plasma concentration of the hormone, this effect is observed several hours after a meal (Koopmans et al., 1998; Szkudelski, 2007); in contrast, fasting decreases blood leptin values (Szkudelski et al., 2004). Leptin production and release are also enhanced by estrogen, and glucocorticoids, while androgens, norepinephrine release and stimulation of β-adrenergic receptors have an opposite effect (Gettys et al., 1996; Mantzoros et al., 1996; Ryan and Elahi, 1996; Watanobe and Suda, 1999).

1.2.3. Leptin receptors and signaling pathways

Six leptin receptor isoforms (Ob-Ra-f) have been identified with the structure of the class I cytokine receptor family, generated by alternative splicing from the *db* (or *lepr*) gene. The receptors are categorized into three groups: secreted (Ob-Re), short (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf) and long (Ob-Rb) isoforms (Kwon et al., 2016).

Ob-Re is a soluble receptor that binds circulating leptin with high affinity. In lean individuals up to 50 % of leptin is present in the bound form, whereas the majority of leptin is in free form in obese people (Sinha et al., 1996).

The other isoforms are transmembrane receptors (Baumann et al., 1996; Lee et al., 1996), short and long types are distinguished by their intracellular domain size (Kwon et al., 2016). Short form receptors are found mainly in the choroid plexus and brain microvessels, and have their primary role in leptin transport through the blood-brain barrier

and in lysosomal degradation of the protein (Kastin et al., 1999; Uotani et al., 1999; Hileman et al., 2002).

Leptin's effects on energy homeostasis are mediated by the long isoform of leptin receptor (Ob-Rb). Leptin binding promotes a conformational change of the receptor that results in the autophosphorylation and activation of Janus kinase 2 (JAK2). JAK2 phosphorylates itself and three tyrosine residues of Ob-Rb (Tyr985, Tyr1077, and Tyr1138) to induce different signaling pathways with distinct functions (Park and Ahima, 2014) (Fig. 3).

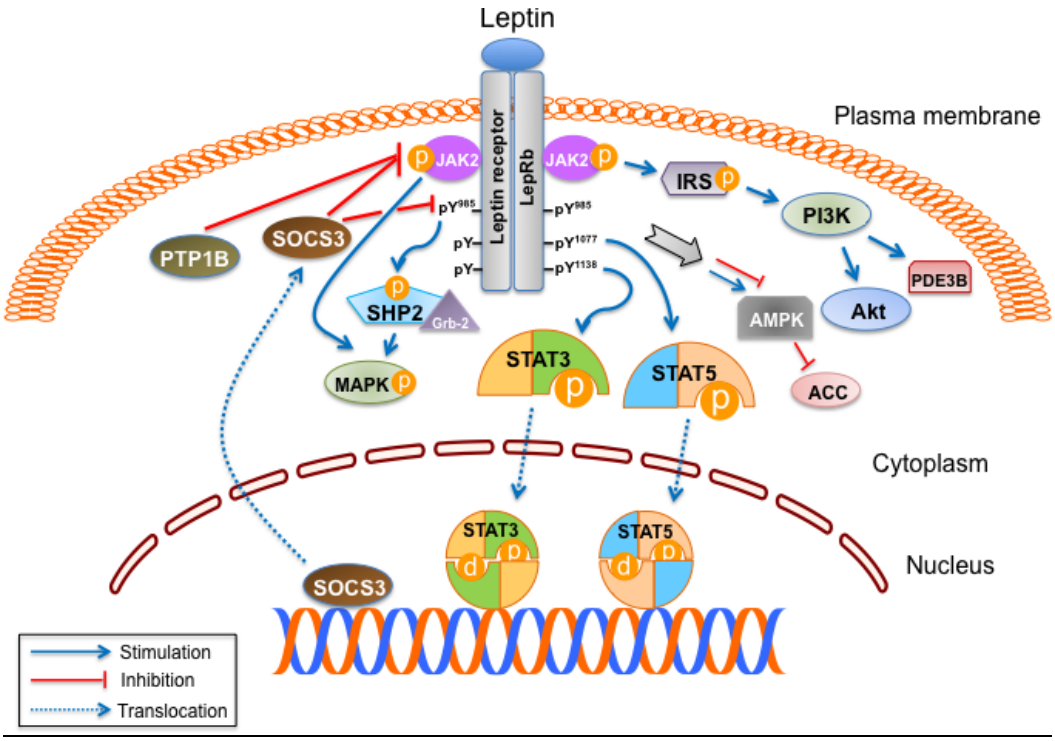

Fig. 3 *Leptin signaling pathways. LepRb: long isoform of leptin receptor (Ob-Rb), JAK2: Janus kinase 2, pY: phosphorylated tyrosine residue, STAT3 and STAT5: signal transducer and activator of transcription 3 and 5, SOCS3: suppressor of cytokine signaling 3, PTP-1B: protein tyrosine phosphatase-1B, SHP2: SH2 domain containing protein tyrosine phosphatase 2, Grb2: adaptor protein growth factor receptor-bound protein 2, MAPK: mitogen-activated protein kinase, PI3K: phosphatidylinositol-3-kinase, IRS: insulin receptor substrate, PDE3B: phosphodiesterase 3B, AMPK: 5'-adenosine monophosphate-activated protein kinase, ACC: acetyl-CoA carboxylase. (Park and Ahima, 2014)*

JAK2/STAT3 signaling: phosphorylation of Tyr1138 results in the recruitment of signal transducer and activator of transcription 3 (STAT3) to permit its phosphorylation and dimerization. Activated STAT3 translocates to the nucleus, where it mediates changes in the transcription of target genes, including neuropeptides (e.g. POMC, AgRP) and suppressor of cytokine signaling 3 (SOCS3, a feedback inhibitor of Ob-Rb signaling) (Bjørbaek et al., 1999). STAT3 mediates the main effects of leptin on energy balance (Myers et al., 2008). Disruption of its signaling results in hyperphagia, decreased energy expenditure and morbid obesity (Bates et al., 2003).

JAK2/STAT5 signaling: phosphorylation of Tyr1077 promotes the recruitment and activation of signal transducer and activator of transcription 5 (STAT5). This type of signaling plays a minor role in leptin's regulation of feeding and BW (Lee at el., 2008), but also mediates reproductive effects of leptin (Münzberg and Morrison, 2015).

SHP2/ERK signaling: phosphorylation of Tyr985 recruits SH2 domain containing protein tyrosine phosphatase 2 (SHP2) to mediate the activation of the extracellular signal-regulated kinase (ERK) pathway. This type of signaling is related to thermogenesis and suppression of FI; inhibition of this pathway is known to reduce BAT thermogenesis and leptin-induced anorexia in mice (Rahmouni et al., 2009; Zhou and Rui, 2013). Tyr985 serves as the binding site for SOCS3 playing a prominent role in the feedback inhibition of Ob-Rb (Bjørbaek et al., 1999).

IRS/PI3K signaling: insulin receptor substrate (IRS) phosphatidylinositol-3-kinase (PI3K) signaling mediates leptin effects on FI (modulating the expression of neuropeptides) and sympathetic nerve activity (increasing renal sympathetic outflow) in the hypothalamus (Harlan et al., 2013). Leptin also exerts rapid effects through the PI3K pathway regulating neuronal firing rates. These actions are critical for modulating immediate release of hypothalamic neuropeptides from nerve terminals (stimulation of POMC neurons, inhibition of NPY, AgRP neurons) (Cowley et al., 2001; Hill et al., 2008).

AMPK signaling: 5'-adenosine monophosphate-activated protein kinase (AMPK) regulates feeding and enhances catabolic mechanisms. In the hypothalamus leptin inhibits AMPK activity in order to suppress FI (Minokoshi et al., 2008). Conversely, in the periphery, leptin activates AMPK thereby stimulating fatty acid oxidation and glucose uptake [through glucose transporter type 4 (GLUT4) translocation] in the skeletal muscle (Winder and Hardie, 1999).

SOCS3 and PTP-1B: SOCS3 and protein tyrosine phosphatase-1B (PTP-1B) are negative feedback regulators of leptin signaling by inhibiting JAK2. Overexpression of

either of them leads to leptin resistance in mice (Zabolotny et al., 2002; Howard et al., 2004).

Leptin receptor expression has been identified in the ARC, VMN, PVN, LHA and in the dorsomedial nucleus of the hypothalamus (DMH), in the hippocampus among other brain areas and in the NTS (Mercer et al., 1996; Elmquist et al., 1998; Burguera et al., 2000). It is also found in the adipocytes, in lung, liver, kidney or reproductive tissues and in immune cells (Hoggard et al., 1997; Zamorano et al., 1997; Kielar et al., 1998) suggesting widespread actions for the hormone.

1.2.4. Effects of leptin

Energy balance

During the past decades, leptin has emerged as probably the most important peripheral feedback signal to hypothalamic nuclei involved in the long-term central regulation of energy balance (Benoit et al., 2004). Leptin has coordinated catabolic activity: it does not only induce anorexia, but it also increases MR and Tc (Hwa et al., 1996; Sahu, 2004; Steiner and Romanovsky, 2007).

Increasing leptin levels are assumed to suppress overeating and to enhance energy expenditure in order to prevent further energy (fat) accumulation, while the effects are the opposite in case of decreased leptin levels (e.g. suppression of energy loss during starvation). Congenital lack of leptin, or structural defects of the hormone or of its receptors are accompanied by severe obesity (Zhang et al., 1994; Friedman and Halaas, 1998; Rosenbaum and Leibel, 2014).

The hormone passes through the blood-brain barrier via a special saturable transport system (Zlokovic et al., 2000) and binds to its receptors. These central catabolic leptin actions are mainly mediated by alterations in the expression of neuropeptides in the ARC. On the one hand it activates anorexigenic–catabolic mechanisms conveyed by POMC-derived melanocortins (primarily by alpha-MSH) and by CART, while on the other hand it inhibits orexigenic–anabolic pathways connected with NPY and perhaps also with AgRP. Peptides of the second order neurons (orexins, CRH, etc.) downstream to the cells of the ARC contribute to these mechanisms (Baskin et al., 1999; Székely and Szelényi, 2005; Valassi et al., 2008; Berglund et al., 2012).

The metabolic and thermogenic effects of leptin are partly mediated via the sympathetic nervous system through different hypothalamic nuclei (Pandit et al., 2017).

Leptin-responsive neurons in the DMH stimulate BAT thermogenesis and energy expenditure wihout influencing FI (Rezai-Zadeh et al., 2014). Activation of BAT thermogenesis involves the increased expression of uncoupling protein (UCP)-1 (Haynes et al., 1997; Scarpace et al., 1997), that „uncouples" electron transport and oxidative phosphorylation, promoting energy wasting and heat production (Muoio and Lynis Dohm, 2002). Leptin administration into the VMN increases renal sympathetic activity and plasma catecholamine levels (Montanaro et al., 2005); leptin-responsive POMC neurons in the ARC also regulate energy expenditure via the sympathetic nervous system (Rahmouni et al., 2003).

In the periphery, leptin also acts on different sites of the afferent vagus (Wang et al., 1997; Shiraishi et al., 1999; Buyse et al., 2001; Gaigé et al., 2002), transmitting information to the brainstem (e.g. to the NTS) (Buyse et al., 2001; Grill et al., 2002; Székely and Szelényi, 2005).

Reward system

Feeding is a complex motivational process controlled by various inputs including smell, taste, hormonal effects and cognitive state. It has been shown that leptin has a role in regulating the mesolimbic dopaminergic reward system (Opland et al., 2010; Domingos et al., 2011). The Ob-Rb detected in the ventral tegmental area (VTA) indicates that leptin signaling is capable of modulating normal and hedonic feeding behavior (Figlewicz and Sipols, 2010). Central administration of leptin into the VTA decreases FI, reduces the value of a sucrose reward (Domingos et al., 2011), while reduction of leptin receptors within VTA neurons increases FI, and also increases acute intake of palatable high-fat food (Davis et al., 2010, Balaskó et al., 2014).

Gastrointestinal system

Gastric epithelial cells are able to secrete leptin either towards the blood circulation or into the gastric juice. This secretion responds very rapidly (within minutes) to stimuli like FI or peptide hormones such as secretin and CCK (Cammisotto and Bendayan, 2012). The increase in the plasma concentration is relatively small, however, the change is sufficient to modify the effects of CCK with a synergistic interaction (Matson et al., 1997). Leptin is involved in CCK-mediated regulation of gastrointestinal function to induce short-term modulation of FI. Ob-Rb is coexpressed with CCK1-receptors in a subset of vagal afferent neurons (Buyse et al., 2001). Leptin enhances CCK signaling via increasing

receptor sensitivity to potentiate the inhibitory action of the protein on FI (Barrachina et al., 1997; Peters et al., 2006; de Lartige et al., 2012).

Lipid and glucose homeostasis

Leptin plays an important role in controlling lipid and glucose metabolism independently of FI or BW regulation. It stimulates ß-oxidation and lipolysis via sympathetic activation and decreases triglyceride stores in the white adipose tissue and in the liver (Gallardo et al., 2007). The hormone increases oxidation of fatty acids in peripheral tissues and reduces intracellular lipid content in skeletal muscle protecting nonadipocytes from lipotoxicity (Muoio et al., 2002). Leptin has direct effects on glucose homeostasis primarily through glucose-sensing neurons of the ARC and VMN. The hormone suppresses hepatic glucose production partly by decreasing glucagon level, and increases peripheral glucose uptake in skeletal muscle, BAT and other organs (German et al., 2011; Berglund et al., 2012; Fernández-Formoso et al., 2015). Leptin increases insulin sensitivity and, in turn, insulin stimulates leptin production and secretion in the adipose tissue (Seufert, 2004).

Cardiovascular system

Leptin effects to maintain normal blood pressure seem to be balanced in lean individuals with leptin sensitivity. These effects refer to blood pressure lowering mechanisms (vasodilation by promoting nitric oxide release from the endothelium) (Nakagawa et al., 2002) but also to blood pressure elevating actions. The pressor effects mainly involve the sympathetic drive (Beltowski, 2006) and also the release of vasoconstrictive substances, such as angiotensin II and endothelin (Quehenberger et al., 2002; Kershaw and Flier, 2004). In chronic hyperleptinemia, the interaction among the vasoconstrictor and vasodilatory effects of leptin may be disrupted, resulting in hypertension. Stimulation of renal sympathetic nerve activity appears to be intact in the presence of resistance to the weight reducing effects of the hormone, supporting the concept of selective central leptin resistance in obesity (Tune and Considine, 2007; Kshatriya et al., 2011).

Central nervous system

There is growing evidence that leptin has neuroprotective properties and favorable effects in various neurodegenerative diseases. Leptin can induce neurotrophic signals via

the increased expression of brain-derived neurotrophic factor (BDNF, Komori, et al., 2006; Weng et al., 2007), and prevents neuronal death (Dicou et al., 2001; Greco et al., 2008; Martins et al., 2013) with potential beneficial effects in Alzheimer's or Parkinson's disease (Signore et al., 2008).

Neuroendocrine systems

Leptin is necessary for the regulation of the hypothalamic-pituitary axes regulating puberty, fertility and energy metabolism (Prolo et al., 1998). Congenital leptin deficiency or mutation in the leptin receptor gene results in neuroendocrine dysfunction including infertility, while leptin treatment stimulates pulsatile gonadotropin secretion, puberty onset and development of secondary sexual characteristics (Farooqi et al., 2002). Leptin acts centrally via multiple neuronal pathways involving the kisspeptin system and ARC AgRP neurons to modulate the activity of gonadotropin-releasing hormone (GnRH) neuronal network (Quennell et al., 2009; Cravo et al., 2011; Sheffer-Babila et al., 2013).

Leptin has a crucial role in the biosynthesis and secretion of TRH in the PVN (Harris et al., 2001). In addition, the hormone increases the growth of the thyroid gland tissue, and stimulates the secretion of thyroid hormones. Prolonged fasting (with the fall of circulating leptin) lowers circulating thyroid hormone levels, which are restored to normal after leptin injections (Légrádi et al., 1997).

Immune system

Leptin is implicated in the immune response, with effects in both innate and adaptive immunity. It acts as a proinflammatory agent. Leptin upregulates the expression of several proinflammatory cytokines, such as tumor necrosis factor alpha, interleukin-6 (IL)-6, and IL-12, while it enhances T helper 1 response and suppresses T helper 2 pathways (Otero et al., 2006). Patients with leptin deficiency have an increased incidence of infections and marked abnormalities of T cell number and function (Farooqi et al., 2002).

1.2.5. Leptin resistance with aging and obesity

Rodents or humans with congenital leptin deficiency or with chronically low leptin levels (e.g. in lipodystrophy) are hyperphagic and obese. Leptin administration in these individuals results in a marked decrease in FI and BW (Farooqi et al., 2002; Paz-Filho et

al., 2014). In contrast, the vast majority of obese people are insensitive to leptin treatment, indicating the existence of “leptin resistance” in common forms of obesity (Hukshorn et al., 2002; Moon et al., 2011).

Leptin resistance has been attributed to multiple factors, including impaired leptin transport across the blood-brain barrier, tachyphylaxis (receptor downregulation) in response to chronically elevated hormone levels, or disruption of leptin signaling in the hypothalamus (Knight et al., 2010; Myers et al., 2012; Park and Ahima, 2014). Resistance can also develop downstream to leptin target neurons (e.g. disrupted melanocortin signaling) (Lu et al., 1994) or upregulation of anabolic mediators (e.g. NPY or AgRP) also has to be taken into account (Baskin et al., 1999; Berglund et al., 2012; Balaskó et al., 2017).

It has long been recognized that the responsiveness to leptin changes both with adiposity (Lin et al., 2000) and with age (Scarpace et al., 2000b). The combination of aging and obesity, *i.e.* age-related obesity is characterized by a tendency towards progressive weight gain starting at a younger age in humans and mammals reaching a peak in late middle-aged or aging groups. Such a weight gain has been associated with the development of progressive peripheral and, later on, also with central leptin resistance (van Heek et al., 1997; Scarpace et al., 2000a, 2000b; Shek and Scarpace, 2000; Sahu, 2004). In particular, suppression of FI by leptin was demonstrated to decrease (with consequent hyperleptinemia) (Scarpace et al., 2000c) during the course of age-related weight gain (Scarpace et al., 2000a) and in obesity of other etiologies at any age (Lin et al., 2000; Myers et al., 2012).

However, several questions remain unresolved in this field. For example, evidence is not conclusive whether age *per se* or rather the accompanying obesity leads to the development of leptin resistance. Moreover, very old age-groups of humans and mammals tend to lose weight [aging anorexia (Morley, 2001)] that cannot be explained on the basis of aging-induced leptin resistance.

Previously, Scarpace and coworkers showed that leptin-induced anorexia was strong in the young but not in old rats (Scarpace et al., 2000b). These observations support the role of aging in the development of leptin resistance. However, resistance to the hormone did already appear at a young age in diet-induced obese rats rather supporting the primary role of obesity in this phenomenon (Soós et al., 2010). It may be assumed that caloric restriction that reduces fat mass would prevent leptin resistance. However, some studies failed to confirm the efficacy of caloric restriction in the restoration of leptin

responsiveness when tested on FI alone (Gabriely et al., 2002a). In contrast, others reported that a 3-month food deprivation successfully re-established leptin responsiveness in old rats, at least regarding suppression of FI (Fernández-Galaz et al., 2002).

Previously, different, even inverse leptin effects have been shown upon central chronic and acute application of the protein (García-Cáceres et al., 2011). Moreover, diverse intracellular pathways of chronic and acute leptin actions have been identified in hypothalamic POMC neurons (Hill et al., 2008). It would be therefore important to also investigate the age-related pattern of acute and chronic central leptin effects with regard to energy metabolism.

Such studies injecting or infusing leptin directly into the brain may be of importance, as central leptin responsiveness appears to be maintained longer than the peripheral one during the development of obesity (van Heek et al., 1997). Moreover, synthesis of this hormone has also been detected in the brain of humans and mammals (Morash et al., 1999; Wiesner et al., 1999; Eikelis et al., 2007). Investigation of the development of age- and obesity-related changes in central leptin resistance may therefore implicate later therapeutic possibilities (e.g. regarding intranasal application of leptin in obesity, Schulz et al., 2012; Spetter and Hallschmid, 2015).

In addition, previous animal studies focused mainly on anorexigenic leptin effects while other parameters, in particular those related to the hypermetabolic actions like MR, locomotor activity (ACT) and Tc were left largely unexplored.

2. Objectives

2.1. We aimed to investigate age-related changes in acute central leptin effects on parameters of energy balance.

We tested the anorexigenic and hypermetabolic responsiveness to intracerebroventricular (ICV) injections of the hormone in different age-groups of rats (3, 6, 12, 18 or 24 months old). In addition, we carried out a detailed thermoregulatory analysis of hypermetabolic leptin effects. Regarding the mechanism of age-related changes in leptin-induced anorexia and hypermetabolism, expression of the long isoform of the leptin receptor (Ob-Rb) and that of the signal transduction inhibitor SOCS3 gene in the ARC was studied by quantitative real-time polymerase chain reaction (qRT-PCR). The influence of high-fat diet-induced obesity on the anorexigenic effects of leptin was also analyzed in the 6- and 12-month age-groups.

2.2. We also aimed to investigate the influence of aging and that of nutritional states on chronic central leptin effects on parameters of energy balance.

The effects of a 7-day large dose ICV leptin infusion on FI, BW, heart rate [HR, representing MR (Butler, 1993)], Tc and spontaneous ACT were investigated in normally fed (NF) male Wistar rats aged 3, 6, 12, 18 or 24 months. Regarding nutritional states, 6-, 12-, 24-month calorie-restricted (CR) age-groups were established (rats were maintained on a reduced energy diet from age 2 months). For further comparison, 6- and 18-month old obese rats (maintained on high-fat diet from age 2 months) were also infused ICV with leptin.

3. Materials and methods

3.1. Animals

Different age-groups of male Wistar rats from the Colony of the Institute for Translational Medicine of the Medical School, University of Pécs, Hungary were used in the present study. After they reached the appropriate age, rats were maintained individually in plastic home-cages (375 mm × 215 mm, height 149 mm) that contained wood-chip bedding covered with steel grid, equipped with feeder and bottle container at an ambient temperature (Ta) of 22–25 °C (up to 27-28 °C, thermoneutral in the nest). Although long-term isolation may alter the behavior of the animals, in order to measure the daily FI and to provide the allotted portion of food in the CR groups, it was necessary to use individual caging. Lights were on between 06.00 and 18.00 h. The following age-groups were established: 3 (young adult), 6 (younger middle-aged), 12 (older middle-aged), 18 (aging) and 24 (old) months old. (The maximal life-span of our colony reaches 30 months, about 50 % of rats survive 26 months, but after the age of 24 months surgical interventions involve very high mortality.)

At certain age-groups, animals were divided into subgroups: NF, high-fat diet-induced obese (HF), or CR. NF rats were fed standard laboratory rat chow ad libitum (11 kJ/g; CRLT/N rodent chow, Szindbád Kft., Gödöllő, Hungary), HF rats received IPS TestDiet (Diet-Induced Obesity Rodent Purified Diet with 60 % Energy from Fat, 21.6 kJ/kg) from 2 months of age. CR animals received 2/3rd of the normal daily amount of standard powdered chow (16 g/day) from age 2 months, with vitamin and mineral supplementation. Tap water was continuously available in all groups. All animals were accustomed to regular handling. Spontaneous daily FI and BW were measured every day at 09.00 h.

The following groups were tested in the experiments: (1) NF animals at ages 3, 6, 12, 18 and 24 months (NF3, NF6, NF12, NF18 and NF24); (2) HF 6-, 12- and 18-month old rats (HF6, HF12 and HF18 – the HF rats usually died before the age of 24 months); (3) CR animals of three age-groups (CR6, CR12 and CR24).

3.2. Acute experiments

3.2.1. Surgical interventions

Rats were operated on for the purpose of implanting a 22 gauge stainless-steel leading cannula into the right lateral cerebral ventricle for ICV injections. Surgeries were performed according to our previous studies (Soós et al., 2010; Pétervári et al., 2011) under intraperitoneal (IP) ketamine-xylazine [78 mg/kg (Calypsol, Richter) + 13 mg/kg (Sedaxylan, Eurovet)] general anesthesia and 2 mg IP Gentamicin was used to prevent infections. The head was fixed in a stereotaxic apparatus, the skin over the skull was incised, the bone was cleaned and three holes were drilled for two miniature screws and for the leading cannula, with its tip being at A: 1.0 mm (anterior to bregma), L: 1.5 mm (right lateral to bregma), V: 3.5 mm (ventral to dura). Dental cement fixed the complex to the skull. A stylet closed the lumen of the guide cannula which was replaced during the experiments with a 28 gauge injection cannula outreaching the guide cannula by about 0.5 mm. In order to check the appropriate location of the guide cannula angiotensin II (Sigma, A9525, 20 ng/5 μl) was injected through a polyethylene (Portex) tube attachment around 3 days after the implantation of the guide cannula. Appropriate location was assumed if at least 5 ml water was consumed within 30 min (Pétervári et al., 2010).

3.2.2. Administration of substances

When measuring acute anorexigenic effects, the animals received leptin (1 μg, recombinant leptin, Bachem) or pyrogen-free saline (PFS, solvent 0.9 % NaCl) after 48-h fasting. Leptin dissolved in PFS in a volume of 5 μl or 5 μl PFS alone filled the proximal end of the 20-25 cm-long polyethylene tube attached to the injection cannula. A small bubble separated the contents of the proximal part from the PFS that filled the distal part of the tube.

In acute thermoregulatory experiments an amount of 1, 4 or 10 μg of the protein was dissolved in PFS in a volume of 5 μl and was injected similarly. Control rats received PFS in 5 μl volume. In these studies leptin was injected after the animals reached a thermal steady state (usually 60-90 min after closing the metabolic chamber). All injections were given remotely, without causing any discomfort to the animals and administered at around

09.00 h, early in the inactive phase of the circadian activity at a slightly subthermoneutral or thermoneutral temperature.

3.2.3. Assessment of food consumption

For 10-14 days before the experiments rats were transferred to the automated Feed-Scale system (Columbus, OH) to get habituated to the environment and to the powdered form of rat chow (Fig. 4). Rats were given a powdered form of chow for 10-14 days before and also during the experimental procedures in order to avoid hoarding behavior and to allow the continuous measurement of FI. The powdered version of the high-fat diet contained 10 % normal powdered chow admixed to the powdered high-fat pellets (20.54 kJ/g). Standard or high-fat rat chow and tap water were provided ad libitum, except for the 48-h fasting period when only water was available for the appropriate groups.

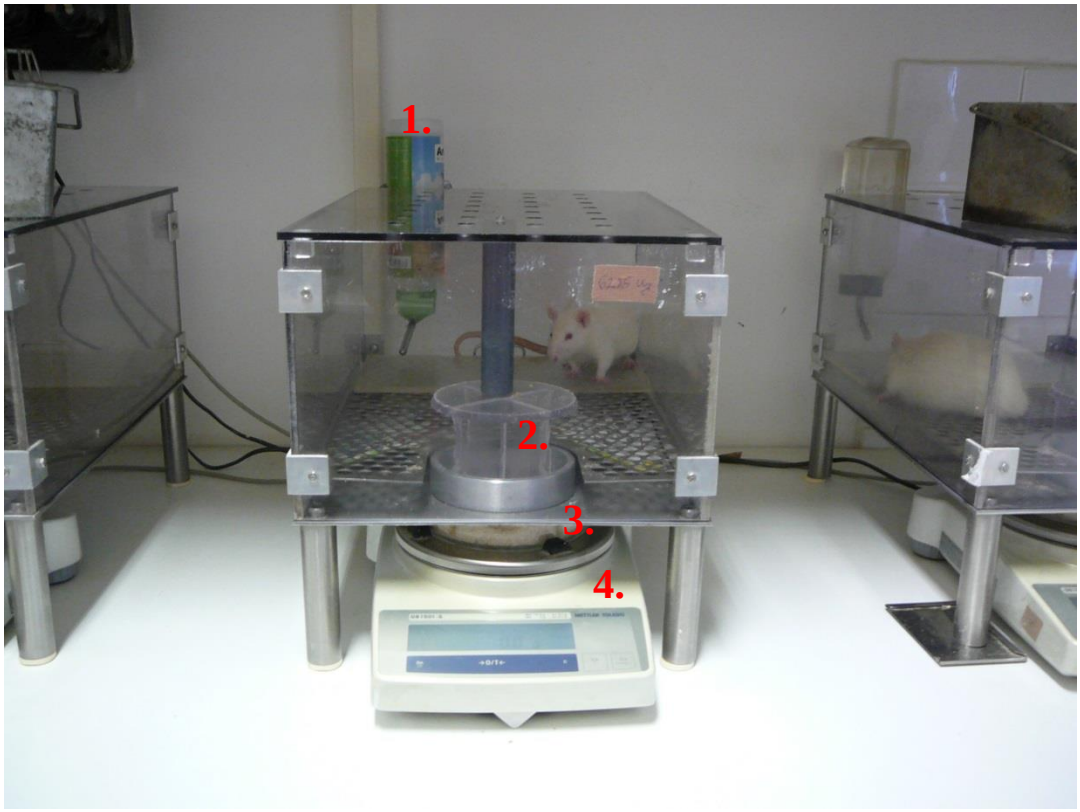

Fig. 4 *Feed-Scale system: 1. water bottle, 2. feeder, 3. food tray containing powdered rat chow, 4. digital scale*

The system allowed continuous recording of the amount of consumed food, data were registered every 10 minutes. On day 1 at 09.00 h food was removed for 48-h. Five

minutes before the re-feeding started (on day 3 at 09.00) assigned rat groups received 1 μg ICV leptin injection (in 5 μl volume) to measure the inhibitory effect of the hormone on 4-h cumulative FI. In control experiments PFS (5 μl) was given. Manual measurements of BW were carried out daily. Normally fed animals at ages 3, 6, 12, 18 and 24 months and two groups of HF rats (HF6 and HF12) were tested in these experiments.

3.2.4. Assessment of metabolic and thermoregulatory functions

During the experiments oxygen consumption (VO_2, representing MR), Tc and tail skin temperature (Ts, indicating heat loss) were measured. The tests were performed on semi-restrained rats, singly enclosed in cylindrical wire-mesh confiners (Fig. 5) in separate metabolic chambers (size: 20 x 30 x 18.5 cm). As the animals were previously accustomed to the semi-restraining cages for at least a week, we could minimize the stress during the experiments.

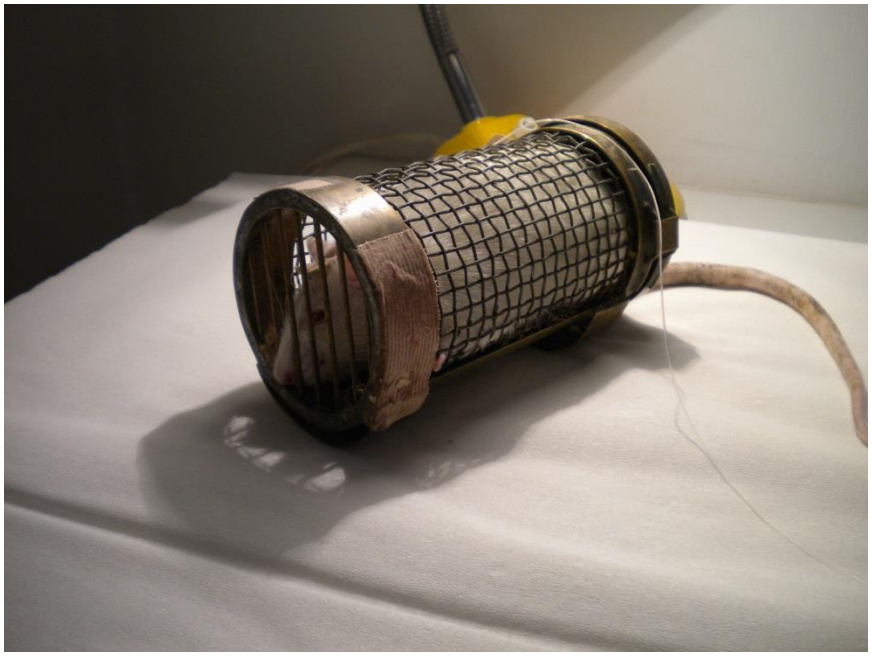

Fig. 5 *Semi-restrained rat in the cylindrical wire-mesh confiner*

The measurements were performed between 09.00 h and 15.00 h, during this period the animals could not eat or drink. The rats in their confiners were placed singly into a tightly sealed plexiglass metabolic chamber continuously ventilated with room air. Four chambers were used simultaneously that were immersed into a thermostatically controlled

water-bath (Fig. 6). For thermoregulatory analysis of leptin, different Ta-s were applied: thermoneutrality (28 ºC) allows the activation of vasodilation (heat loss), a slightly subthermoneutral environment (25 ºC) that elicits a constant skin vasoconstriction without fluctuations in the Ts, but also leads to a slight decrease in initial Tc that facilitates the observation of hyperthermic responses, as well (Romanovsky et al., 2002).

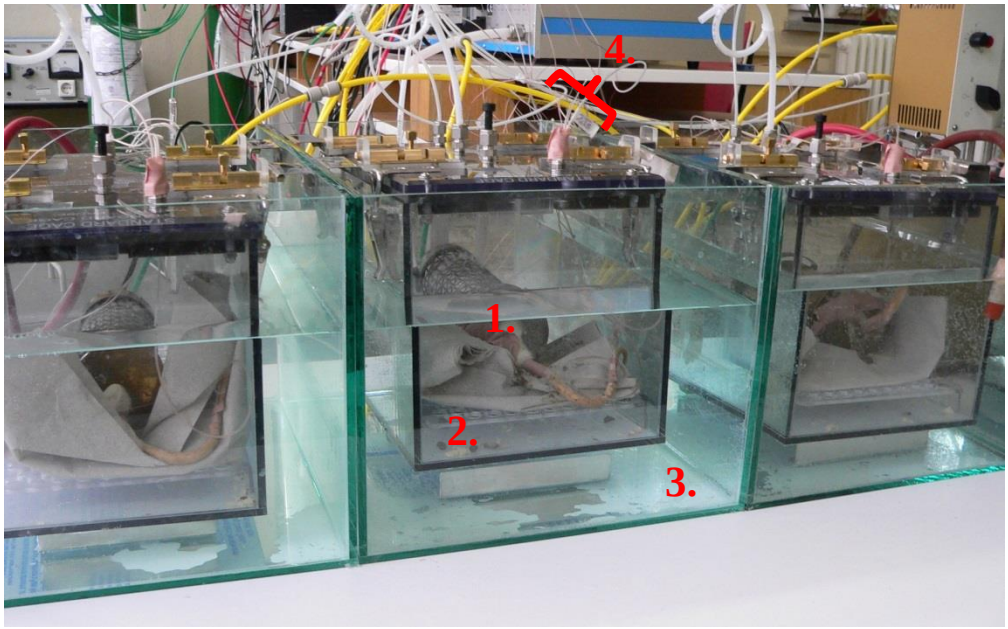

Fig. 6 *Indirect calorimetry: 1. rat in the semi-restraining cage, 2. metabolic chamber ventilated with room air, 3. thermostatically controlled water-bath, 4. extensions of the intracerebroventricular cannula and thermocouples*

The injection cannula was attached to the proximal end of the 20-25 cm-long polyethylene tube. At the distal part of the tube, 5 µl PFS was slowly injected that resulted in the ICV administration of the 5 µl leptin solution or PFS via the injection cannula.

Following the ICV injection the VO_2 was registered in 10 min intervals for 3 hours. For measuring Tc and Ts copper-constantan thermocouples were attached to the animals. The colon thermocouple was inserted 10 cm beyond the anal sphincter and fixed by tape to the tail, the tail skin thermocouple was fixed on the dorsal skin of the distal part of the tail. One thermocouple recorded Ta of the chamber. The rate of heat loss (heat loss index, HLI) was calculated from the relationship of the monitored temperatures: HLI = (Ts-Ta) / (Tc-Ta) (Romanovsky and Blatteis, 1996). HLI near 0 (Ts values approaching Ta) suggests vasoconstriction as a heat conserving mechanism, HLI near 1 (Ts values near Tc) indicates vasodilation as an appearance of heat loss activity. In the semi-restraining cages the

animals could not turn around or reach the thermocouples. The thermocouples and the extensions of the ICV cannula were pulled through a tightly sealed port of the metabolic chamber allowing us to inject the animals without causing any acute stress or discomfort.

Oxygen consumption (ml O_2/kg/min) and carbon-dioxide production (ml CO_2/kg/min) from the air perfusing the chamber were determined by indirect calorimetry (Oxymax, Equal Flow, Columbus, OH). Temperature data were collected by a Digi-Sense Benchtop Thermomether (Cole-Parmer) for electronic processing and evaluation.

Thermoregulatory functions were tested in NF animals at ages 3, 6, 12, 18 and 24.

3.3. Chronic experiments

3.3.1. Surgical interventions

In chronic experiments, an IP transmitter was implanted under IP ketamine + xylazine [78 mg/kg (Calypsol, Richter) + 13 mg/kg (Sedaxylan, Eurovet)] general anesthesia after an at least 7-day adaptation of the animals in the biotelemetric MiniMitter system (Fig. 7). After one week of recovery they had a second operation under similar anesthesia (day 0, between 09.00 and 15.00 h). This time an ICV cannula (Alzet BrainKit) was implanted into the right lateral cerebral ventricle as it was described earlier in details (3.2.1). At the same time an Alzet osmotic minipump filled with leptin or PFS was inserted underneath the skin of the nape which was connected to the outer end of the BrainKit. The anesthesia and the surgery severely influenced all parameters, therefore values of day 0 (except for BW measured on the morning preceding the operation) were excluded from all analyses.

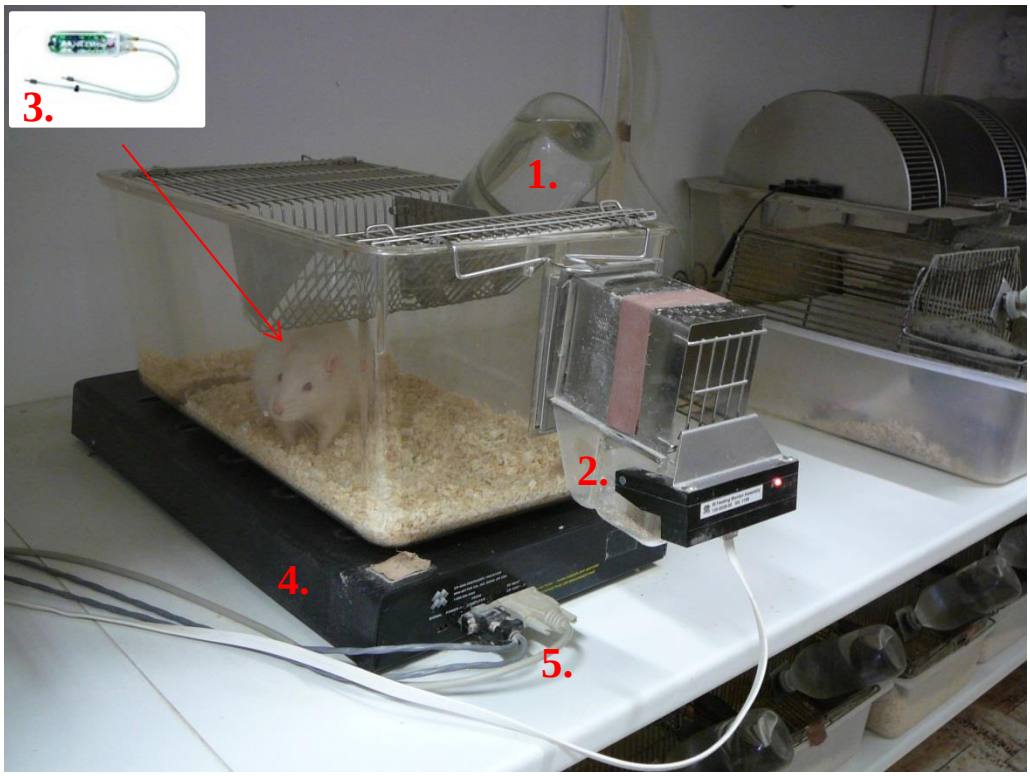

Fig. 7 *Biotelemetry: 1. water bottle, 2. feeder containing powdered rat chow, 3. intraperitoneal transmitter with heart rate electrodes (website of Starr Life Sciences), 4. receiver, 5. connections to the computer*

After the experiments rats were euthanized by an overdose of IP injection of urethane (3-5 g/kg, Reanal). The site of the brain's injection cannula was checked macroscopically by coronal sections of the removed and fixed brains in all cases. Only rats with appropriate cannula location were included in the analysis.

3.3.2. Administration of substances

The osmotic minipump was filled with leptin or PFS. The infusion reached the brain after 8-10 h and secured a standard slow ICV infusion (1 μg/μl/h leptin or 1 μl/h PFS) for a period of 7 days.

3.3.3. Assessment of food consumption and energy expenditure

The responsiveness to ICV leptin infusion regarding the regulation of energy expenditure was tested in a biotelemetric system (MiniMitter VMFH series 4000, Sunriver, OR). Animals from each group had a transmitter implanted IP, then they were placed into

the MiniMitter cage. The transmitters conveyed signals of Tc, HR [for indirect assessment of MR, (Butler, 1993)] and spontaneous (horizontal) ACT of freely moving animals. The receiver was placed underneath the MiniMitter chamber. The automatically recorded 5-min data were collected and integrated into two mean 12-h values per day, one characterizing values of the daytime (resting) period (photophase), another one the nighttime (active) period (scotophase). For data analysis the VitalView software provided by the manufacturer was used (Harkin et al., 2002).

By the help of an attached food container the daily FI was manually measured every day, together with BW.

Normally fed animals at ages 3, 6, 12, 18 and 24 months, two groups of HF rats (HF6 and HF18), and three CR groups (CR6, CR12 and CR24) were tested in these experiments.

To test the validity of HR monitoring for assessment of MR, measurements of VO_2 were performed by indirect calorimetry (Oxymax, Columbus, OH). Freely moving rats from NF12 and CR12 groups were placed singly into ventilated plexiglass metabolic chambers at Ta of 25 °C, with food (according to their feeding schedule) and water available *ad libitum*. From the air perfusing the chamber the O_2-consumption, CO_2-production were measured in 10-min intervals for a few days prior to and throughout the 7-day PFS or leptin infusion period (except for a 20-min period/day when the chamber was opened for cleaning at 09.00 h). The results were collected on a computer and the data were averaged for day-and nighttime 12-h periods according to the circadian rhythm.

3.4. Post mortem examinations

3.4.1. Body composition measurements

At the autopsy following the ICV infusion (day 8) indicators of body composition of NF, CR and HF rats were determined: the wet weights of the anterior tibial muscle, the retroperitoneal fat tissue and the epididymal fat pad were measured and expressed as percentage of the actual BW (Soós et al., 2010; Balaskó et al., 2013).

3.4.2. Studies on gene expressions

Intact NF male Wistar rats of each age-group (n = 6-7/group) were decapitated. The brains were quickly dissected, frozen in liquid nitrogen. ARC samples were punched from 1 mm thick slices [-2 to -3 mm from the bregma (Paxinos and Watson, 2006)] of the brains cut on a brain matrix (Ted Pella, CA, USA) by two razor blades. Sections were placed on a chilled mat and the mediobasal hypothalamic area containing the ARC was microdissected by a 1 mm diameter Harris punching needle (Sigma-Aldrich Budapest, Hungary). Samples were stored at -70 °C until further processing.

The total ribonucleic acid (RNA) was isolated with the Pure Link^{TM} RNA Mini Kit (Life Sciences, Carlsbad CA, USA) according to the protocol suggested by the manufacturer. Samples were homogenized, RNA was purified by ethanol treatment, and eluted from the membrane. The total amount of RNA was determined by NanoDrop (Thermo Scientific). High-capacity complementary deoxyribonucleic acid (cDNA) kit was applied (Applied Biosystems, Foster City, CA, USA) to perform cDNA synthesis, using 1 μg of total RNA sample according to the official protocol.

For gene expression analysis, qRT-PCR was performed using SensiFast SYBR Green reagent (BioLine). Amplifications were run on ABI StepOnePlus system. StepOne software was used to analyze gene expressions, which was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. Based on the quality of previous PCR reference curves in male Wistar rats, the GAPDH was chosen as a reference gene (Füredi et al., 2016). The primer sequences are shown in Table 1. PCR conditions were also set according to previous studies (Füredi et al., 2016): one cycle 95 °C for 2 minutes, 40 cycles at 95 °C for 5 seconds and 60 °C for 30 seconds. The amplification of PCR products were calculated according to the $2^{-\Delta\Delta Ct}$ method.

Table 1 *Primer sequences*

Primers	Forward (5'-3')	Reverse (5'-3')
Ob-Rb	TCTGGAGCCTGAACCAGTTT	GGAAGTGCTCCACCCGATAG
SOCS-3	GGATTCTACTGGAGTGCCGT	CTCAGTGTGAAGAAGTGGCG
GAPDH	TGCCATCACTGCCACTCAGA	GTCAGATCCACAACGGATACATTG

3.5. Statistical analysis

Data were statistically analyzed by one-way, two-way and repeated-measures ANOVA tests, complemented by Tukey's or Scheffe's *post hoc* tests, when more than two groups were compared (SPSS 11.0 for Windows). Regarding analysis of MR (VO_2), ANCOVA was applied with covariate $BW^{0.75}$ (Tschöp et al., 2011). Experimental results are expressed as mean values ± S.E.M. in the figures. All experimental groups contained at least 6-8 rats. The level of significance was set at $p < 0.05$.

3.6. Ethical issues

In all experimental interventions and procedures both the general regulations and the special rules of the permit obtained from the University of Pécs Ethical Committee for the Protection of Animals in Research (BA 02/200-11/2011) were strictly observed, in accordance with the main directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC, Directive 2010/63/EU of the European Parliament and of the Council).

4. Results

4.1. Acute leptin administration

4.1.1. Characteristics of the experimental groups including mean BW values before and following 48-h fasting and 4-h re-feeding energy intake values

Table 2 shows BW of the different age-groups of treated vs. control animals. Initial BW-s of treated and those of control animals did not differ within age-groups, as shown by one-way ANOVA ($p > 0.05$ in all cases, Table 2).

Mean BW values of different NF age-groups (Table 2) were in accord with those observed in our previous studies (Balaskó et al., 2013): up to 18 months of age BW showed a rising tendency, then it started to decline slightly in the oldest animals. Mean BW values of HF rats (Table 2) exceeded those of age-matched NF groups. In HF12 BW values were significantly higher than those of all NF rats. Although HF6 weighed less than HF12, their BW was comparable to NF12, twice their age (Table 2).

Upon 48-h fasting weight loss of NF age-groups ranged from 20-45 g corresponding to 7 % to 11 % of initial BW. Weight loss of HF animals reached 25-35 g corresponding to 4-6 % of initial BW. In NF rats, the subsequent cumulative 4-h energy intake (during re-feeding) expressed in kJ ranged from 80 to 110 kJ (Table 2). Re-feeding expressed in kJ was largest in the HF animals, values of HF12 exceeded those of HF6 (Table 2).

Table 2 *Body weight (BW) values before and after a 48-h fasting and cumulative energy intake (FI) during the consequent 4-h re-feeding of rats belonging to different age-groups and nutritional states*

group (age and nutritional state):	BW (g) before fasting	BW (g) before re-feeding	4h cumulative FI (kJ)
NF3 control:	336.2 ± 8.7	307.3 ± 9.1	84.3 ± 7.3
NF3 leptin:	339.0 ± 8.3	310.5 ± 6.7	38.5 ± 7.9 [c]
NF6 control:	443.6 ± 10.6	410.1 ± 11.8	90.4 ± 5.4
NF6 leptin:	434.1 ± 11.7	396.7 ± 13.8	62.3 ± 6.4
HF6 control:	590.3 ± 16.6 [*]	562.0 ± 19.9 [*]	159.2 ± 9.8 [b]
HF6 leptin:	616.3 ± 30.3 [*]	588.8 ± 63.5 [*]	92.4 ± 17.8
NF12 control:	531.2 ± 20.3	487.5 ± 18.9	106.3 ± 8.4
NF12 leptin:	514.7 ± 22.5	471.8 ± 20.3	73.3 ± 14.4
HF12 control:	701.6 ± 51.7 [#]	667.8 ± 50.8 [#]	246.5 ± 20.5 [a]
HF12 leptin:	658.8 ± 50.9 [#]	636.8 ± 48.8 [#]	115.0 ± 13.9 [c]
NF18 control:	521.3 ± 30.6	486.6 ± 31.4	91.1 ± 4.6
NF18 leptin:	530.8 ± 24.5	490.6 ± 26.1	66.0 ± 3.5
NF24 control:	517.8 ± 26.5	469.8 ± 24.3	100.8 ± 16.2
NF24 leptin:	494.8 ± 24.9	450.5 ± 21.9	35.8 ± 11.3 [c]

Values are expressed as the mean ± S.E.M. for at least six-eight rats in each group. For statistical analysis of the data shown in this table one-way ANOVA with Tukey's post hoc test was applied.

BW values of control vs. leptin-treated groups of the same age and nutritional state did not differ. Concerning initial BW values the following statistically significant differences were denoted in the table: [#] *HF12 vs. all NF groups ($p < 0.05$),* [*] *HF6 vs. age-matched NF and younger NF groups ($p < 0.05$). The above described differences also appear among after-fasting BW values.*

Regarding 4-h cumulative FI values of control groups the following statistically significant differences were denoted in the table: "a" HF12 vs. all NF groups ($p < 0.05$), "b" HF6 vs. age-matched NF and younger NF group ($p < 0.05$).

Leptin treatment reduced 4-h cumulative FI significantly compared to controls of the same age and nutritional state in the following groups: "c" NF3 ($p < 0.05$), NF24 ($p < 0.01$), HF12 ($p < 0.001$).

4.1.2. Effects of central leptin injection on FI

In young adult rats (NF3) the ICV administered acute leptin injection (1 μg) caused a strong suppression of 4-h cumulative FI during re-feeding following 48-h fasting [repeated-measures ANOVA for the 4-h re-feeding period from 30 to 240 min: $F(1,10) = 11.700$, $p = 0.007$, Fig. 8]. Leptin failed to induce a significant anorexigenic response in younger and older middle-aged (NF6: $F(1,16) = 4.152$, $p = 0.058$; NF12: $F(1,10) = 4.240$, $p = 0.066$, Fig. 10) and also in aging rats (NF18: $F(1,10) = 1.514$, $p = 0.254$, not shown). Interestingly, the leptin-induced anorexigenic response became significant again in old rats (NF24: $F(1,14) = 6.517$, $p = 0.023$, not shown). Fig. 9 summarizes the age-related pattern of acute central leptin administration-induced anorexia: the rates of suppression of the cumulative FI (240 min) given in % of the corresponding control FI value are shown for all NF age-groups. These results suggest that the anorexigenic effects of an acute central administration of leptin show non-linear changes with aging: young adult animals show the strongest, while middle-aged rats show the weakest response.

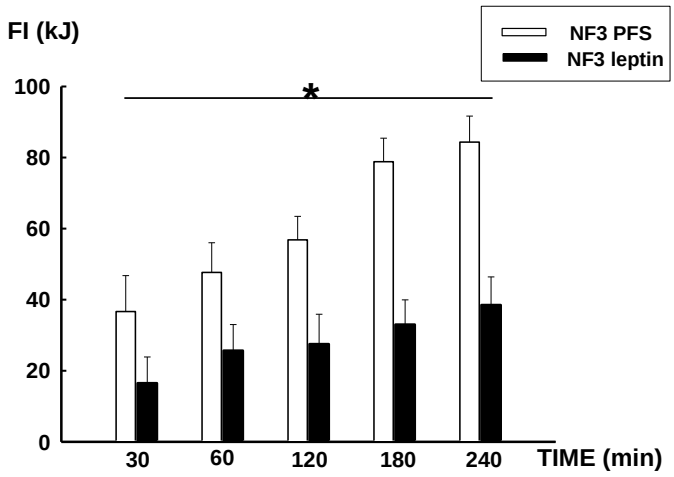

Fig. 8 *Reduction in the cumulative 4-h food intake (FI) induced by 48-h fasting expressed in kJ-s in normally fed young adult (NF3) rats following acute intracerebroventricular (ICV) leptin (1 μg) or pyrogen-free saline (PFS) treatment. Asterisk (*) indicates significant difference between re-feeding of ICV leptin-treated and control rats (repeated-measures ANOVA for the 4-h re-feeding period from 30 to 240 min: p = 0.007; n = 6-8 rats/group).*

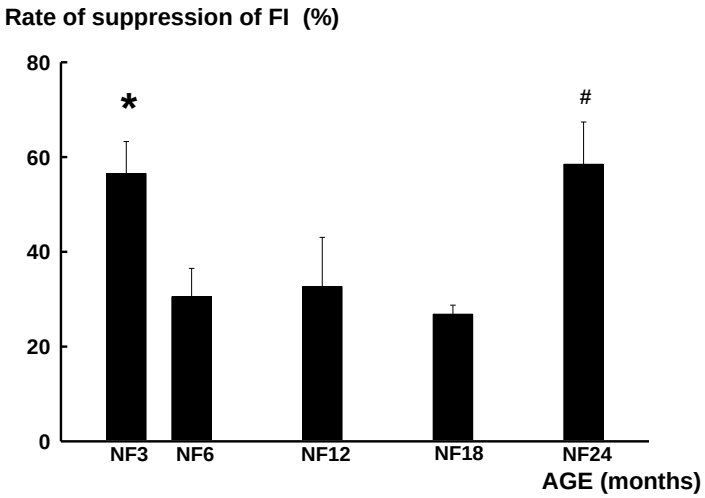

Fig. 9 *The rate of leptin-induced suppression in the cumulative 4-h food intake (FI) induced by 48-h fasting (expressed in percentage of control FI) in different age-groups of normally fed (NF) rats. Asterisk (*) indicates significant differences between the rates of suppression in NF3 vs. NF6 or NF18 (one-way ANOVA with Tukey's post hoc test, $p < 0.05$), the number sign (#) indicates significant differences between values of NF24 vs. NF6 or NF18 (one-way ANOVA with Tukey's post hoc test, $p < 0.05$; n = 6-8 rats/age-group).*

Regarding the obese animal groups (HF6, HF12), alterations in the nutritional state influenced the responsiveness to the hormone. The younger obese group failed to show a significant reduction in re-feeding FI (HF6: 19.64 ± 9.7 % suppression by 240 min, $F(1,10) = 1.062$, $p = 0.337$, Fig. 10). Surprisingly, older middle-aged obese rats, demonstrated a significant anorexigenic responsiveness to leptin (HF12: 51.93 ± 7.3 % suppression by 240 min, $F(1,10) = 8.340$, $p = 0.02$, Fig. 10).

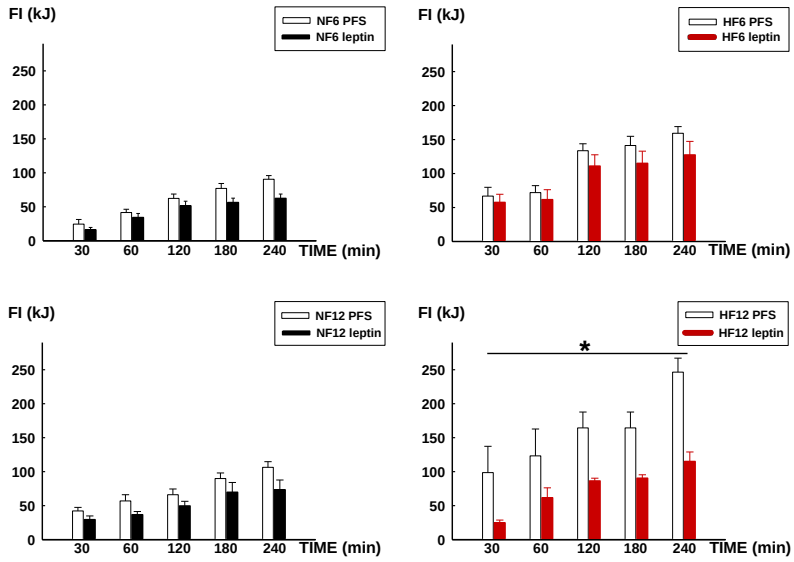

Fig. 10 *Reduction in the cumulative 4-h food intake (FI) induced by 48-h fasting, expressed in kJ-s in normally fed younger and older middle-aged (NF6 and NF12, respectively) and high-fat diet-induced obese younger and older middle-aged (HF6 and HF12, respectively) rats following intracerebroventricular (ICV) leptin (1 μg) or pyrogen-free saline (PFS) treatment. Asterisk (*) indicates significant difference between re-feeding values of ICV leptin-treated and control HF12 rats (repeated-measures ANOVA for the 4-h re-feeding period from 30 to 240 min: p = 0.02; n = 6-8 rats/group).*

These findings suggest that obesity may influence age-related central leptin resistance in a complex way, i.e. obesity may not always elicit or aggravate leptin resistance, it may promote some features of central leptin responsiveness in some age-groups.

4.1.3. Effects of central leptin injection on MR and thermoregulation

Although initially, three different doses of leptin (1, 4 and 10 μg) were tested in young adult rats and all doses elicited similar, significant hyperthermic responses, the 4 μg dose was chosen for the thermoregulatory analysis, because this dose elicited the largest increase in VO_2 ($p < 0.05$, comparison of different doses not shown).

For thermoregulatory analysis, two different Ta-s, thermoneutrality (28 ºC, results not shown) and a slightly subthermoneutral environment (25 ºC) were applied. The young adult age-group (NF3) showed a leptin-induced significant increase in MR (represented by VO_2, at both Ta-s), with a consequent significant rise in Tc. The rise in VO_2 developed 40 min following the ICV injection, the increase in Tc started at 60 min postinjection. Since leptin-induced hypermetabolism and hyperthermia was stronger at 25 °C Ta, thereafter this Ta was applied in all other age-groups. Fig. 11 demonstrates leptin-induced hypermetabolic/hyperthermic effects (4 μg dose) at 25 °C in young adult rats [ANOVA repeated measures for VO_2: $F(1,11) = 9.331$, $p = 0.011$; Tc: $F(1,11) = 155.797$, $p = 0.001$].

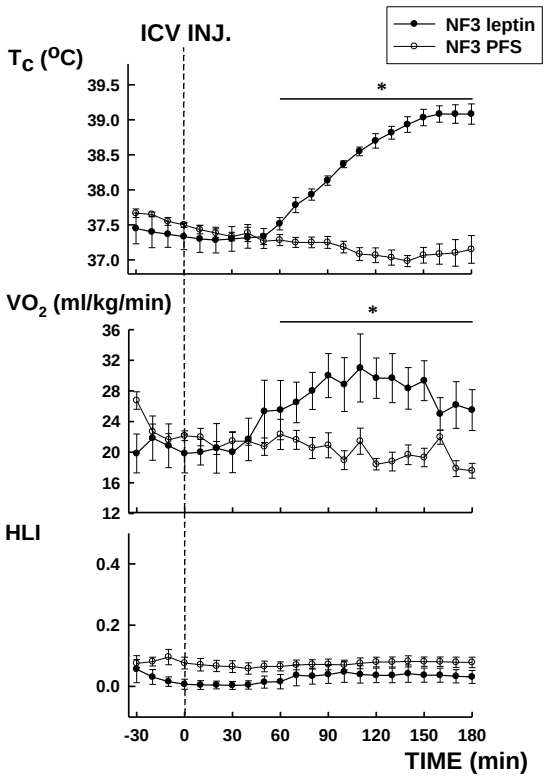

Fig. 11 *Effects of an intracerebroventricular (ICV) leptin (4 μg) injection on metabolic rate and body temperature of normally fed young adult rats (NF3) at an ambient temperature of 25 ºC. The curves represent recordings of core body temperature (Tc), oxygen consumption (VO_2) and heat loss index (HLI). Full symbols represent changes following leptin-treatment, empty symbols represent controls [effects of pyrogen-free saline (PFS) injection]. Asterisks (*) indicate significant differences between Tc or VO_2 changes of leptin-treated vs. control rats (repeated-measures ANOVA from 60 to 180 min: $p = 0.001$ for Tc, $p = 0.011$ for VO_2; $n = 6$-8 rats/group).*

Vasodilation, indicated by HLI, failed to develop even at a thermoneutral Ta (28 °C), despite the hyperthermia, suggesting a coordinated hyperthermic response to the hormone, similar to febrile reactions, such as prostaglandin E-induced hyperthermia or experimental endotoxin fever. The anorexic effect of leptin also fits the pattern of sickness behavior adjoining fever.

The maximal increase in Tc (Fig. 12) and VO_2 (Fig. 13) varied with aging. Young adult animals showed the biggest leptin-induced hyperthermic and hypermetabolic response ($p < 0.001$ for Tc, $p < 0.001$ for VO_2). Younger- and older middle-aged animals showed more moderate leptin-induced increases in Tc (NF6: $p = 0.001$, NF12: $p < 0.001$, respectively) and VO_2 (NF6: $p = 0.023$, NF12: $p = 0.047$, respectively) as compared with their respective controls. In contrast, in aging and old rats leptin failed to increase Tc or VO_2 to a significant extent. The maximal leptin-induced rises in Tc and that of VO_2 of the 18-month group differed from those of young adult animals (3- vs. 18-month $p = 0.013$ for Tc and $p = 0.012$ for VO_2, 3- vs. 24-month $p = 0.012$ for Tc, one-way ANOVA for Tc and ANCOVA analysis for VO_2). According to overall statistical analyses, the hypermetabolic/hyperthermic effects of leptin were influenced by aging (two-way ANOVA for Tc and VO_2: $p = 0.031$ and $p = 0.019$, respectively). Heat loss mechanisms did not seem to be activated as no vasodilation developed in any of the groups. The hypermetabolic/hyperthermic effects of leptin seem to show a monotonous decline with aging. The age-dependence of the hypermetabolic/hyperthermic responses appears to be different from that of the anorexigenic ones.

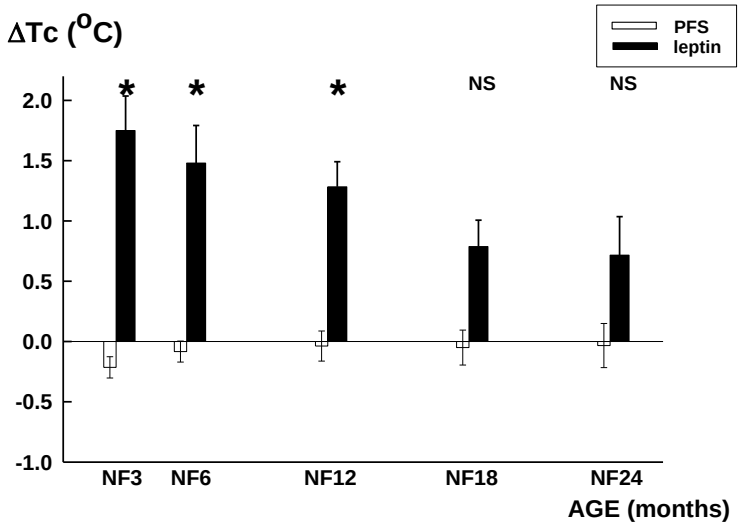

Fig. 12 *Leptin-induced hyperthermia in different age-groups of normally fed (NF) rats. Full columns represent changes in core body temperature (ΔTc) at 180 min following an intracerebroventricular (ICV) leptin injection (4 μg), empty columns indicate similar values of controls following ICV pyrogen-free saline (PFS) injection. Initial core body temperature values were similar in all age-groups (ranging from 37.1 ± 0.1 to 37.5 ± 0.3 ºC). Asterisks (*) indicate significant differences between ΔTc of ICV leptin-treated and control rats of the same age-group (one-way ANOVA: NF3 p < 0.001, NF6 p = 0.001, NF12 p < 0.001, NS: non-significant; n = 6-8 rats/group).*

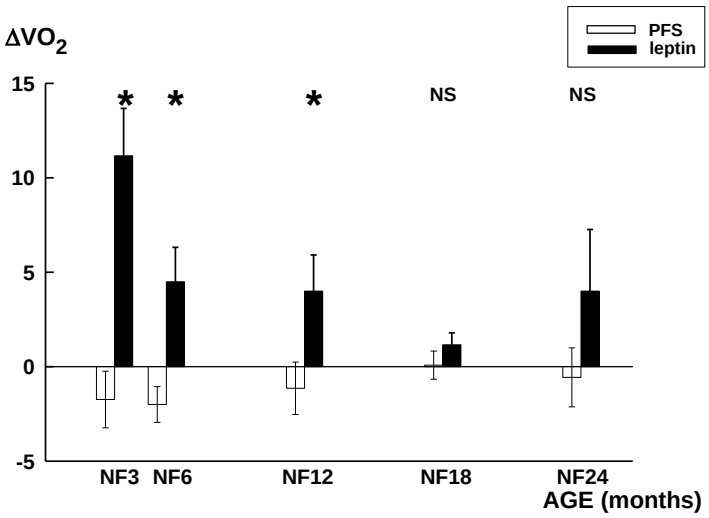

Fig. 13 *Leptin-induced hypermetabolism in different age-groups of normally fed (NF) rats. Full columns represent the maximal changes in oxygen consumption (ΔVO_2) following an intracerebroventricular (ICV) leptin injection (4 μg), empty columns indicate similar values of controls following ICV pyrogen-free saline (PFS) injection. Initial VO_2 values were similar in all age-groups (ranging from 17.8 ± 1.5 to 22.2 ± 1.5 ml/kg/min). Asterisks (*) indicate significant differences between ΔVO_2 of ICV leptin-treated and control rats of the same age (ANCOVA: NF3 p < 0.001, NF6 p = 0.023, NF12 p = 0.047, NS: non-significant; n = 6-8 rats/group).*

4.2. Chronic leptin administration

4.2.1. Effects of central leptin infusion on BW and body composition indicators

Concerning the effects of age on initial BW values of the NF groups, one-way ANOVA with Scheffe's post hoc test showed a difference between the values of NF3 and those of all the older NF age-groups ($p < 0.001$). No further differences were detected from NF6 to NF24.

Regarding all experimental groups, initial BW reached the highest level in the HF18 group followed by the HF6, NF18, NF24, NF12, NF6, the CR groups and finally the NF3 (Fig. 14). Indicators of body composition showed different patterns for fat pads and muscle mass in control groups (Table 3). Epididymal and retroperitoneal fat pads were significantly larger in the HF groups than those of all other animals, followed by the values of NF6-24 with non-significant differences among them and then by those of NF3. Fat pads reached the lowest wet weight in CR animals. Their values were significantly smaller than those of HF or NF rats. The muscle mass indicator suggested the appearance of sarcopenia in the oldest NF24 group, otherwise all other values were similar. Calorie-restriction in our study, therefore, did not cause sarcopenia. (All p-values of one-way ANOVA test were smaller than 0.05.)

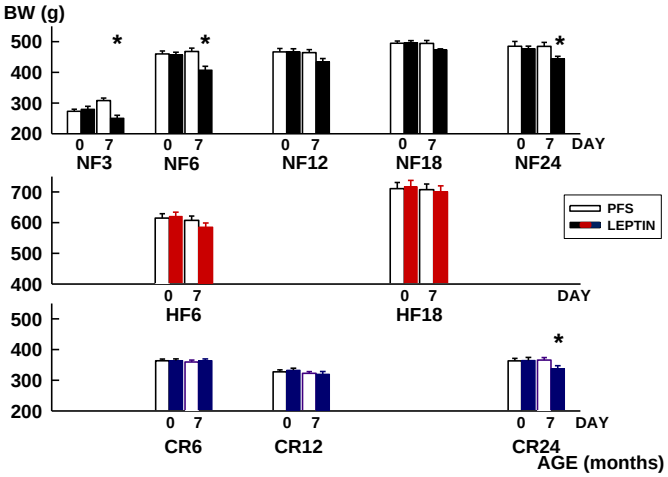

Fig. 14 *Body weight (BW) values on day 0 and day 7 of an intracerebroventricular leptin (closed bars) or pyrogen-free saline (PFS, open bars) infusion. Asterisks (*) indicate significant differences between day 7 BW of leptin- vs. PFS-treated rats (n = 6-8 rats/group). NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted. P values of the one-way ANOVA tests are: p = 0.049 for NF3, p = 0.007 for NF6, p = 0.031 for NF24, p = 0.029 for CR24.*

Table 3 *Indicators of body composition after leptin or pyrogen-free saline (PFS) infusion*

Group (age and nutritional state)	epididymal fat (g/100 g body weight)		retroperitoneal fat (g/100 g body weight)		tibialis anterior muscle (g/100 g body weight)	
	PFS	leptin	PFS	leptin	PFS	leptin
NF3	0.37±0.02	0.01±0.01*	0.32 0.02	0.01±0.01*	0.20±0.01	0.20±0.01
NF6	0.47±0.02	0.12±0.04*	0.48±0.04	0.01±0.01*	0.19±0.01	0.19±0.01
HF6	1.12±0.08	0.75±0.05*	1.90±0.10	1.08±0.13*	0.18±0.01	0.19±0.01
CR6	0.26±0.01	0.17±0.03	0.07±0.03	0.01±0.01	0.20±0.02	0.20±0.01
NF12	0.53±0.05	0.53±0.09	0.47±0.07	0.33±0.07	0.19±0.01	0.19±0.01
CR12	0.25±0.05	0.22±0.02	0.12±0.02	0.10±0.04	0.21±0.02	0.20±0.01
NF18	0.55±0.07	0.48±0.01	0.64±0.14	0.44±0.11	0.17±0.01	0.14±0.02
HF18	0.90±0.12	1.05±0.04	1.50±0.36	1.48±0.24	0.15±0.01	0.14±0.01
NF24	0.41±0.02	0.33±0.06	0.59±0.09	0.21±0.12*	0.13±0.01	0.16±0.01
CR24	0.20±0.09	0.12±0.04	0.02±0.04	0.01±0.01	0.18±0.02	0.19±0.01

Values are expressed as the mean ± S.E.M. for at least six-eight rats in each group.
NF: normally fed, CR: calorie-restricted, HF: high-fat diet-induced obese. Numbers following these abbreviations indicate the age of the animal groups in months. Asterisks (*) indicate significant differences between corresponding values of leptin- vs. PFS-treated rats. All p values of the one-way ANOVA tests for epididymal fat are lower than 0.001. For retroperitoneal fat: NF3 p = 0.028, NF6, HF6 p < 0.001, NF24 p = 0.049.

Controls of the youngest NF3 age-group showed significant growth (from 273.0 ± 7.0 g to 308.0 ± 8.3 g, p = 0.007, i.e. 12.8 ± 0.9 % growth by the 7th day of the PFS infusion, Fig. 14) corresponding to their normal growth rate that was missing in all older animals.

The 7-day leptin infusion suppressed BW in the two youngest and the oldest NF age-groups compared with the corresponding 7th-day value of their control animals, while the smaller weight loss of the NF12, NF18 leptin-treated groups did not reach statistical significance (Fig. 14). The weight loss calculated as percentage of the initial day 0 BW

also showed a characteristic age-related pattern: compared to the values of the three youngest age-groups [NF3: −10.3 ± 2.7 % (cf. +12.6 ± 0.9 % growth in their controls), NF6: −10.8 ± 1.0 %, NF12: −7.1 ± 0.5 %] the BW decline of the NF18 became more moderate (−4.7 ± 1.6 %) and finally NF24 old rats exhibited a more pronounced weight reduction again (−7.0 ± 0.6 %). Regarding body composition indicators following leptin infusion muscle mass failed to change significantly in any groups (Table 3). Both fat pads were found to be significantly reduced in the two youngest NF age-groups, corresponding to changes in BW. While leptin failed to reduce fat pads in the late middle-aged NF12 and in aging NF18 groups, in the old NF24 rats leptin decreased the retroperitoneal fat by more than 50 %.

Groups of altered nutritional states failed to lose BW during the leptin infusion as compared with their PFS-treated controls with the exception of CR24 (Fig. 14). These old CR rats lost 7.5 ± 0.3 % of their initial BW (cf. the similar 7.0 ± 0.6 % loss in NF24). Leptin-induced loss of fat mass was significant in HF6 but not in HF18 (Table 3). Although in CR groups, fat pads practically disappeared upon leptin administration, statistical tests failed to show significance due to the very low fat content of PFS-treated CR rats. No infusion-induced change in muscle mass was observed. (All p-values of one-way ANOVA were smaller than 0.05.)

4.2.2. Effects of central leptin infusion on FI

Among NF rats no differences of FI were observed in the pre-infusion period. The ICV leptin administration suppressed their daily FI values throughout the infusion without any sign of recovery. The reduction of the 7-day cumulative energy intake (in kJ, from day 1 to day 7) was found to be significant in all age-groups as compared with PFS-infused controls (Fig. 15, one-way ANOVA). This suppression (the difference between the values of corresponding control and leptin-treated groups expressed as percentage of the control energy intake also shown in Fig. 15) was similar (though kept decreasing gradually) in the three youngest age-groups (NF3: 54.4 %, NF6: 43.6 %, NF12: 39.5 %), became weak in NF18 (19.0 %) but returned again to a higher value in old NF24 rats (38.8 %). In a different approach (not shown in figures), using daily FI values of NF age-groups throughout the infusions (from day 1 to day 7), two-way repeated-measures ANOVA revealed an age-related effect on leptin-induced anorexia [$F(4, 61) = 3.171$, $p = 0.020$] with

a significantly weaker effect in NF18 rats. Their values differed from all other NF age-groups ($p < 0.05$, Scheffe's posthoc test).

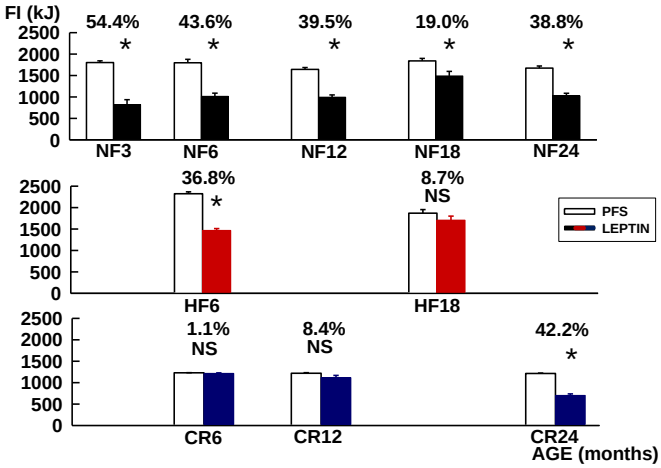

Fig. 15 *Cumulative food intake (FI in kJ) values from day 1 to day 7 in the course of a 7-day intracerebroventricular leptin (closed bars) or pyrogen-free saline (PFS, open bars) infusion. Asterisks (*) indicate significant differences between leptin vs. PFS-treated rats (n = 6-8 rats/group). Above the bars the rate of leptin-induced suppression (the difference between the values of corresponding control and leptin-treated groups expressed as percentage of the control energy intake) is also indicated. NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted. P values of the one-way ANOVA tests are: $p < 0.001$ for NF3, NF6, NF12 and NF24, $p = 0.009$ for NF18, $p < 0.001$ for CR24, $p < 0.001$ for HF6.*

High-fat diet-induced obesity diminished the leptin-induced anorexia in HF6 (36.8 % vs. 43.6 % in NF6) and abolished it completely in HF18 (8.7 % vs. 19.0 % in NF18) (Fig. 15).

In contrast, in CR animals the age-dependence was different: no leptin-induced anorexia was found in the 6- and 12-month-old animals (despite leptin administration, these hungry animals consumed practically all the provided food), but the suppression of cumulative FI became significant in CR24 (42.2 %) exceeding even the corresponding NF24 value (38.8 %) (Fig. 15).

4.2.3. Effects of central leptin infusion on HR and VO_2

Basic HR (indirectly indicating changes of MR) gradually declined with aging in NF rats (Fig. 16). During the course of the ICV leptin infusion the mean daytime HR

values (inactive period, nadir of the circadian rhythm) rose significantly in the three youngest NF age-groups. When comparing leptin vs. PFS-treated animals, repeated-measures ANOVA showed significant differences from day 1 to day 6: NF3 [$F(1, 11) = 13.102$, $p = 0.004$], NF6 [$F(1, 13) = 8.227$, $p = 0.013$], NF12 [$F(1, 27) = 866.69$, $p < 0.001$]. Regarding the mean nighttime HR data (active period, maxima of the circadian rhythm), in NF3 moderate transient rise (from the day 3 to day 6 of the infusion), in NF12 a more sustained elevation (from day 1 to day 6) was observed: NF3 [$F(1, 11) = 5.298$, $p = 0.042$] and NF12 [$F(1, 27) = 879.40$, $p < 0.001$]. Mean HR values did not change in the two oldest NF18 or NF24 animals.

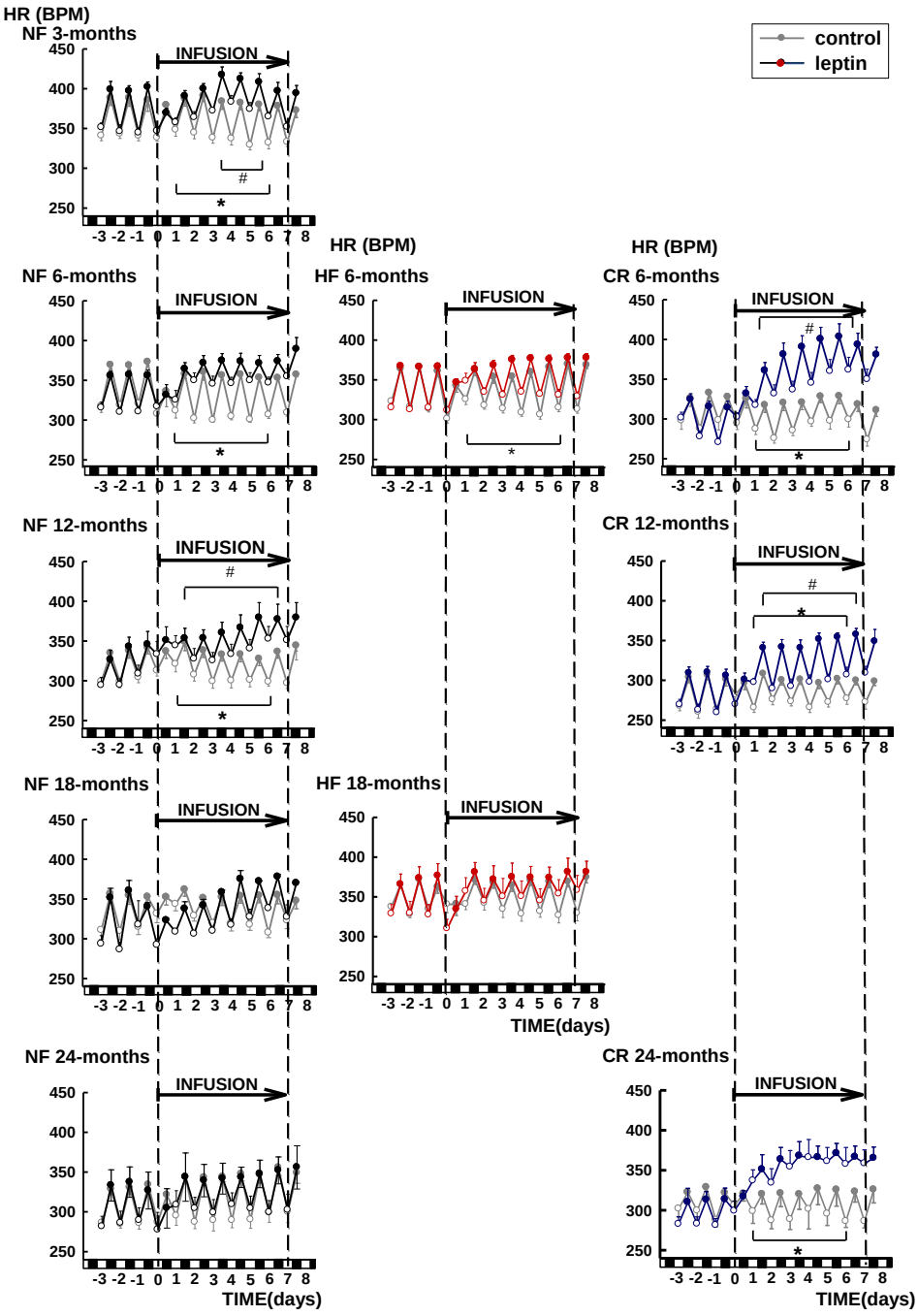

Fig. 16 *Circadian changes of heart rate (HR) expressed in beats per minute (BPM): 12-h averaging (mean ± S.E.M.) for the active (nighttime, closed symbols) and inactive (daytime, open symbols) periods during intracerebroventricular infusion of leptin (colored) or pyrogen-free saline (control, gray). Asterisks (*) indicate significant differences for daytime mean HR values, number signs (#) indicate differences for the nighttime HR values calculated by repeated-measures ANOVA (n = 6-8 rats/group). NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted.*

High-fat diet-induced obesity tended to increase the basal HR values (Fig. 16). Obesity decreased leptin-induced daytime tachycardia in HF6 to a more moderate level from that of age-matched NF [HF6: from day 1 to day 6, $F(1, 19) = 8.173$, $p = 0.010$] and abolished it completely in HF18.

In contrast, calorie-restriction tended to decrease the basal HR values (Fig. 16). CR rats exhibited a different pattern concerning leptin-induced tachycardia: a pronounced HR-rise seen in CR6 rats (day and night, exceeding those seen in NF6 rats) was attenuated in middle-aged CR12 (moderate rise in day- and nighttime values) and became pronounced again for daytime values in old CR24 rats (elevation of daytime minima). Results of repeated-measures ANOVA tests for leptin vs. corresponding control groups were: HR minima from day 1 to day 6 in CR6 [$F(1, 17) = 11.537$, $p = 0.003$], in CR12 [$F(1,20) = 7.714$, $p = 0.012$] and in CR24 rats [$F(1, 7) = 8.306$, $p = 0.024$]; HR maxima from day 1 to day 6 in CR6 [$F(1, 17) = 17.579$, $p = 0.001$] and in CR12 [$F(1, 20) = 25.803$, $p < 0.001$]. In CR24 the elevation of HR maxima failed to reach statistical significance.

Oxygen consumption (VO_2) data (used for direct assessment of MR) of NF12 and CR12 rats obtained during the infusion periods (in control or leptin-treated rats) showed close correlation with the respective infusion-induced changes in HR (Table 4). Significant differences were detected between daytime and nighttime VO_2 values of leptin-treated rats on day-5 of the infusion and corresponding initial (pre-infusion) reference values or those of their respective control PFS-treated animals. When comparing daytime and nighttime leptin-hypermetabolism in different nutritional states, the rise was significantly higher in CR12 than in NF12 rats ($p < 0.001$ for daytime results, $p = 0.028$ regarding nighttime data) (Table 4).

Table 4 *Leptin-induced rise in daytime and nighttime oxygen consumption (VO_2) of normally fed (NF) and calorie-restricted (CR) 12 month-old rats*

Daytime VO_2			
Group	Initial reference values	Day-5 of the control (PFS) infusion	Day-5 of the leptin infusion
NF12	20.44 ± 0.42 (100 %)	21.31 ± 0.41 (104 %)	24.25 ± 0.50* (119 %)
CR12	19.52 ± 0.39 (100 %)	19.66 ± 0.33 (101 %)	27.90 ± 0.48*# (143 %)

Nighttime VO_2			
Group	Initial reference values	Day-5 of the control (PFS) infusion	Day-5 of the leptin infusion
NF12	25.40 ± 0.45 (100 %)	26.85 ± 0.56 (106 %)	29.35 ± 0.58* (116 %)
CR12	24.58 ± 0.47 (100 %)	24.80 ± 0.51 (101 %)	31.15 ± 0.58*# (128 %)

Values are expressed as the mean ± S.E.M. for at least six-eight rats in each group.

The oxygen consumption (VO_2 expressed in ml/kg/min) of the normally fed (NF12) and calorie-restricted (CR12) 12 month-old groups of rats at daytime vs. nighttime (upper vs. lower panel). Initial reference values were recorded before implantation of the intracerebroventricular cannula. Results obtained on day-5 of the infusion periods (in control or leptin-treated rats) are also expressed as a percentage of the corresponding initial reference values. Asterisks (*) show significant differences (one-way ANOVA) between VO_2 values of leptin-treated rats and corresponding initial reference values or those of control animals gained at corresponding time points of the infusions (in all cases $p < 0.001$). Number signs (#) indicate significant differences between corresponding values of CR12 vs. NF12 rats on day-5 of the leptin infusion (for daytime results $p < 0.001$, regarding nighttime data $p = 0.028$).

4.2.4. Effects of central leptin infusion on Tc

In NF3 rats, ICV leptin infusion – as compared with controls – was accompanied by an elevation of both mean daytime and nighttime temperatures (Fig. 17): for Tc minima from day 1 to day 6 [$F(1,9) = 7.912$, $p = 0.020$]; Tc maxima from day 1 to day 3 [$F(1, 9) = 5.21$, $p = 0.048$]. In NF6 rats only the daytime (resting) body temperatures rose significantly from day 1 to day 6 [$F(1, 12) = 8.267$, $p = 0.014$]. This hyperthermia was still present, though less pronounced and of shorter duration in NF12 and NF18 rats, and was

completely missing in the oldest group. Results of repeated-measures ANOVA tests for leptin vs. corresponding control groups were: from day 1 to day 4 for Tc minima in NF12 [$F(1, 27) = 4.84$, $p = 0.037$], from day 1 to day 5 for their Tc maxima [$F(1, 27) = 3.84$, $p = 0.047$]; from day 1 to day 3 for Tc minima in NF18 [$F(1, 6) = 7.111$, $p = 0.037$], for their Tc maxima [$F(1, 6) = 8.201$, $p = 0.029$]. In the oldest NF24 animals Tc values did not show any change to leptin infusion.

High-fat diet-induced obesity abolished leptin-induced hyperthermia in both HF groups (Fig. 17).

Calorie-restriction enhanced leptin-induced day- and nighttime hyperthermia in comparison with those of their NF counterparts (Fig. 17). Results of repeated-measures ANOVA tests for leptin vs. corresponding control groups were: from day 1 to day 6 for Tc minima in CR6 [$F(1, 11) = 32.581$, $p < 0.001$], for their Tc maxima [$F(1, 9) = 52.933$, $p < 0.001$]; for Tc minima in CR12 [$F(1, 6) = 7.111$, $p = 0.037$], for their Tc maxima [$F(1, 6) = 8.201$, $p = 0.029$]; for Tc minima in CR24 [$F(1, 7) = 8.55$, $p = 0.022$], for the first two days of Tc maxima [$F(1, 7) = 6.582$, $p = 0.037$].

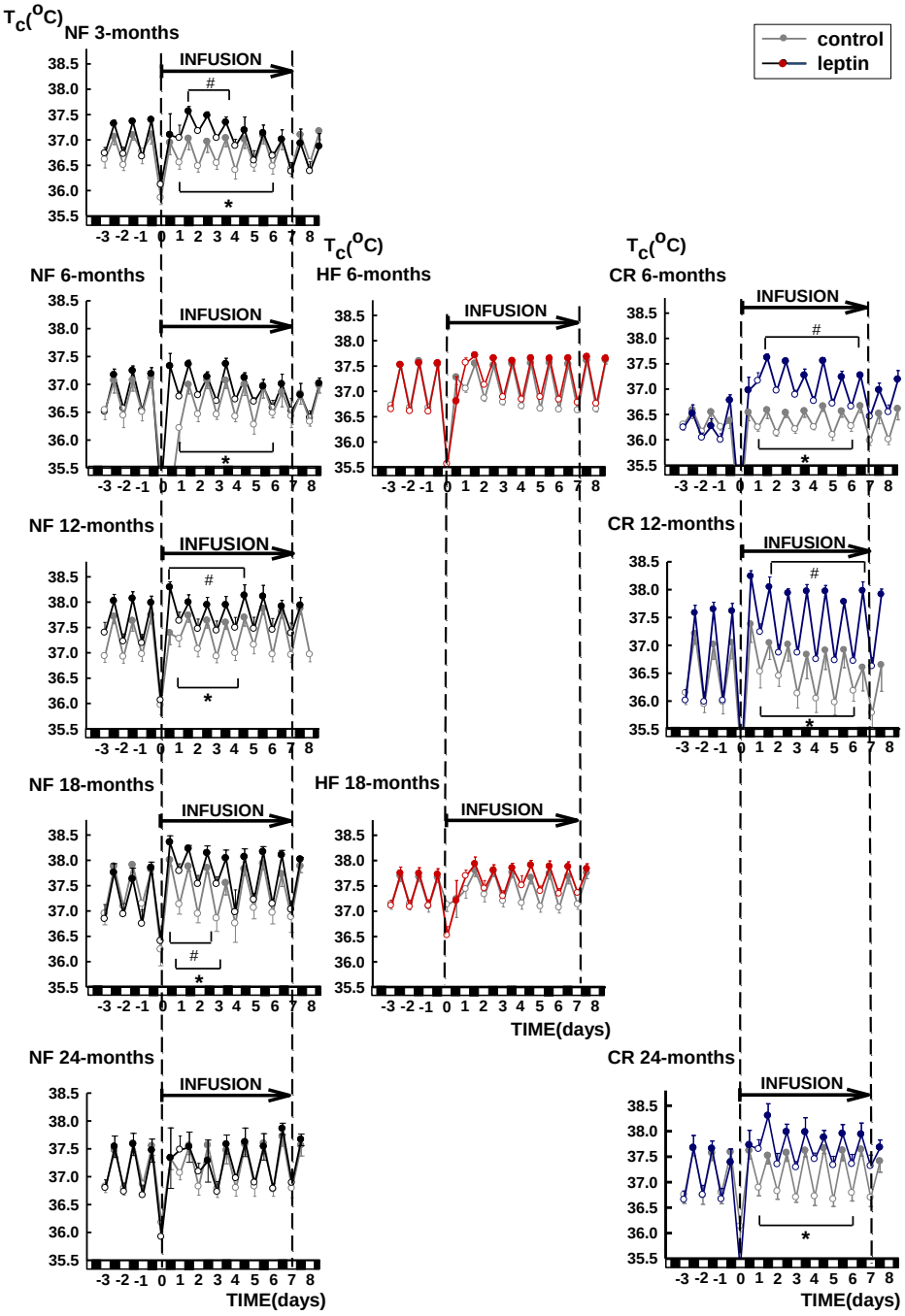

Fig. 17 *Circadian changes of core body temperature (Tc): 12-h averaging (mean ± S.E.M.) for the active (nighttime, closed symbols) and inactive (daytime, open symbols) periods during intracerebroventricular infusion of leptin (colored) or pyrogen-free saline (control, gray). Asterisks (*) indicate significant differences for daytime mean Tc values, number signs (#) indicate differences for the nighttime temperatures calculated by repeated-measures ANOVA (n = 6-8 rats/group). NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted.*

4.2.5. Effects of central leptin infusion on spontaneous ACT

Leptin-induced hyperthermia can not be explained by enhanced ACT, considering that daytime or nighttime ACT failed to show a significant rise during the infusion in any of the groups of rats (data are shown in Fig. 18).

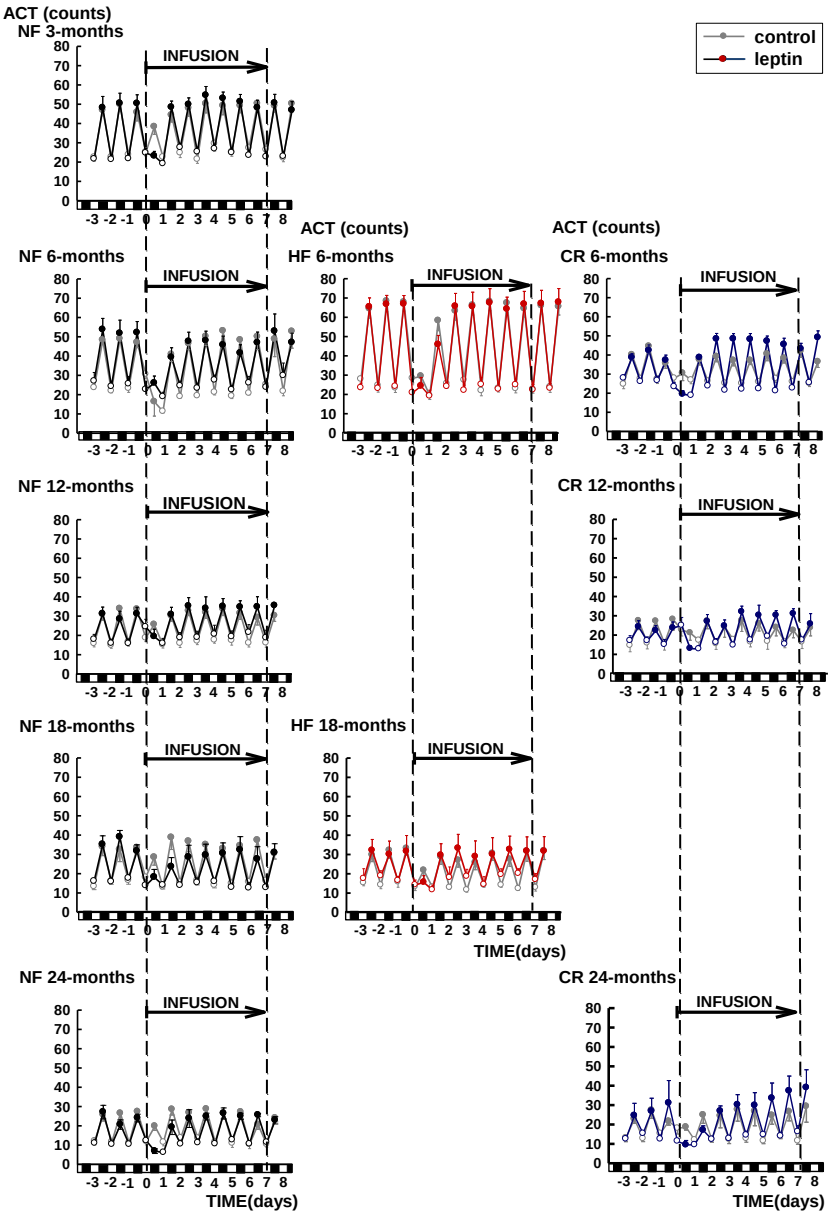

Fig. 18 *Circadian changes of spontaneous horizontal locomotor activity (ACT): 12-h averaging (mean ± S.E.M.) for the active (nighttime, closed symbols) and inactive (daytime, open symbols) periods during intracerebroventricular infusion of leptin (colored) or pyrogen-free saline (control, gray). No significant differences were detected during the infusion periods (n = 6-8 rats/group). NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted.*

4.3. Age-related alterations in gene expression of Ob-Rb and SOCS3 in the ARC

In our study qRT-PCR measurements revealed that in the ARC of different age-groups of NF rats, Ob-Rb expression was maximal in the young adult group and declined severely by the age of 12 months, to rise again somewhat in the aging and old animals (Fig. 19A). Thus, NF12 rats have the lowest value that is significantly different from all other NF groups (NF12 vs. NF3 and NF6 $p < 0.001$, NF12 vs. NF18 and NF24 $p < 0.01$, one-way ANOVA with Tukey's post hoc test). In addition, although Ob-Rb expressions of NF18 and NF24 groups were higher than that of NF12, their values still showed a tendency to remain decreased as compared with that of young animals [these differences have not reached statistical significance, but showed strong tendencies, especially in the oldest age-group (NF3 vs. NF18 $p = 0.090$, NF3 vs. NF24 $p = 0.051$, one-way ANOVA with Tukey's post hoc test)]. Our findings suggest that Ob-Rb expression in the ARC show non-linear changes with aging, similar to those seen in case of acute central anorexigenic leptin effects.

Regarding inhibitory SOCS3 gene expression in the ARC, it was highest in NF18 and NF6 followed by NF24, while it remained low in NF3 and NF12 (Fig. 19B). Significant differences were found between NF18 versus NF3, NF12 and NF24 ($p < 0.05$ for NF3 and NF24, $p < 0.001$ from NF12) and also between NF6 and NF12 ($p < 0.05$). These findings may contribute to the explanation of a weak anorexigenic responsiveness to leptin in NF6 and NF18, despite their relatively high Ob-Rb expression. In addition, the relatively lower SOCS3 expression of NF24 might help explain the significant anorexigenic responsiveness to leptin in old NF rats.

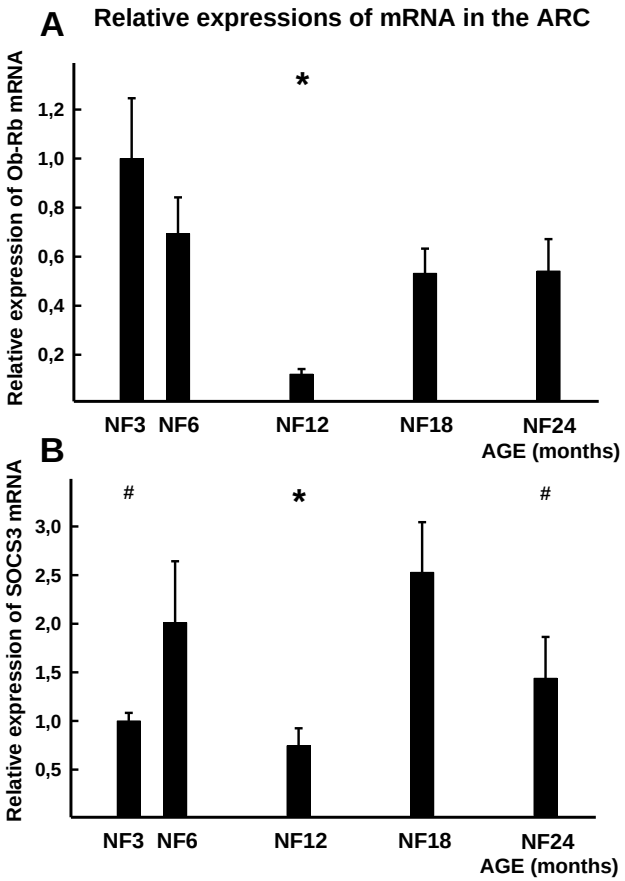

Fig. 19

Panel A *Relative messenger ribonucleic acid (mRNA) expression of the long isoform of leptin receptor (Ob-Rb) gene by quantitative real-time polymerase chain reaction (qRT-PCR) in the arcuate nucleus of the hypothalamus (ARC) in different age-groups of normally fed rats (NF3 to NF24). Data were evaluated by the delta delta Ct method for qRT-PCR analysis. Ob-Rb mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Asterisk (*) indicates significant differences between NF12 and all other age-groups (n = 6-7 rats/age-group).*

Panel B *Relative messenger ribonucleic acid (mRNA) expression of the signal transduction inhibitor suppressor of cytokine signaling 3 (SOCS3) gene by quantitative real-time polymerase chain reaction (qRT-PCR) in the arcuate nucleus of the hypothalamus (ARC) of the same rats as in Panel A. Data were evaluated by the delta delta Ct method for qRT-PCR analysis. SOCS3 mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Asterisk (*) indicates significant differences between NF12 and NF6 or NF18 groups, # indicates significant differences of values of NF3 or NF24 from that of NF18 (n = 6-7 rats/age-group).*

5. Discussion

Leptin is one of the most potent adiposity signals and hormone regulators of long-term energy balance with a complex catabolic activity involving anorexia and hypermetabolism (Sahu, 2004; Steiner and Romanovsky, 2007). Age-related obesity is characterized by progressive weight gain (starting at a younger age) that is accompanied by the development of progressive leptin resistance (Scarpace et al., 2000a and 2000b; Sahu, 2004) further aggravating obesity. However, elderly persons and old mammals tend to lose weight (aging anorexia leading to cachexia and sarcopenia) (Morley, 2001), that cannot be explained on the basis of leptin resistance.

Leptin resistance affecting energy metabolism is a complex phenomenon that may occur at multiple levels (Myers et al., 2012). It may involve reduced capacity of leptin to cross the blood-brain barrier that has been unequivocally proven in obesity (Banks, 2006), but controversial reports have been published concerning its role in aging-associated leptin resistance (Ma et al., 2002; Banks and Farrel, 2003). It may also appear as resistance to leptin-induced signaling cascade via phosphorylation of Ob-Rb or as leptin receptor downregulation (Myers et al., 2012). It may also be based on suppressed activation of the intracellular signaling pathways. These potential pathways of varying importance include: JAK2/STAT3, JAK2/STAT5, SHP2/ERK, IRS/PI3K, and AMPK (Villanueva and Myers, 2008; Rahmouni et al., 2009; Zhou and Rui, 2013). In addition, induction of target genes that negatively regulate leptin response, SOCS3 and PTP-1B (Mori et al., 2004; Bence et al., 2006; Morris and Rui, 2009)] may also be found in the background.

Previous studies in old rats reported functional resistance to the anorexigenic effect to peripherally or centrally applied leptin (Scarpace et al., 2000a; Shek and Scarpace, 2000). As aging is frequently associated with obesity, this resistance was often attributed to the hyperleptinemia originating from the enlarged fat mass. In aged-obese rats, leptin expression in the subcutaneous fat tissue decreased following reduction of visceral fat mass (Gabriely et al., 2002b). In elderly humans of normal BW plasma leptin was smaller than in middle-aged obese persons (raising some doubt about exclusively age-induced leptin resistance in humans), and it decreased further (together with BW) in centenarians (Baranowska et al., 2006). However, it has also been suggested (Gabriely et al., 2002a) that resistance to leptin-induced anorexia developed in 20 months old (aged) rats even if the

age-related fat accumulation was reversed by restricted calorie intake. Other studies reported diminished leptin receptor immunoreactivity and gene expression (Fernández-Galaz et al., 2002), as well as increased SOCS3 gene expression in the ARC of old Wistar rats (Peralta et al., 2002). These age-related changes in gene expression were reverted by food restriction (Fernández-Galaz et al., 2002; Peralta et al., 2002).

The question arises, whether acute and chronic central leptin actions show a similar age-related pattern, as previously, central acute and chronic leptin effects have been shown to be different, even inverse in the central nervous system (García-Cáceres et al., 2011). Moreover, diverse intracellular pathways of chronic and acute leptin actions have been identified in hypothalamic POMC neurons (Hill et al., 2008).

In the present study central acute and chronic anorexigenic and hypermetabolic/hyperthermic leptin effects were investigated in NF, HF or CR male Wistar rats of different age-groups. These tests were complemented by investigation of Ob-Rb and SOCS3 gene expressions in the ARC of different age-groups of NF rats.

5.1. Variations of resistance to the anorexigenic effect of acute central leptin injection

Regarding anorexigenic responsiveness to leptin, we found that aging does not cause a continuous progressive decline in the efficacy of the protein. Instead, characteristic age-related shifts were demonstrated: significant leptin-induced reduction in re-feeding was recorded in the normally fed young adult (NF3) group, while the anorexigenic response failed to reach a significant level in younger or older middle-aged (NF6 or NF12) and also in aging (NF18) rats. This response became significant again in old (NF24) animals. Age-related changes in the gene expression of Ob-Rb (high in NF3, NF6, very low in NF12 and moderately increased in NF18 and NF24) and that of inhibitory SOCS3 (high in NF6 and NF18 followed by NF24 and low in NF3 and NF12) in the ARC may provide some explanation for this phenomenon. Results concerning gene expression contribute to the explanation of the low responsiveness of NF18 (diminished Ob-Rb and high SOCS3) and to some extent to that of NF12 (very low Ob-Rb and low SOCS3). Our findings cannot fully explain the relatively higher anorexigenic responsiveness of NF24, since diminished Ob-Rb expression (similar to that of NF18, but higher than that of NF12) appears to be coupled with an intermediate level of SOCS3 expression (higher than that of NF3, but lower than that of NF18).

Our results are in accord with previous reports showing a decrease in Ob-Rb expression and an increase in SOCS3 expression in old rats when their values are compared with those of young ones (Fernández-Galaz et al., 2002; Peralta et al., 2002). In addition, we have demonstrated a strong decrease of Ob-Rb expression in middle-aged animals, even exceeding that seen in the oldest groups. Moreover, a strong increase in SOCS3 expression in younger middle-aged (NF6) and aging (NF18) groups were also found. The above demonstrated age-related shifts in acute central anorexigenic leptin responsiveness may contribute to the explanation of middle-aged obesity and provide some indications of the background of aging anorexia observed in humans and other mammals (Scarpace et al., 2000b; Morley, 2001).

Although, our study provided some evidence concerning age-related alterations in Ob-Rb receptor and SOCS3 gene expression in the ARC that may contribute to the explanation of age-related shifts in the anorexigenic responsiveness to central leptin administration, many questions concerning this phenomenon, especially regarding changes in old rats remain unresolved. Changes in messenger ribonucleic acid (mRNA) expression are not necessarily associated with equivalent alterations of protein levels. Thus, in the future quantitative analysis of age-related changes affecting leptin receptors in the ARC has to be carried out to confirm our preliminary results. Regarding signal transduction pathways, quantitative measurements of other activating or inhibitory pathways (in addition to SOCS3) would be needed in order to clarify the mechanisms of our in vivo findings.

Investigation of potential mechanisms should also focus on age-related alterations of neuropeptides downstream to leptin, such as the anorexigenic melanocortin system or CART. Although, in case of the melanocortin system a similar age-related pattern in the central anorexigenic responsiveness has been reported (Pétervári et al., 2011), and hypothalamic CART expression has also been shown to increase during aging (Kappeler et al., 2003; Wolden-Hanson et al., 2004), controversial or no data concerning age-related changes in their intrinsic activity, in the rate of mediator production (Kappeler et al., 2003), receptor expression, or activity of their signal transduction pathways is available. Concerning orexigenic hypothalamic peptide systems (NPY or AgRP) that are inhibited by leptin, no unequivocal evidence is available (Kappeler et al., 2003; Wolden-Hanson et al., 2004), although the well-documented age-related decline in orexigenic NPY production and responsiveness (Sahu et al., 1988; Akimoto and Miyasaka, 2010; Botelho and

Cavadas, 2015) may contribute to the explanation of the phenomena described by our study.

In our study, high-fat diet-induced obesity appeared to accelerate the development of the above described age-related regulatory alterations, since leptin-induced anorexia failed to occur in the younger middle-aged obese group, but became strong in the older middle-aged obese rats (HF12). Although they were merely 12 months old, their response rather resembled that of the oldest NF24 rats. Our present findings appear to be contradictory to those observations that describe a strong correlation between obesity and leptin resistance (Lin et al., 2000; Myers et al., 2012). However, similar age-related pattern of anorexigenic effects and similar obesity-induced acceleration of these age-related regulatory alterations were described in case of peripherally administered CCK (Balaskó et al., 2013), as well. In that study, IP injection of CCK induced strong suppression of fasting-induced re-feeding in normally fed young (NF4-NF6) and old (NF18-NF24), but not in middle-aged (NF12) rat groups. CCK-anorexia was non-significant in HF6 (similar to that in NF12), but became strong in the middle-aged obese HF12 rats, resembling the anorexigenic response of NF18 or NF24. In addition, adaptive reduction in hypothalamic NPY expression in prolonged (5-month) high-fat diet-induced obesity described by previous reports (Stricker-Krongrad et al., 1998; Beck, 2006) may also contribute to the explanation of the enhanced anorexigenic responsiveness to centrally administered leptin in our HF12 rats.

Our present results are also in agreement with previous observations describing equivalent central anorexigenic actions of leptin in lean and diet-induced obese middle-aged rats, when administered via intranasal application (Schulz et al., 2012). Furthermore, conclusions from our data are in line with those of other studies reporting efficient hypothalamic leptin-gene therapy in obese rodents (Turner et al., 2015), i.e. long-term obesity appears to enhance anorexigenic actions of centrally applied leptin. This enhanced responsiveness to centrally applied leptin in chronically obese middle-aged animals may be, at least in part, explained by a possible hypothalamic leptin receptor upregulation. This, as yet hypothesized, receptor upregulation may be a result of the long-term suppression of leptin transport from the periphery to the brain via the blood-brain barrier (Banks et al., 1996). This assumed upregulation may be similar to that found in genetically obese mice (Huang et al., 1997). Moreover, it has been demonstrated that a suppression of leptin transport via the blood-brain barrier develops already in the very early phases of obesity,

when despite peripheral leptin resistance centrally applied leptin still elicits significant responses (Morash et al., 1999).

5.2. Variations of resistance to the hypermetabolic/hyperthermic effect of acute central leptin injection

With regard to the hypermetabolic effects, leptin injection into the lateral cerebral ventricle induced significant increase in MR accompanied by a simultaneous tail vasoconstriction leading to strong hyperthermia in young adult animals (NF3). This response appears to be coordinated from a thermoregulatory point of view. In a coordinated or fever-like hyperthermia heat production increases (indicated by a rise in VO_2) and heat loss decreases (indicated by tail skin vasoconstriction, even at a thermoneutral environment that facilitates peripheral vasodilation). In this sense, leptin-hyperthermia resembles experimental endotoxin fever or CCK-induced hyperthermia (Székely and Szelényi, 1979; Balaskó et al., 2013). The coordinated feature of leptin-induced hyperthermia supports the potential role of leptin in thermoregulation (Steiner and Romanovsky, 2007). This acute central hyperthermic effect did not show dose-dependence (results not shown).

Hypermetabolic/hyperthermic actions declined with aging. Thus, acute central hypermetabolic/hyperthermic and anorexigenic actions of leptin show disparate age-related patterns. Whereas anorexigenic actions are diminished by middle-age and become relatively stronger by old age, hypermetabolic actions show a monotonous decline with aging. Diminished capacity of heat production of old humans and mammals (Thörne and Wahren, 1990; Scarpace and Matheny, 1996), as shown also by the decreased capacity of old patients to develop fever, may explain the lack of hypermetabolic leptin actions in old rats. However, pronounced hyperthermic response of old Wistar rats to centrally applied alpha-MSH argues against this explanation (Rostás et al., 2015). In addition, Münzberg and coworkers (2004) described a region-specific feature of the development of leptin resistance within the hypothalamus of diet-induced obese mice. Resistance appears first within the ARC. The possibility of different hypothalamic regions regulating different components of the leptin response has also been raised (Correia et al., 2002). It could explain how disparate age-related alterations of different leptin actions may develop. Moreover, recently different signal transduction pathways of the anorexigenic versus hypermetabolic leptin actions in the neurons (Zhou and Rui, 2013) were identified that

may also contribute to the explanation of these disparate age-related patterns: JAK2/STAT3 and JAK2/STAT5 signaling appears to be linked predominantly to anorexigenic effects of leptin, while SHP2/ERK signaling was shown to contribute to hyperthermic, as well as, anorexigenic actions of the protein (Rahmouni et al., 2009). Future investigations of age-related changes in these signal transduction pathways may provide evidence concerning disparate age-related changes in leptin-induced anorexia and hyperthermia.

Due to the multifaceted endocrine effects of leptin, the mechanisms behind this phenomenon may also involve other central and peripheral hormonal changes. Hypothalamic anorexigenic and orexigenic peptides downstream to leptin that have been discussed in connection with the anorexigenic leptin effects, may also contribute to changes in the hypermetabolic actions, as well. Moreover, additional neuroendocrine effects of leptin also have to be taken into consideration. During fasting or long-term food deprivation (such as hypothalamic amenorrhoea), low leptin level has been associated with suppressed peripheral thyroid and sex hormone levels, which recover upon leptin supplementation (Ahima et al., 1996; Chan et al., 2003; Welt et al., 2004; Park and Ahima, 2015). As the levels of these hormones, with well-documented hypermetabolic effects, decline during aging, their diminished release upon central leptin administration may also help explain the lower hypermetabolic responsiveness in older age-groups.

5.3. Variations of resistance to the anorexigenic effect of chronic central leptin infusion

All NF groups maintained significant responsiveness to the anorexigenic effects of leptin throughout the 7-day infusion. Although previous observations revealed chronic (28-day-long) ICV leptin infusion-induced leptin resistance (Sahu, 2002), in our study the anorexigenic effect of ICV leptin persisted throughout the infusion period. Regarding age-related alterations, this effect of leptin was strong in NF3, NF6 and NF12 rats, it was attenuated by the age of 18 months, to become more pronounced again in the oldest group. It appears, that similarly to central catabolic melanocortin agonist alpha-MSH (Pétervári et al., 2010), the anorexigenic action of leptin varies with age in a non-linear fashion: it exhibits weaker effects in aging animals (NF18), despite the high leptin dose applied. Accordingly, with aging, a certain leptin resistance developed. However, the really old age-group reacted to leptin strongly again. A similar age-related pattern has been

demonstrated not only for central melanocortins, but also for the anorexigenic effect of peripheral catabolic mediator CCK (Balaskó et al., 2013).

These findings are in accord with the above described decline of the anorexigenic responsiveness to ICV administered acute leptin injection from the young adult to the middle-aged and aging groups, followed by enhancement of sensitivity in the oldest rats.

Leptin effects on BW gradually declined in older animals (falling just below statistical significance in NF12) indicating age-related leptin resistance. No reduction in BW of the aging NF18 animals was seen, while the oldest NF24 age-group regained the responsiveness suggested by their weight loss. In these oldest rats the significant reduction of retroperitoneal fat also underlined their somewhat enhanced responsiveness.

Regarding the effects of diet-induced obesity, HF6 rats failed to lose weight in response to the leptin infusion in contrast to the NF6 rats, despite a comparable fall in cumulative energy intake, i.e. despite their young age these rats exhibited some loss of leptin effects – probably those affecting MR (see later). These findings support the earlier observations of our group (Soós et al., 2010). This functional leptin resistance was further aggravated in older (HF18) obese rats that failed to show any leptin-induced anorexia, weight or fat loss. (Incidentally, the same age-group of the NF category showed the smallest leptin-induced anorexia.) These results are in accord with previous reports on obesity-induced leptin resistance (Lin et al., 2000; Scarpace et al., 2000c; Levin and Dunn-Meynell, 2002), while they also underline the important contribution of aging to leptin resistance.

Life-long calorie-restriction in 6- or 12-month old age-groups appeared to completely prevent the anorexigenic effect. Additionally, leptin did not induce any fall in BW in CR6 or CR12 rats, possibly suggesting leptin resistance affecting anorexigenic actions already in middle-aged animals. However, chronic calorie-restriction may induce such an extreme orexigenic tone due to upregulation of orexigenic NPY and AgRP, and simultaneous downregulation of POMC, CART and CRH gene expressions of the hypothalamus (Dallman et al., 1999; McShane et al., 1999; Shimokawa and Higami, 2001; Chiba et al., 2009) that even a high-dose leptin infusion cannot overcome it (Soós et al., 2010). The enhanced feeding response to NPY in CR6 rats in our previous experiments also supports the idea of their elevated orexigenic activity (Soós et al., 2010). Therefore, the apparent leptin resistance in CR6 or CR12 rats may only be virtual. These findings also suggest that leptin sensitivity may be maintained, only it could be hidden by the extremely high orexigenic tone.

Moreover, in the oldest CR24 group (following life-long calorie-restriction), leptin suppressed energy intake significantly that led to significant weight loss slightly exceeding that of NF24 animals. These latter results appear to support earlier observations (Fernández-Galaz et al., 2002) of maintained anorexic leptin effects in old CR animals. This enhanced responsiveness may not be explained by any diminishment of NPY expression in old rats, since calorie-restriction raised NPY expression in the hypothalamus of young, middle-aged or old rats alike (McShane et al., 1999).

5.4. Variations of resistance to the hypermetabolic/hyperthermic effect of chronic central leptin infusion

The anorexia does not cover all the effects of leptin on energy balance: the hormone also enhances MR and Tc (Luheshi et al., 1999; Wang et al., 1999; Székely et al., 2013). In the present work – in accordance with earlier data from the literature and with our observations – MR was assessed indirectly on the basis of HR (Butler, 1993; Astrand et al., 2004; Pétervári et al., 2011) or directly on the basis of VO_2.

Resting MR may rather be associated with the day-time HR and Tc values (circadian minima) characterized by low physical activity in rodents, whereas nighttime values (circadian maxima) may be strongly influenced by spontaneous ACT.

Unlike basal Tc data, basal HR values (maxima and minima) of NF rats gradually declined with aging confirming previous observations (Da Silva et al., 2005). During the course of the leptin infusion the HR and Tc values rose in the younger NF age-groups, whereas in the oldest rats the leptin-induced tachycardia or hyperthermia failed to develop. These findings may suggest the development of leptin resistance in the course of aging (Shek and Scarpace, 2000; Scarpace et al., 2002) and/or it may be explained by the diminished sympathetic activity and molecular pathways of thermogenesis (Scarpace et al., 2000a; Eikelis et al., 2003), which cannot be enhanced by leptin in NF rats (unlike in CR ones with *ab ovo* low sympathetic activity).

In diet-induced obese rats the hypermetabolic/hyperthermic leptin effects were strongly attenuated. Only daytime HR minima showed a moderate increase in the young obese animals that disappeared altogether in the older obese rats.

In CR animals the hypermetabolic/hyperthermic effects were very pronounced as compared with their age-matched NF counterparts (also confirmed more directly by calorimetry). This extreme hypermetabolic response was maintained even in the CR24

group in which the thermogenic capacity would otherwise be expected to be limited due to their old age (Horan et al., 1988), in accordance with their low basal HR values (and sympathetic activity). While in some CR rats the resistance to leptin-induced anorexia may be virtual, the enhanced hypermetabolic effects of the protein are certainly not hypothetical.

5.5. Age- and nutritional state-associated changes in the catabolic effects of a chronic central leptin infusion

In the present studies different components of the catabolic leptin effects showed non-parallel changes with aging: the non-linear age-related alterations in the anorexigenic leptin actions were coupled by age-dependent decline in the hypermetabolic/hyperthermic leptin effects across the NF age-groups. Another example of disparate age-related alterations on catabolic peptidergic regulation has been provided by the central melanocortin system (Pétervári et al., 2011). Moreover, dissociation of the anorexigenic and thermogenic effects of leptin has already been described in a different experimental model. In chronic experimental hyperleptinemia causing leptin resistance, the anorexic effect was attenuated after 25 days, while the hypermetabolic effect dissolved only after 83 days (Scarpace et al., 2002). In the present study the overall catabolic effect of leptin caused weight loss in the two younger NF groups and moderately in the oldest one, but not in NF12 and NF18 animals.

Obesity abolishes catabolic leptin responsiveness via suppression of hypermetabolic responses, while allowing maintenance of some anorexigenic effects in HF6 rats. In older HF18 animals even the anorexigenic effects were completely abolished. These findings are in accord with previous observations concerning obesity-induced leptin resistance (Levin and Dunn-Meynell, 2002). In addition, the present study indicates that the diet-induced obesity affects hypermetabolic actions prior to the anorexigenic ones.

The partial leptin resistance of younger CR rats and the reinforced full-scale responsiveness of the old ones suggest that aging *per se* does not have an exclusive and aggravating role in the development of leptin resistance. These findings also underline the potential importance of CR in the prevention or reversal of leptin resistance in animals (Wilsey and Scarpace, 2004).

It may be concluded that different nutritional states – whether obesity or calorie-restriction – influence predominantly the hypermetabolic mechanisms.

Experimental data presented here seem to shed some light on the progressive feature of obesity and leptin resistance: the early appearance of obesity leads to the gradual loss of hypermetabolic effects that in turn decreases protective mechanisms against further weight gain and leads to progressive fat accumulation. Furthermore, aging aggravates this obesity-induced leptin resistance by abolishing even the anorexigenic effects, producing a self-perpetuating process. Life-long calorie-restriction prevents deterioration of hypermetabolic leptin-effects in animals. However, these experimental findings have restricted significance with regard to nutritional treatments in humans, as therapeutic dietary restrictions are usually temporary.

Surprisingly, the oldest CR rats exhibit full-scale leptin-responsiveness including very pronounced anorexigenic and hypermetabolic effects. In addition, in NF rats following a gradual decrease in leptin responsiveness up to age 18 months, the leptin-induced anorexia and weight loss become pronounced again in the oldest age group. Incidentally, such old rats start losing muscle. Thus, apart from the decreased catabolic leptin effects promoting middle-age obesity, our data has some implication for leptin responsiveness also in the later development of aging anorexia and sarcopenia.

6. Summary

6.1. Central acute hypermetabolic/hyperthermic leptin effects are coordinated from a thermoregulatory point of view (increased heat production is accompanied by suppressed heat loss), i.e. they resemble fever-like hyperthermia.

6.2. Central anorexigenic and hypermetabolic leptin effects (acute and chronic) show disparate age-dependent changes: anorexigenic actions become diminished in middle age and increase again in old age, while hypermetabolic/hyperthermic actions decline with aging.

6.3. Aging *per se* does not have an exclusive and aggravating role in the development of leptin resistance regarding anorexia. Age-related changes in the central catabolic effects of leptin may contribute to the explanation of middle-aged obesity and aging anorexia.

6.4. Obesity suppresses hypermetabolic/hyperthermic leptin effects in all age-groups. However, central anorexigenic leptin responsiveness remains maintained in middle-aged groups.

6.5. Life-long calorie-restriction enhances hypermetabolic/hyperthermic central leptin-effects in all age-groups and enhances central anorexigenic effects in old rats.

6.6. Different nutritional states – whether obesity or calorie-restriction – influence predominantly the hypermetabolic/hyperthermic central leptin effects.

7. Perspectives

Age-related shifts in central anorexigenic leptin responsiveness may not only contribute to the explanation of middle-aged obesity, but also to that of aging anorexia and cachexia of humans and other mammals.

Our findings concerning central anorexigenic leptin effects in middle-aged obese rats may support the possibility of efficient central (intranasal) leptin treatment of obesity of long standing. In addition, some leptin release of the human brain has also been demonstrated (Wiesner et al., 1999; Li et al., 2001), although the extent of it appears to be rather small, and specific sites have not been identified (Wiesner et al., 1999). Such central leptin production has been shown to be higher in female subjects than in males, moreover obesity appeared to enhance it (Eikelis et al., 2007) instead of suppressing it. As central leptin receptor expression was also shown to remain unimpaired in obese subjects (Eikelis et al., 2007), potential activation of leptin production of the central nervous system may also offer future therapeutic choices in age-related obesity.

8. References

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9. List of publications

9.1. Peer-reviewed articles related to this thesis

1. Pétervári E, **Rostás I**, Soós S, Tenk J, Mikó A, Füredi N, Székely M, Balaskó M. (2014) Age versus nutritional state in the development of central leptin resistance. Peptides, 56C: 59–67.
   IF: 2.618

2. **Rostás I**, Tenk J, Mikó A, Füredi N, Soós S, Solymár M, Lengyel A, Székely M, Gaszner B, Feller D, Pétervári E, Balaskó M. (2016) Age-related changes in acute central leptin effects on energy balance are promoted by obesity. Exp Gerontol, 85: 118–127.
   IF: 3.350 (in 2015)

9.2. Peer-reviewed articles unrelated to this thesis

1. Balaskó M, **Rostás I**, Füredi N, Mikó A, Tenk J, Cséplő P, Koncsecskó-Gáspár M, Soós S, Székely M, Pétervári E. (2013) Age and nutritional state influence the effects of cholecystokinin on energy balance. Exp Gerontol, 48(11): 1180–1188.
   IF: 3.529

2. **Rostás I**, Füredi N, Tenk J, Mikó A, Solymár M, Soós S, Székely M, Pétervári E, Balaskó M. (2015) Age-related alterations in the central thermoregulatory responsiveness to alpha-MSH. J Therm Biol, 49–50: 9–15.
   IF: 1.621

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   IF: 2.352 (in 2015)

4. Mikó A, Füredi N, Tenk J, **Rostás I,** Soós S, Solymár M, Székely M, Balaskó M, Brunner SM, Kofler B, Pétervári E. (2016) Acute central effects of alarin on the regulation on energy homeostasis. Neuropeptides, S0143-4179(16): 30087–30097.
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5. Tenk J, **Rostás I**, Füredi N, Mikó A, Soós S, Solymár M, Gaszner B, Székely M, Pétervári E, Balaskó M. (2016) Acute central effects of corticotropin-releasing factor (CRF) on energy balance: Effects of age and gender. Peptides, 85: 63–72.
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Cumulative impact factor (without citable abstracts): 32.837

9.3. Conference presentations related to this thesis

1. **Ildikó Rostás**, Péter Novinszky, Judit Tenk, Szilvia Soós, Miklós Székely, Erika Pétervári, Márta Balaskó. Central catabolic effects of leptin during the course of aging. IBRO Workshop, Debrecen, January 16-17, 2014.

2. **Rostás Ildikó**, Rimai Tamás. Centrális energetikai leptin hatások életkor-függő eltérései. XIX. Korányi Frigyes Tudományos Fórum, Budapest, March 06-07, 2014.

3. **Ildikó Rostás**, Tamás Rimai, Eszter Varga, Judit Tenk, Szilvia Soós, Miklós Székely, Erika Pétervári, Márta Balaskó. Age- and nutritional state-related catabolic effects of a central leptin infusion. Joint Meeting of the Federation of European Physiological Societies (FEPS) and the Hungarian Physiological Society, Budapest, August 27-30, 2014.
   Acta Physiologica Hungarica (2014) 211(S697): 130.
   IF: 0.734

4. **Ildikó Rostás**, Eszter Varga, Tamás Rimai, Judit Tenk, Szilvia Soós, Erika Pétervári, Márta Balaskó. Catabolic effects of central leptin infusion during aging. 9th Young European Scientist Meeting, Porto, Portugal, September 18-21, 2014.

5. **Ildikó Rostás**, Rebeka Pagáts, Péter Klespitz, Judit Tenk, Márta Balaskó. Leptin in age-related metabolyc dysregulation. Third International Symposium on Hypertension, Osijek, Croatia, November 28-30, 2014.

6. **Ildikó Rostás**, Zsófia Csernela, Judit Tenk, Szilvia Soós, Erika Pétervári, Miklós Székely, Márta Balaskó. Leptin in metabolic dysregulation: the influence of age and nutritional state. 5th Central European Congress on Obesity, Budapest, October 1-3, 2015.

7. **Rostás Ildikó**, Szakács Zsolt, Serényi Dóra, Soós Szilvia, Pétervári Erika, Balaskó Márta. Az akut centrális leptin injekció életkor- és elhízás-függő energetikai hatásai. FAMÉ Tudományos Konferencia, Pécs, June 1-4, 2016.

8. Márta Balaskó, Péter Nagy, **Ildikó Rostás**, Judit Tenk, Szilvia Soós, Miklós Székely, Erika Pétervári. Age-related central leptin-reistance in different nutritional states. IBRO Workshop, Debrecen, January 16-17, 2014.

9. Rimai Tamás, **Rostás Ildikó**. A leptin centrális katabolikus hatásainak korfüggő eltérései. VI. Nemzetközi és XII. Országos Interdiszciplináris Grastyán Konferencia, Pécs, March 18-20, 2014.

10. Márta Balaskó, Zsófia Csernela, Melanie Ehlers, **Ildikó Rostás**, Judit Tenk. Leptin in age-related metabolic dysregulation: the influence of nutritional states. Third International Symposium on Hypertension, Osijek, Croatia, November 28-30, 2014.

11. Márta Balaskó, **Ildikó Rostás**, Alexandra Mikó, Nóra Füredi, Judit Tenk, Szilvia Soós, Miklós Székely, Erika Pétervári. Age-related shifts in the acute anorexic effects of leptin: the influence of body composition. 15th Biannual Conference of the Hungarian Neuroscience Society (MITT), Budapest, January 22-23, 2015.

12. Pétervári Erika, Mikó Alexandra, **Rostás Ildikó**. Centrális anorexigén leptin hatások korfüggő változásai: a testösszetétel szerepe. Magyar Gerontológiai és Geriátriai Társaság XXXVIII. Kongresszusa, Gyula, May 28-29, 2015.

13. Erika Pétervári, **Ildikó Rostás**, Nóra Füredi, Margit Gáspár-Koncsecskó, Szilvia Soós, Miklós Székely, Márta Balaskó. Age-related shifts in the responsiveness to centrally applied leptin.
    Experimental Gerontology (2015) 68: 101.
    IF: 3.485

9.4. Conference presentations unrelated to this thesis

1. **Ildikó Rostás**, Veronika Sipos, Szilvia Soós, Miklós Székely, Márta Balaskó. The thermoregulatory effects of alpha-melanocyte stimulating hormone and its antagonist HS024. IBRO International Workshop, Pécs, January 21-23, 2010.

2. **Rostás Ildikó**, Sipos Veronika, Barcza Zsófia. Az alpha-melanocyta stimuláló hormon és az antagonista HS024 hatása a hőszabályozásra. XVI. Nemzetközi Marosvásárelyi TDK konferencia, Marosvásárhely, Romania, March 18-21, 2010.

3. **Rostás Ildikó**. A melanocortin rendszer hőszabályozási hatásai. PTE ÁOK Házi TDK Konferencia, Pécs, April 15-17, 2010.

4. **Rostás Ildikó**, Szabad Árpád Olivér. Az alpha-melanocyta stimuláló hormon hőszabályozási hatásai. XV. Korányi Frigyes Tudományos Fórum, Budapest, April 29-30, 2010.

5. **Rostás Ildikó**, Szabad Árpád Olivér, Sipos Veronika, Andreas Thomas Schmidt, Barcza Zsófia, Tore Schjottelvik. Az alpha-melanocyta stimuláló hormon hatása a hőszabályozásra. HMAA balatonfüredi diákkonferencia, Balatonfüred, August 20-21, 2010.

6. **Rostás Ildikó**, Szabad Árpád Olivér, Sipos Veronika. Az alpha-melanocyta stimuláló hormon hőszabályozási hatásainak vizsgálata patkányokon. IV. Intézményi Tudományos Grastyán konferencia, Pécs, December 2-3, 2010.

7. **Rostás Ildikó**, Sipos Veronika, Szabad Árpád Olivér. Életkorfüggő eltérések az alpha-melanocyta-stimuláló-hormon hőszabályozási hatásaiban. Nemzetközi Marosvásárelyi TDK konferencia, Marosvásárhely, Románia, March 17-20, 2011.

8. **Rostás Ildikó**, Szabad Árpád Olivér. Akut centális α-MSH injekció hőszabályozási hatásai az életkor függvényében. XVI. Korányi Frigyes Tudományos Fórum, Budapest April 14-15, 2011.

9. **Rostás Ildikó**, Sipos Veronika, Soós Szilvia, Balaskó Márta, Pétervári Erika, Székely Miklós. Age-related alterations in the thermoregulatory effects of alpha-melanocyte-stimulating hormone. Farmakológiai, anatómus, mikrocirkulációs, élettani társaságok közös tudományos konferenciája (FAMÉ), Pécs, June 8-11, 2011.
   Acta Physiologica (2011) 202(S684): 79-80.
   IF: 3.090

10. **Ildikó Rostás**, Árpád Olivér Szabad, Veronika Sipos. Aging modifies the thermoregulatory effects of alpha-melanocyte-stimulating hormone. HMAA balatonfüredi diákkonferencia, Balatonfüred, August 19-20, 2011.

11. **Ildikó Rostás**, Nóra Füredi, Judit Tenk, Alexandra Mikó. Thermoregulatory effects of alpha-melanocyte-stimulating hormone depend on age. János Szentágothai Memorial Conference & Student Competition, Pécs, October 29-30, 2012.

12. **Ildikó Rostás**, Alexandra Mikó, Nóra Füredi, Judit Tenk. Complex effects of neuropeptide alpha-melanocyte stimulating hormone on energy homeostasis during the course of aging. Leiden International Medical Student Conference, Leiden, The Netherlands, March 13-17, 2013.

13. **Rostás Ildikó**, Mikó Alexandra, Füredi Nóra, Tenk Judit. Az alpha-melanocyta-stimuláló-hormon szerepe az energiaháztartás szabályozásában. V. Nemzetközi és XI. Országos Interdiszciplináris Grastyán Konferencia, Pécs, April 17-19, 2013.

14. **Ildikó Rostás**. Complex effects of neuropeptide alpha-melanocyte-stimulating-hormone during the course of aging. 5th International Student Medical Congress in Kosice, Kassa, Slovakia, June 26-28, 2013.

15. **Ildikó Rostás**, Judit Tenk, Alexandra Mikó, Nóra Füredi. Complex effects of alpha-MSH during the course of aging. 2nd International Doctoral Workshop on Natural Sciences, Pécs, September 11-12, 2013.

16. **Ildikó Rostás**, Judit Tenk, Alexandra Mikó, Nóra Füredi. Complex effects of alpha-melanocyte-stimulating-hormone during the course of aging. 3rd Congress of the

Croatian Physiological Society and 1st Regional Congress of the Physiological Societies, Rijeka, Croatia, September 13-15, 2013.
Periodicum Biologorum (2013) 115(S2): 50.
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17. **Rostás Ildikó**, Mikó Alexandra, Soós Szilvia, Balaskó Márta, Pétervári Erika, Székely Miklós. Centrális cholecystokinin receptorok korfüggő energetikai hatásai. Egymást értő szakemberek nemzetközi konferencia, Budapest, October 01-02, 2013.

18. Veronika Sipos, **Ildikó Rostás**, Szilvia Soós, Miklós Székely, Erika Pétervári. Effects of central alpha-MSH infusion in middle aged rats of different nutritional states. IBRO International Workshop, Pécs, January 21-23, 2010.

19. Sipos Veronika, **Rostás Ildikó**, Szabad Árpád Olivér. Centrális alpha-MSH infúzió energetikai hatásai változnak a tápláltsági állapottal. Nemzetközi Marosvásárelyi TDK konferencia, Marosvásárhely, Romania, March 18-21, 2010.

20. Szabad Árpád Olivér, **Rostás Ildikó**. A hosszútávú energetikai reguláció életkorfüggő eltéréseinek vizsgálata centrálisan adott neuropeptidekkel. XV. Korányi Frigyes Tudományos Fórum, Budapest, April 29-30, 2010.

21. Barcza Zsófia, Szabad Árpád Olivér, **Rostás Ildikó**. A szomatosztatin 4 receptor altípus szerepe az energiaháztartás szabályozásában. Nemzetközi Marosvásárelyi TDK konferencia, Marosvásárhely, Romania, March 18-21, 2010.

22. Márta Balaskó, **Ildikó Rostás**, Árpád Olivér Szabad, Margit Koncsecskó-Gáspár, Szilvia Soós, Erika Pétervári, Miklós Székely. Age versus regulation of energy homeostasis in rats: shifts in the activity neuropeptides. The 7th Joint Meeting of the European Neuropeptide Club and the American Summer Neuropeptide Conference, Pécs, June 21-24, 2010.
Neuropeptides (2010) 44: 533.
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23. Márta Balaskó, Veronika Sipos, **Ildikó Rostás**, Szilvia Soós, Erika Pétervári, Miklós Székely. Nutritional state influences effects of central alpha-MSH infusion in middle-aged rats. 6th International Melanocortin Meeting, Utrecht, the Netherlands, July 8-11, 2010.

24. Márta Balaskó, Veronika Sipos, **Ildikó Rostás**, Szilvia Soós, Miklós Székely, Erika Pétervári. Obesity versus age in the regulation of energy homeostasis: melanocortins and leptin. 11th International Congress on Obesity, Stockholm, Sweden, July 11-15, 2010.
    Obesity Reviews (2010) 11(S1): 204.
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25. Sipos Veronika, **Rostás Ildikó**, Szabad Árpád Olivér, Andreas Thomas Schmidt, Tore Schjottelvik. Centrális alpha-MSH infúzió energetikai hatásainak változása a tápláltsági állapottal. HMAA balatonfüredi diákkonferencia, Balatonfüred, August 20-21, 2010.

26. Szabad Árpád Olivér, Sipos Veronika, Andreas Thomas Schmidt, **Rostás Ildikó**, Barcza Zsófia. Centrális neuropeptidek életkorfüggő testtömeg szabályozási tendenciákban betöltött szerepének vizsgálata patkányokon. HMAA balatonfüredi diákkonferencia, Balatonfüred, August 20-21, 2010.

27. Tore Schjottelvik, **Ildikó Rostás**, Veronika Sipos. Effects of central alpha-MSH infusion in rats of various nutritional states. V YES Meeting, Fundacao Eng. Antonia de Almeida, Portugal, September 24-26, 2010.

28. Balaskó Márta, **Rostás Ildikó**, Sipos Veronika, Soós Szilvia, Székely Miklós, Pétervári Erika. Az életkor hatásai az energia-háztartás centrális szabályozására. A Magyar Gerontológiai és Geriátriai Társaság XXXIII. Kongresszusa, Budapest, November 26-27, 2010.

29. Sipos Veronika, **Rostás Ildikó**, Szabad Árpád Olivér. A testösszetétel hatása a centrális melanocortin rendszer energetikai hatásaira. IV. Intézményi Tudományos Grastyán konferencia, Pécs, December 2-3, 2010.

30. Szabad Árpád Olivér, Sipos Veronika, **Rostás Ildikó**. Az életkor szerepe centrális peptiderg szabályozó rendszerek energetikai hatásaiban. IV. Intézményi Tudományos Grastyán konferencia, Pécs, December 2-3, 2010.

31. Sipos Veronika, **Rostás Ildikó**. Centrális alpha-MSH injekció akut energetikai hatásai különböző tápláltsági állapotú patkányokban. PTE-ÁOK Tudományos Diákköri Konferencia, Pécs, February 17-18, 2011.

32. Szabad Árpád Olivér, **Rostás Ildikó**, Sipos Veronika. Az orexigén és anorexigén aktivitás életkorfüggő eltéréseinek vizsgálata patkány modellben. Nemzetközi Marosvásárelyi TDK konferencia, Marosvásárhely, Romania, March 17-20, 2011.

33. Sipos Veronika, Szabad Árpád Olivér, **Rostás Ildikó**. Az alpha-MSH hőszabályozási hatásainak vizsgálata különböző tápláltsági állapotú patkányokban. Nemzetközi Marosvásárelyi TDK konferencia, Marosvásárhely, Romania, March 17-20, 2011.

34. Sipos Veronika, Szabad Árpád Olivér, **Rostás Ildikó**. Akut centrális a-MSH injekció hőszabályozási hatásai a testösszetétel függvényében. IX. Országos Interdiszciplináris Grastyán Konferencia, Pécs, March 29 - April 01, 2011.

35. Szabad Árpád Olivér, Sipos Veronika, **Rostás Ildikó**. A táplálékfelvétel szabályozás életkorfüggő eltéréseinek vizsgálata patkányban. IX. Országos Interdiszciplináris Grastyán Konferencia, Pécs, March 29 - April 01, 2011.

36. Andreas Schmidt, **Ildikó Rostás**. Age-associated alterations in the thermoregulatory effects of alpha-melanocyte stimulating hormone (alpha-MSH). International Students' Conference CROSS7, Zagreb, Croatia, April 13-16, 2011.

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