

Hypothalamic melanocortin system in the regulation of the energy homeostasis: studies in aging rats and in SHR animals

DOCTORAL (Ph.D.) THESIS



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INTRODUCTION

Obesity, as a common risk factor for numerous diseases became in the last decades a major epidemiological and general healthcare challenge. While middle-aged populations tend to become obese, old age is rather characterized by anorexia and consequent loss of active tissues (muscle) leading to sarcopenia. As both trends are also observed in other mammals, common endogenous regulatory alterations in the energy balance may contribute to their development (Székely et al. 2016). Although these are well-known epidemiological observations, our knowledge on its neurobiological background is still insufficient.

1. Regulation of the energy balance: hypothalamus and the main regulatory peptides

The main components of energy balance are food intake (FI), metabolic rate (MR) and heat loss (HL). The balance between FI and MR determines on the short-term the actual feeding state (hunger vs. satiety), while on the long-term it determines the body weight (BW, nutritional state). The balance between the MR and the HL provides the stability of the core temperature (T_c). These two closely related regulatory circles control together the energy homeostasis. The regulatory circles interact with each other: environmental temperature and internal thermoregulatory changes influence the FI, while the nutritional state and the BW affect the control of T_c. If the balance between FI and the MR is overturned, BW change occurs, resulting either in weight gain/obesity or in malnutrition.

Both, the regulation of the BW and that of T_c are orchestrated by hypothalamic neuropeptidergic centers. Peptides based on their effects on the FI may be classified as orexigenic (increase the FI) or anorexigenic (decrease the FI) mediators. From the aspect of energy balance, the orexigenic/anorexigenic effects on FI can be evaluated as coordinated, if they are coupled with concomitant regulatory changes in the MR and T_c, which act in the same direction of energy balance. Hyperphagia with hypometabolism (decreased MR) is an anabolic state (results in weight gain), in contrast to the catabolic effect, i.e. hypophagia with hypermetabolism (increased MR) resulting in weight loss (Székely and Szelényi 2005).

2. The main catabolic system of the hypothalamus: the melanocortins

The hypothalamic melanocortin (MC) system including adrenocorticotrop-hormone (ACTH), beta-endorphin, beta-lipotropine, corticotropin-like intermediate-lobe-protein (CLIP) as well as alpha-, beta-, and gamma-melanocyte-stimulating hormones (MSH) has well established and important role in the regulation of energy homeostasis. Excitatory ligands (i.e. alpha-, beta-, gamma-MSH and ACTH) of the MC receptors are derived from a post-translational processing of pro-opiomelanocortin (POMC), which is coded by the *Pomc* gene. The main expression site is the hypothalamic arcuate nucleus (ARC). Two naturally present endogenous MC antagonists, more precisely inverse antagonists are the agouti protein and agouti-related peptide (AgRP) occurring in the central nervous system (Garfield et al. 2009).

Five distinct types of MC receptors (MC1R-MC5R) have been identified. All the MC receptors are coupled to G_s proteins and signalize the cyclic AMP (cAMP) pathway. The MC4R, expressed primarily in the paraventricular nucleus of the hypothalamus (PVN) is the most important receptor type concerning the regulation of energy homeostasis. Production of alpha-MSH in the ARC is triggered by peripheral signals (i.e. leptin, expressed mainly by adipose tissue and insulin) providing information about the feeding/nutritional state and BW. Stimulation of MC4R by alpha-MSH induces a catabolic effect: weight loss induced by

decreased FI and increased MR via activation of sympathetic nervous system with simultaneously elevated Tc (Krashes et al. 2016).

3. Age-related alterations in the central peptidergic regulation of energy homeostasis

Changes in body composition (BC) associated with aging in men from middle to old age include a progressive increase in fat mass. Especially visceral adiposity associated with elevated plasma insulin and leptin levels as well as leptin resistance should be pointed out. With old age and the onset of age-related pathology, more severe anorexia, malnutrition and weight loss associated with a decline in muscle mass and strength (sarcopenia) may occur and result in significant morbidity and mortality. The mechanisms underlying these important age-associated alterations involve neuropeptides in the control of energy balance, BW and BC (Balaskó et al. 2014; Kmiec et al. 2013; Pétervári et al. 2011; Székely et al. 2016).

Our research group found characteristic age-dependent changes in the responsiveness to centrally applied leptin injection/infusion in male Wistar rats: anorexigenic and weight reducing effects of leptin diminished in middle-aged and aging (12- and 18-month-old) animals, but they were more pronounced again in the oldest (24-month-old) group. This pattern may contribute to the explanation of both middle-aged obesity and aging anorexia. In contrast, hypermetabolic/hyperthermic effects of leptin gradually declined with aging (Pétervári et al. 2014; Rostás et al. 2016). Leptin inhibits the orexigenic neuropeptide Y (NPY), the major anabolic regulator of the ARC, which has also been reported to decline with aging (Kmiec et al. 2013). The other main target of the leptin in the ARC is the MC system. In previous *in vivo* studies of our research group the anorexigenic and weight reducing effects of centrally applied alpha-MSH in male Wistar rats showed non-linear changes with aging, similar to those seen in case of central anorexigenic leptin: the effects of the peptide were strong in young and again in old rats but they were weak in the middle-aged groups (Pétervári et al. 2010; 2011). However, other previous *in vitro* studies failed to reveal an unequivocal age-related pattern in the endogenous activity of the hypothalamic MC system as indicated by gene expression of POMC: either decreased (Gruenewald and Matsumoto 1991; Kappeler et al. 2003; Nelson et al. 1988; Arens et al. 2003; Lloyd et al. 1991; Rigamonti et al. 2006) or unchanged MC activity was described in old rodents (McShane et al. 1999; Wolden-Hanson et al. 2004; Zhang et al. 2004). Acute hypermetabolic/hyperthermic effects of a central alpha-MSH injection in different age-groups of rats have not been investigated yet. To date, no detailed systematic analysis on hypothalamic MC system was carried out across more than three age-groups. Therefore, we aimed to systematically investigate the age-related dynamics of the MC system including those of alpha-MSH, AgRP and MC4R across different age groups of rats from young to old age.

4. Interaction between the regulation of energy homeostasis and blood pressure (BP)

Besides regulating energy balance, certain hypothalamic neuropeptides are involved in the BP regulation. The most potent hypothalamic neuropeptides such as alpha-MSH and NPY are involved in the regulation of metabolic rate and sympathetic nervous system activity, possibly contributing to BP elevation (Barzel et al. 2016).

Hypertension is defined as BP persistently higher than 140/90 mmHg in adults, even at rest. This condition leading to serious cardiovascular, cerebrovascular, renal complications affects about one third the Hungarian population, thereby it means a burden of public health (Bodo et al. 2008). These epidemiological data support both its priority in research and the high importance of reliable animal models.

5. Dysregulation of the energy homeostasis and blood pressure in spontaneously hypertensive rat (SHR) strain

Spontaneously hypertensive rats are the most often used rodent models to study hypertension and antihypertensive agents (Pinto et al. 1998). They have high sympathetic tone and progressive hypertension (Judy et al. 1976). Chronic calorie-restriction was able to prevent their hypertension (Dolinsky et al. 2010). Their FI and BW are lower than in normotensive controls, even on a high-fat diet, suggesting a dysregulation of energy homeostasis in these animals (Oliveira et al. 2009). The activity of the hypothalamic MC system in SHR seems to be enhanced: a MC3/4R antagonist could reduce mean arterial pressure to a greater extent in SHR than in controls. This suggests that high endogenous activity of the MC system contributes to the maintenance of adrenergic tone and elevated BP in SHR even though the mRNA levels for POMC and for MC4R in the mediobasal hypothalamus were not increased as compared to controls (da Silva et al. 2008). In contrast, POMC mRNA was found to be increased in the ARC of SHR that may be related to the genesis of their spontaneous hypertension (Yin et al. 1997). Central NPY eliciting hypotension and bradycardia counteracts the MC-mediated effects (Williams et al. 2004). In our study we aimed to investigate the potential role of the MC system and NPY in the dysregulation of feeding and BW of SHR strain.

AIMS AND HYPOTHESES

Our aim was to investigate the key role of the hypothalamic MC system in the regulation of energy homeostasis.

Hypothesis I.

Activity of the MC system shows characteristic age-related pattern. Decreased activity of the hypothalamic MC system contributes to the development of middle-aged obesity, but later the MC activity increases leading to anorexia and weight loss in old age.

We aimed to test this hypothesis in our male Wistar rat strain in 3 settings of experiments.

1) In order to demonstrate middle-aged obesity and aging sarcopenia in our rat model, we measured BW and assessed body composition (BC) of different age-groups.

We aimed to provide evidence on changes of BC during aging by applying three different methods for the assessment of BC. In addition to *post mortem* BC analysis (PMA), we aimed to assess BC also *in vivo*. We evaluated abdominal micro-computed tomography (CT) scan restricted to the L1-L3 region (micro-CT_(L1-L3), a scan of previously untested abdominal region in rats) and a skinfold thickness-based method (STM, in small rodents still not validated) with regard to the widely used whole-body micro-CT scan in rats. We also put forward to compare and validate these three approaches for future *in vivo* studies where the aging-related dynamics of BC should be quantified. With regard to the above described techniques, we aimed to test the following assumptions:

- Abdominal micro-CT_(L1-L3) with short scanning time is an equally useful tool as whole-body micro-CT.
- The simple and non-invasive total STM is also suitable to detect differences and follow changes in fat mass of rats.
- Visceral fat measured by micro-CT_(L1-L3) and subcutaneous fat assessed by STM would show similar age-related differences.

- *Post mortem* measurement of retroperitoneal fat reflect age-related differences in body fat better than measurement of epididymal fat.

2) *In vivo* investigation of hypothalamic responsiveness to exogenous MC in Wistar rats:

We hypothesized that the acute short-term metabolic/thermoregulatory effects of alpha-MSH may also show age-related alterations similar to those of the anorexigenic MC effects. Therefore, we analyzed the acute hypermetabolic/hyperthermic effects of a central alpha-MSH injection in different age-groups of rats. In addition, we aimed to analyze complex acute catabolic (hypermetabolic and anorexigenic) effects of this exogenous MC agonist in five age-groups of male Wistar rats from young adult to old age with special regard to the BW development curve.

3) *In vitro* analysis of the endogenous hypothalamic MC system in Wistar rats:

We investigated the age-related dynamics of the endogenous MC system including those of alpha-MSH, AgRP and MC4R in the ARC and PVN using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and a semiquantitative immunohistochemical approach. Then, dynamics of these age-related changes were systematically compared with those of the *in vivo* results.

Hypothesis II.

Spontaneously hypertensive rats **do not show characteristic middle-aged obesity** seen in Wistar rats: their BW is lower than that of normotensive (NT) controls, even on a high-fat diet, suggesting a dysregulation of energy homeostasis. *We assumed, that enhanced activity of hypothalamic anorexigenic MCs and diminished tone of orexigenic NPY may contribute to their BW dysregulation.*

We aimed to test this hypothesis in 2 settings of experiments.

1) *In vivo* investigation of hypothalamic responsiveness to exogenous peptides in SHR:

To test our hypothesis, we treated male 3-months-old SHR and NT rats with central alpha-MSH, AgRP or NPY injections in an acute model and with MC4R antagonist (HS024) infusion in a chronically (7-day long) treated setup. We aimed to analyze the effects of the peptides on the FI and BW.

2) *In vitro* analysis of the endogenous hypothalamic systems in SHR:

To semi-quantify the (endogenous) activity of the above described peptidergic system, immunohistochemistry was applied.

MATERIALS AND METHODS

1. Animals

Five age-groups [*i.e.* young adult (3-month-old), adult (6-month-old), middle-aged (12-month-old), aging (18-month-old) and old (24-month-old)] of male Wistar rats were used to test *Hypothesis I*. In certain thermoregulatory experiments, a juvenile (2-month-old) group was also applied. For investigating *Hypothesis II*, 3-month-old NT and SHR rats were subjected. All rats were bred at the Animal Facilities of the Institute for Translational Medicine and the Department of Anatomy, University of Pécs, Hungary. All interventions were in accord with institutional (University of Pécs) and international standards (86/609/EEC, Directive 2010/63/EU of the European Parliament and of the Council).

Hypothesis I:

Cohorts of non-treated male Wistar rats were used for body composition (micro-CT, PMA, STM) measurements. Two further cohorts of rats were subjected to intracerebroventricular (ICV) cannula implantation surgery for FI-assessment and metabolic/thermoregulatory measurements. Other two cohorts of intact rats were used for immunohistochemistry and qRT-PCR.

Hypothesis II:

For *in vivo* experiments rats with ICV cannula were used. Cohorts of NT and SHR rats were subjected to implantation of an ICV cannula connected to Alzet osmotic minipump in order to measure the effect of HS024 (infusion) on the cumulative FI and BW. Three further cohorts of NT and SHR were subjected to ICV cannula implantation surgery in order to measure the orexigenic effect of AgRP (injection) on the daytime FI, to measure the orexigenic effect of NPY (injection) on the daytime FI and to measure the anorexigenic effect of alpha-MSH (injection) on 12-h cumulative spontaneous nighttime FI. In case of *in vitro* investigations, cohorts of non-treated male NT and SHR rats were used for qRT-PCR to measure the relative expression of POMC mRNA in the ARC or for immunohistochemistry to measure the number of alpha-MSH producing cells as well as the peptide level of alpha-MSH and NPY in ARC. Alpha-MSH signal density was also evaluated in PVN.

2. Body composition assessment

2.1. Micro-CT

Analyses were done in Janos Szentagothai Research Centre, University of Pécs. Micro-CT scan was performed in rats under intraperitoneal (IP) ketamine-xylazine [78 mg/kg (Calypsol, Richter) + 13 mg/kg (Sedaxylan, Eurovet)] anesthesia that provided 1.5-2 hours of deep sleep in the micro-CT scanner (Skyscan 1176 high resolution *in vivo* micro-CT, Kontich, Belgium). The center of the scanned field of view was aligned at the level of the L2. Overall scan time was 14-15 minutes during analyses of abdominal region between L1-L3 and 60-75 minutes when whole body micro-CT (from the base of the skull to the distal end of the tibia) scan was applied. Three components (fat and bone percentage, other soft tissue) of the body composition were measured. For the statistical analysis only fat percentage values were used.

2.2. Skinfold thickness measurement (STM)

For STM a short ether anesthesia was sufficient. Skinfold thickness were measured in five locations with Lange caliper: four landmarks were determined according to Marshall (1969) (right scapular, right abdominal, right *triceps brachii*, right leg) and an additional site was chosen at the right iliac crest. All ST values were added to calculate the total ST. Repeatability and reproducibility were also tested.

2.3. Post mortem analysis (PMA)

After an overdose of urethane (3 g/kg, IP), indicators of body fat (epididymal, retroperitoneal fat pads) and indicator of muscle mass (wet weight of *m. tibialis anterior*, *m. soleus*, *m. extensor hallucis longus*, *m. extensor digitorum longus*) from the left side of the animals were removed and weighed with centesimal accuracy in gramm (g). These indicators were calculated for 100 g BW.

3. Surgeries

Rats were operated for the purpose of implanting a leading guide cannula into the right lateral cerebral ventricle for ICV injections or an ICV cannula for ICV infusion. Animals were

anesthetized by IP ketamine + xylazine (s. above) and they also received Gentamycin injection (2 mg IP) for prophylaxis. Correct position of the tip of the cannula: A: -1.0 mm, L: 1.5 mm, V: 3.5 mm. For chronic administration, Alzet osmotic minipump filled with a solution was placed under the skin simultaneously and connected to the ICV cannula.

4. Metabolic and thermoregulatory assessment

Oxygen consumption (ml O₂/kg/min, VO₂ representing metabolic rate) was determined by indirect calorimetry (Oxymax). Simultaneously, core temperature (T_c) and tail skin temperature (T_s, indicating heat loss) were also measured by thermocouples attached to Digi-Sense Benchtop Thermometer. Measurements were performed on semi-restrained rats singly placed in cylindrical wire-mesh confinements in four separate, thermostatically controlled metabolic chambers (25°C). At this temperature there is a constant skin vasoconstriction without fluctuations in T_s, but vasodilation (an important thermoregulatory reaction to alpha-MSH) can be evoked. This ambient temperature also allows the observation of hyperthermic responses. The VO₂ was registered in 10-min intervals for 3 hours following the ICV alpha-MSH and PFS injection.

5. Measurement of food consumption of rats

The orexigenic and anorexigenic effects of peptides were measured by an automated FeedScale System in freely moving animals. Two weeks before the tests, rats were transferred individually to chambers of the system. Thus, they were habituated to the environment and to the powdered rat chow. The system measured the consumed food in grams every 10 minutes following the injections and every 30 minutes during the 7-day infusion, and a computer recorded the data for the statistical analysis.

6. Immunohistochemistry

To perform immunohistochemistry, rats were deeply anesthetized by an IP overdose of urethane, then transcardially perfused with ice-cold 50 ml of 0.1M phosphate buffered saline (PBS, pH 7.5) followed by 300 ml chilled 4% paraformaldehyde in 0.1M Millonig buffer for 20 min. Besides alpha-MSH-MC4R double labeling, single immunofluorescence for AgRP and NPY were performed. Sections were digitalized using a confocal laser scanning microscope.

7. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Rats were removed from their home cages and decapitated within 2 minutes. Brains were instantaneously dissected and quickly frozen in liquid nitrogen and finally stored at -70°C until further use. PVN and ARC samples were punched from 1 mm thick slices (-2 to -3 mms from the Bregma) of the brains cut on a brain matrix by two razor blades. Sections were placed on an ice-chilled mat. The total amount of RNA was isolated with the Pure LinkTM RNA Mini Kit according to protocol suggested by the manufacturer. High capacity cDNA kit was applied to perform cDNA synthesis, using 1 µg of total RNA sample according to the official protocol. SensiFast SYBR Green reagent (BioLine) was used to perform qRT-PCR, for gene expression analysis. 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.

8. Statistical analysis

For the statistical analyses, Pearson correlation test, repeated-measures-, two-way- and one-way analysis of variance (ANOVA) were applied followed by Fisher's *post hoc* analysis. SPSS 11.0 for Windows and Statistica 8.0 for Windows softwares were used.

RESULTS AND DISCUSSION

Hypothesis I.

1.1. Results of body composition analysis

Fat mass of Wistar rats of different BW values was assessed by abdominal micro-CT_(L1-L3), skinfold thickness-based method (STM) and wet weight of epididymal, retroperitoneal fat pads determined by *post mortem* analysis (PMA). These methods of body fat assessment in the same animals were evaluated with regard to whole-body micro-CT (as the most accurate measurement). The strongest correlation was observed between the abdominal and whole-body micro-CT. The total skinfold thickness showed a surprisingly close correlation with fat percentage determined by whole-body scan. Fat mass indicators (PMA) showed weaker associations with whole-body micro-CT. Correlations between fat percentage values based on abdominal micro-CT_(L1-L3) and wet weights of epididymal and retroperitoneal fat pads measured by PMA also proved to be significant. Regarding total skinfold thickness, correlations were also strong. Repeatability analysis showed good relationship between the two within-day measurements of STM. Reproducibility between observers also showed good relationship. Thus, total ST could be regarded as a reliable parameter.

Age-related changes in fat percentages of Wistar rats assessed by abdominal micro-CT_(L1-L3) were remarkably similar to those in retroperitoneal fat pads (for 100 g BW) determined by PMA. Both methods showed age-related increase in fat mass until 18 month of age, followed by a decline in the oldest rats. Epididymal fat pads determined by PMA reached its peak in the middle-aged 12-months-old rats. In contrast with age-related patterns shown by the other methods, total skinfold thickness increased during the course of aging without any decline.

With regard to the differentiation between visceral and subcutaneous fat percentages assessed by abdominal micro-CT_(L1-L3), age-related changes in visceral fat percentages were similar to those (above mentioned) in retroperitoneal fat pads determined by PMA or total fat assessed by micro-CT_(L1-L3). In contrast, the age-related pattern characterizing subcutaneous fat resembled that of total ST (without any decline in the oldest rats).

The muscle indicator using PMA showed lower value in the aging 18-month-old rats than in younger ones and a further decline in the oldest group. These results suggest the appearance of sarcopenia in the aging and old groups.

1.2. Discussion of body composition measurements

We demonstrated the efficacy of different methods in the assessment of body fat in rats of different age-groups. Our results revealed that the micro-CT_(L1-L3), scanning this previously untested abdominal region in rats could be an equally useful tool as whole-body micro-CT regarding visceral fat percentage assessment. Our results are in accordance with earlier reports claiming that scanning the abdomen with micro-CT between vertebrae L1-L6, L1-L5 or L4-L5 (Hillebrand et al. 2010; Judex et al. 2010; Lubura et al. 2012) may provide sufficient basis to estimate total body fat in rodents as judged by comparison with whole-body micro-CT. Reducing the size of the scanned region decreased the duration of the scan and that of the

necessary anesthesia markedly. It is especially important in old, obese rats that show especially high mortality risk during anesthesia.

The total STM is simple, quick cost-effective and non-invasive *in vivo* technique, moreover it can be carried out repeatedly without deep anesthesia. However, it requires technical training and only measures subcutaneous fat. In rats this method has not been sufficiently validated. According to our data, total STM is also suitable to detect differences and follow changes in fat mass in rats.

Our result confirmed our hypothesis that visceral fat assessed separately by micro-CT_(L1-L3) and retroperitoneal fat determined by PMA show similar age-related pattern: both methods indicated a gradual increase from 3- to 18 months of age, followed by a slight decline in the oldest rats. In contrast, PMA based on epididymal fat pad indicated the peak earlier, already at 12 months of age. These findings support our hypothesis, that *post mortem* retroperitoneal fat measurement reflects age-related differences in body fat better than measurement of epididymal fat. Subcutaneous fat measured by micro-CT_(L1-L3) and by STM showed a monotonous age-related increase without any late decline. Therefore, loss of fat in previously more obese animals may not be followed by this latter method especially in very old rats characterized by age-related accumulation of connective tissue. Our new assessment of muscle mass indicated the appearance of age-related muscle loss already in aging 18-month-old animals with the highest BW and body fat. These data are in accord with human observations: muscle mass declines at a greater rate with age than body mass, reducing muscle strength leading to disability and loss of independence (Keller et al. 2014).

2.1. Age-related changes in the hypothalamic MC system: Results of *in vivo* investigation of catabolic responsiveness to exogenous MC in Wistar rats

The BW development of male Wistar rats of our colony shows a marked continuous age-related rise until 18 months of age. Thereafter a pronounced decline is observed. In our *in vivo* study acute metabolic/thermoregulatory and anorexigenic effects of ICV injected alpha-MSH were investigated in different age-groups of rats. Hypermetabolic and anorexigenic components of the catabolic effects of ICV alpha-MSH-injection showed characteristic age-related changes. The hypermetabolic effect (elevations in VO₂ with consequent rise in Tc) remained significant in all age-groups except for older middle-aged rats. Accordingly, this effect reached its nadir in the 12-month-old group followed by an increase in older animals. In old rats, the significant increase in VO₂ was associated with a significant rise in Tc again despite the strong vasodilation. Similarly, the anorexigenic effect was also significant in all age-groups (except for older 12-month-old rats). Accordingly, this effect of the peptide also reached its minimum in the 12-month-old group followed by an increased efficacy in older animals.

2.2. Discussion of *in vivo* experiments

The age-related shifts in the acute hypermetabolic/hyperthermic responses to alpha-MSH appear to be similar to those of the anorexigenic effects of the peptide (Pétervári et al. 2010). Our results suggest that decreased catabolic efficacy of alpha-MSH precedes middle-aged weight gain and obesity (i.e. the highest BW and visceral fat percentage observed by 18 months of age), and later on an increased efficacy (in the 18-month-old group) precedes weight/muscle loss in old (24-month-old) rats. These observed age-related shifts in catabolic alpha-MSH-effects may contribute to the development of middle-aged obesity and later to that of weight loss (sarcopenia) of old age.

3.1. Age-related changes in the hypothalamic MC system: Results of *in vitro* analysis of the endogenous activity in Wistar rats

Our *in vitro* findings revealed potential mechanisms of the age-related *in vivo* trends in BW regulation in male Wistar rats. In middle-aged rats peptide contents of alpha-MSH and MC4R decreased both in the ARC and PVN (without matching changes in gene expression), while in aging animals the increase in alpha-MSH and MC4R peptide contents were associated with parallel increases in POMC and MC4R mRNA expressions. These shifts in MC4R in the PVN may contribute to the explanation of the changes in the responsiveness to exogenously administered alpha-MSH. Although AgRP mRNA expression in both nuclei showed some increase in aging animals, its protein content remained unchanged across all age-groups.

3.2. Discussion of *in vitro* results in view of our *in vivo* observations

Our study is the first to analyze age-related shifts in the endogenous tone of the hypothalamic MC system across five age-groups with parallel analysis of gene expression and immunohistochemistry. Previous studies have usually compared POMC and/or AgRP gene expressions of only a young and an old group (Zhang et al. 2004; Kappeler 2003; Nelson et al. 1988; Arens et al. 2003; Rigamonti et al. 2006), only a few of them included a third (middle-aged) group in the comparison (Gruenewald et al. 1991; Wolden-Hanson et al. 2004; McShane et al. 1999; Lloyd et al. 1991). They showed controversial results probably due to use of different rat and mouse strains, gender differences or diverse methodologies.

Our results prove that the alpha-MSH and MC4R peptide immunoreactivity along with the POMC and MC4R mRNA expression are affected by age in a way that may contribute to the explanation of aging anorexia. Moreover, the earlier occurring decline in alpha-MSH and MC4R peptide immunoreactivity may contribute to the explanation of the weight gain and obesity of middle-aged animals. Our results suggest that reaching a critical BW or fat mass may provide the trigger for the activation of the MC system, that in turn aggravates aging anorexia.

Hypothesis II.

4.1. Dysregulation in SHR rats: results of *in vivo* and *in vitro* experiments

The ICV alpha-MSH injection reduced the cumulative spontaneous FI in both NT and SHR groups, but the rate of the reduction was significantly stronger in SHR than in NT animals. The ICV injection of AgRP significantly increased FI without difference between SHR vs. NT rats. The selective MC4R antagonist HS024 increased daily FI in the NT group already from the 2nd day of the ICV infusion, while its orexigenic effect in SHR rats started with a delay, just from the 4th day. As a consequence of increased FI, the BW in both HS024-treated groups also increased but with a different time course (delay in SHR). The NPY-induced cumulative FI (in percentage of daily FI) was significantly lower in SHR than in the age-matched NF group. Cell counting revealed that there is no statistically significant difference between SHR and NT rats in the number alpha-MSH immunoreactive neurons in the ARC. In contrast, assessment of fluorescent light intensity revealed that SHR had 28 % higher alpha-MSH SSD (specific signal density) in the ARC than NT rats. To quantify the SSD of NPY nerve fibers in ARC, we found that there is no significant difference between SHR vs. NT groups in the NPY. No statistical difference was found in the SSD of respective immunosignals in alpha-MSH, NPY and AgRP immunopositive terminals in the PVN.

4.2. Dysregulation in SHR rats: discussion of *in vivo* and *in vitro* experiments

Based on our results, we propose that the characteristic phenotype of SHR may be partly explained by enhanced responsiveness to the MC agonist and by the diminished orexigenic responsiveness to NPY found in young adult animals. Both our immunohistochemical and qRT-PCR findings provide further support for the role of the enhanced activity of the central MC system in the regulation of energy balance in SHR. A previous observation using MC3R/MC4R antagonist reported diminished orexigenic effect in SHR (daSilva et al. 2008). Delayed and diminished orexigenic effects of the MC4R antagonist infusion in our study are still compatible with the concept of increased endogenous MC tone, but the underlying mechanisms require further investigations including, among other factors, the development of innervation of second order nuclei in SHR.

SUMMARY OF NOVEL FINDINGS

Hypothesis I.: Activity of the MC system shows characteristic age-related pattern: decreased activity of the hypothalamic melanocortin system contributes to the development of middle-aged obesity, but later the MC activity increases leading to anorexia and weight loss in old age.

1. Micro-CT-L₁₋₃ is a good and useful method for repeated body fat assessment in rats.
2. The skin thickness measurement (STM) is also useful to follow the body fat changes *in vivo* in rats.
3. The acute catabolic effects of intracerebroventricularly applied alpha-MSH show characteristic age-dependent changes in male Wistar rats: decreased catabolic efficacy of alpha-MSH precedes middle-aged weight gain, and later on an increased efficacy precedes weight loss in old rats. The observed age-related shifts in catabolic alpha-MSH-effects may contribute to the development of middle-aged obesity and later to that of weight loss of old age.
4. Our *in vitro* findings revealed potential mechanisms of these age-related *in vivo* trends in BW regulation. In middle-aged rats peptide contents of alpha-MSH and MC4R decreased both in the ARC and PVN (without matching changes in gene expression), while in aging animals the increase in alpha-MSH and MC4R peptide contents were associated with parallel increases in POMC and MC4R mRNA expressions. Although AgRP mRNA expression in both nuclei showed some increase in aging animals, its protein content remained unchanged across all age-groups.

Hypothesis II.: We assumed, that enhanced activity of hypothalamic anorexigenic melanocortins and diminished tone of orexigenic NPY may contribute to the BW dysregulation of SHR strain.

5. Our results suggest that a higher MC-production/responsiveness and lower NPY-responsiveness may contribute to the BW dysregulation of SHR.

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PUBLICATIONS

List of publications the PhD thesis is based on [Cumulative impact factor (IF): 13.158]

Nóra Füredi, Alexandra Mikó, Balázs Gaszner, Diána Feller, Ildikó Rostás, Judit Tenk, Margit Solymár, Márta Balaskó, Erika Pétervári. Activity of the hypothalamic melanocortin system decreases in middle-aged and increases in old rats. *Journals of Gerontology - Series A: Biological Sciences* 2018;73(4):438-445.

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