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Age and nutritional state influence the effects of cholecystokinin on energy balance

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ABSTRACT

Cholecystokinin (CCK) is anorexic, irrespective whether it is applied intraperitoneally (IP) or intracerebroventricularly (ICV) in male Wistar rats. The metabolic effects depend on the route of administration: by the IP route it elicits hypothermia (presumably by type-1 receptors, CCK1R-s), while ICV administration is followed by fever-like hypermetabolism and hyperthermia via activation of CCK2R-s, which latter response seems to be most important in the postprandial (compensatory) hypermetabolism. The efficacy of the IP injected CCK varies with age: it causes strong anorexia in young adult 4 and 6-months old and again in old rats (aged 18–24 months), but the middle-aged (12-month old) ones seem to be resistant to this effect. Such pattern of effects may contribute to the explanation of age-related obesity observed in middle-aged animals as well as to the aging anorexia and loss of body weight in old ones. Diet-induced obesity accelerates the appearance of CCK-resistance as well as the return of high sensitivity to CCK in further aging, while chronic calorie-restriction prevents the development of resistance, as if the speed of the age-related regulatory changes was altered by the nutritional state. The effects of ICV applied CCK also change with age: the characteristic anorexic and hypermetabolic/hyperthermic effects can be observed in young adult rats, but the effects gradually and monotonically decline with age and disappear by the old age of 24 months. These disparate age-related patterns of CCK efficacy upon peripheral or central administration routes may indicate that although both peripheral and central CCKR-s exert anorexic effects, they may have dissimilar roles in the regulation of overall energy balance.

1. Introduction

A number of peptide hormones have been shown to influence the components of energy balance (food intake, metabolic rate, body weight, body temperature, etc.) by having either an overall anabolic or catabolic effect (Szekely et al., 2010). The roles played by these peptides are not standard in the course of life: in the regulation of energy balance both the role(s) of individual peptides and their interactions change continuously.

In mammals, including humans, aging is accompanied by characteristic alterations in energy balance (Pétervári et al., 2011). At a juvenile age the balance is positive (calorie intake exceeds energy expenditure) in order to serve the growth and development of the body. In young adults the anabolic (orexigenic and hypometabolic) and catabolic (anorexigenic and hypermetabolic) mechanisms are balanced. However, later on in middle-aged subjects the common age-related obesity (Scarpace et al., 2000) suggests a shift towards anabolic processes, while at old age the characteristic aging anorexia (Chapman et al., 2002), that is often accompanied by senile sarcopenia, indicates excess of catabolic mechanisms.

Both obesity and sarcopenia have serious medical consequences even if not related to age. The peptidergic regulation of energy balance and the effects of neuropeptides may vary not only with age but also with pre-existing body composition (obesity, undernutrition).

Cholecystokinin (CCK) has been recognized for over a century as one of the first known hormones, with a function to enhance gallbladder's motility. By now, it is clear that this gastrointestinal peptide has several other functions in forwarding and digesting the consumed food, and also that it causes satiety (Moran et al., 2006). The latter effect indicates that CCK as a peptide hormone of the brain–gut axis can influence cerebral functions related to energy balance. Controversial data have been reported concerning the contribution of CCK to the maintenance of energy balance. Lo and coworkers demonstrated that CCK knockout mice proved to be resistant to high-fat diet-induced obesity (Lo et al., 2010). However, this mouse strain also showed impaired fat absorption and enhanced metabolic rate that some authors attribute to their genetic background (Lacourse et al., 1999). Lack of CCK may also have contributed to the fat malabsorption due to impaired gallbladder function. Although such studies do not contradict the

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potential importance of CCK in the regulation of energy balance, they demonstrate the complexity of CCK effects.

Food intake enhances CCK production not only in the upper gastrointestinal tract but also in the hypothalamus (Schick et al., 1990, 1994), with obvious central effects in this latter case. Although peripheral CCK has certain central actions, the central functions of CCK of peripheral or central origin may not be identical.

Peripheral type-1 CCK receptors (CCK1R-s) are located mainly on the afferent fibers of the abdominal vagus (Smith et al., 1985; South and Ritter, 1988), although some CCK1R-s have been detected also in the brain (Hirosue et al., 1993). The nucleus of the solitary tract (NTS) serves as a portal for assessing and integrating visceral afferent signals (including CCK-related signals), while the dorsal motor nucleus provides outbound signals towards neurons of the rostral medullary raphe to influence efferent responses (Berthoud, 2004; Blessing, 1997). This system seems to function on the basis of a within-meal negative feedback satiety signal and it is important mainly in determining the short-term regulation of food intake (Balaskó et al., 2012; Moran et al., 2006; West et al., 1984) according to the actual feeding state. The role of this system in the long-term regulation of nutritional state (adiposity) is less clear. Peripherally applied CCK in pharmacological doses elicited hypothermia (Kapás et al., 1987) presumably by a vagal reflex effect causing hypometabolism, skin vasodilation and consequently increased heat loss, independent of the role of CCK in the regulation of food intake and energy balance.

In contrast to peripheral administration, centrally applied CCK acts mainly on CCK2R-s of hypothalamic and other nuclei (Mercer et al., 2000). Centrally injected CCK is known to induce fever-like coordinated changes in energy balance: an increase in metabolic rate, a decrease in heat loss, an elevation of body temperature (Szelényi et al., 1994), and it also evokes anorexia (Gibbs et al., 1973). In endotoxin fever CCK2R-s are also involved (Székely et al., 1994).

The aim of the present study was to investigate whether or not, and how, peripheral vs. central CCK-related effects may contribute to changes of energy balance in the course of aging. A further aim was to check whether nutritional state can influence the effects of CCK, since the efficacy of other peptides have been shown to vary not only with age but also with nutritional state (Soos et al., 2011).

2. Materials and methods

2.1. Animals

Male Wistar rats from the colony of the Department of Pathophysiology and Gerontology were used in the experiments. After weaning the animals were kept individually in plastic cages (375 × 215 mm, height 149 mm, covered by steel grid and equipped with feeder and bottle container) with some wood-chip bedding at an ambient temperature of 22–25 °C. The lights were on between 06.00 and 18.00 h. Standard rat chow (CRLT/N rodent chow, Szindbád Kft., Gödöllő, Hungary, 11 kJ/g) and tap water were continuously available (food but not water was removed for a 48-h period in some groups). Among these normally fed (NF) rats different age-groups have been established: 2, 4, 6, 12, 18 and 24 month old animals (NF2, NF4, NF6 and NF12, NF18 and NF24) represented juvenile, young adult, younger and older middle-aged, aging and old age-groups, respectively. (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 are difficult.)

Some animals were calorie-restricted (CR) from age 2 months onwards: they received 2/3 of the normal daily amount of standard chow (16 g/day), with vitamin and mineral supplementation and unlimited water intake. Some other 6 and 12-month-old rats were made obese by using a high-fat diet (HF, using diet-induced obesity rodent purified diet with 60% Energy from Fat, IPS TestDiet®, 21.6 kJ/g) from age 2 months. For 10–14 days before and also during the experimental procedures rats that participated in the assessment of CCK effects on food intake received a powdered form of their respective types of chow and were transferred to an automated FeedScale system which allowed continuous recording of their food consumption and prevented food hoarding. The powdered version of the high-fat diet contained 10% normal powdered chow admixed to the powdered high-fat pellets (20.54 kJ/g). Body weight and spontaneous daily food intake were measured every day at 09.00 h — thereby the animals were also accustomed to regular handling. Rats used in the analysis of metabolic rate and body temperature were habituated for at least a week prior to experiments to semi-restraining boxes in which they were able to move somewhat forward and backward but not to change the head-to-tail position.

All experiments and interventions were undertaken according to the general rules and special approval of the University of Pécs Ethical Committee for the Protection of Animals in Research (BA 02/2000-11/2011), in accordance with the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC).

2.2. Surgical interventions

For intracerebroventricular (ICV) injections a 22-gauge metal leading cannula was stereotaxically implanted into the right lateral cerebral ventricle (parameters: 1 mm posterior and 1.5 mm right lateral to bregma, 3.5 mm ventral to dura) for chronic use, under intraperitoneal (IP) ketamine + xylazine [78 mg/kg (Calypsol, Richter) + 13 mg/kg (Sedaxylan, Eurovet)] anesthesia. The cannula was fixed to the skull by dental cement with the help of 2 miniature screws into the bone. The lumen of this cannula was regularly closed by a stylet — at injections the stylet was replaced by a fitting 28-gauge injection cannula that was connected to a pp10 polythene tube for remote ICV injections. For in vivo testing of the cannula's placement angiotensin II (20 ng/5 μl) was injected and the subsequent water consumption was measured: the test was positive and the location of the cannula was assumed to be optimal if at least 5 ml water was consumed within 30 min (Pétervári et al., 2010).

Following the experiments the animals were euthanized by an IP injection of urethane and the injection sites of their brains were checked macroscopically by coronal sections of the removed and fixed brains. (Data of rats with inappropriately placed cannula were excluded from the statistical analysis.) Simultaneously, the left retroperitoneal and epididymal fat pads were removed and weighed, along with the tibialis anterior muscle, as indicators of body composition (Soos et al., 2010). All body composition indicators were calculated for 100 g body weight.

2.3. Administration of substances

Cholecystokinin-8 (Bachem) or solvent pyrogen-free saline (PFS) was administered either by direct IP injections at a dose of 5 μg (4.4 nmol) in a volume of 0.5 ml for assessment of anorexigenic effects (see Section 2.4), in other cases at a pharmacological dose of 100 μg (88 nmol, as applied in earlier studies, Kapás et al., 1987) in a volume of 0.1 ml in metabolic studies (see Section 2.5), or by ICV injections at a dose of 500 ng (0.44 nmol) in a volume of 5 μl for both anorexigenic and metabolic tests. In the analysis of anorexia the injections were given 5 min prior to presentation of food, while in the metabolic studies CCK was injected after the animals reached a thermal steady state (usually 60–90 min after closing the metabolic chamber). For measurements of metabolic rate and body temperature, the ways of CCK administration were slightly different (see Section 2.5).

2.4. Assessment of CCK effects on food intake in function of age and nutritional state

The anorexigenic responsiveness to IP or ICV CCK injections was assessed in a number of rats (6–8 rats per group) from different populations according to age and nutritional state via measuring their

inhibitory effects on 3-h cumulative food intake (per unit body weight) induced by 48-h food deprivation (from 09.00 on day 1 until 09.00 on day 3). In control experiments PFS was used. The consumed food was measured in a Feed-Scale system (Columbus), which allowed fine assessment of the rate of feeding. The data were collected in 30-min periods. Normally fed animals at ages 2, 4, 6, 12, 18 and 24 months, a group of CR animals (CR12 with unlimited access to powdered chow during the 3-h re-feeding), and two groups of HF rats (HF6 and HF12) were tested in the experiments.

2.5. Assessment of CCK effects on metabolic rate and body temperature

Normally fed rats from groups of various ages were singly enclosed in restraining cylindrical boxes of corresponding size and placed into a tightly sealed plexiglass metabolic chamber (size: 20 × 30 × 18.5 cm) that was perfused by a standard gas mixture (corresponding to "standardized" room air). The chamber was immersed into a thermostatically controlled water-bath to secure standard (25 °C or 28 °C, i.e., lower end of the thermoneutral zone or within thermoneutrality) or cool (20 °C) ambient temperature (T_a) for the experimental animals. Copper-constantan thermocouples were attached to the rats for measuring colonic (core) and tail skin temperatures (T_c and T_s, respectively): these – together with the thermocouple for the chamber – were exteriorized from the sealed chamber.

An injection cannula inserted into the chronically preimplanted ICV cannula was connected to a 20–25-cm-long pp10 polythene (Portex) tube (Szelényi et al., 1994). The tube contained at the cranial end the peptide in a volume of 5 μl separated by a small bubble from the PFS filling the rest of the tube, which was closed and exteriorized together with the thermocouples. At injections, 5 μl PFS was slowly injected at the outer end of the tube, thereby the CCK was injected ICV without disturbing the animal.

For IP injections the cannula was acutely inserted (through the lumen of a needle) prior to the experiment to the abdominal cavity, fixed by sticky tape and the animal was placed into the restraining box. Both the cannula and the thermocouples were exteriorized similarly as described previously.

Oxygen consumption, CO_2 production and respiratory quotient (RQ) were determined by the help of an Oxymax gas analyzer (Columbus, OH) and the data were electronically processed.

All temperature data were collected by a Digi-Sense 12-channel scanning Benchtop thermometer (Cole-Parmer) for electronic evaluation. Heat loss state ("heat loss index", HLI, as used in earlier studies; Romanovsky et al., 2000) was assessed from the relationship of the three monitored temperatures [HLI = (Ts − Ta) / (Tc − Ta)]: Ts values approaching Ta (HLI near 0) suggested vasoconstriction as a sign of heat conservation state, while those Ts values nearer to Tc (HLI near 1) suggested vasodilation as an early manifestation of general enhancement of heat loss activity.

This methodology of indirect calorimetry allowed assessment of metabolic rate (O_2 consumption) and that of RQ, while from the relationship of the measured temperatures HLI was calculated. These data reflect heat production and heat loss states, respectively, together with the consequent core temperature.

2.6. Statistical analysis

Experimental results are presented as mean ± S.E.M. Groups of different ages and body compositions contained at least 6–8 rats. For statistical analysis of the data SPSS 11.0 for Windows software was applied for one-way ANOVA with Scheffe's post hoc tests and SigmaPlot for Windows version 11.0 was applied for regression analysis. Differences reaching $p < 0.05$ were considered to be statistically significant.

3. Results

Body weight (BW) (Table 1) and body composition values (calculated for 100 g BW) of different NF age-groups (Fig. 1 and Table 2) were in accord with those observed in our previous studies (Pétervári et al., 2010): up to 12 months of age BW showed a rising tendency with a plateau phase between 12 and 18 months, then it started to decline slowly. In juvenile rats BW and fat mass indicators were significantly smaller than those of all other groups. No difference in muscle mass was detected in any group, except for the oldest (24 months old) sarcopenic animals.

Body weight (Table 1) and fat content of HF rats exceeded those of age-matched NF controls, while the relative muscle mass remained similar (Fig. 1 and Table 2). Body composition indicators of NF6 vs. HF6 rats were as follows: epididymal fat: 0.35 ± 0.04 vs. 0.77 ± 0.07 g/100 g BW ($p < 0.001$); retroperitoneal fat: 0.39 ± 0.07 vs. 1.07 ± 0.15 g/100 g BW ($p = 0.002$). Similar ratios were observed in the NF12 vs. HF12 groups: epididymal fat: 0.52 ± 0.04 vs. 1.00 ± 0.05 g/100 g BW ($p < 0.001$); retroperitoneal fat: 0.50 ± 0.03 vs. 1.82 ± 0.19 g/100 g BW ($p < 0.001$). Fat mass and BW values of HF12 were significantly higher than those of all other rats. Although HF6 weighed less than HF12, their BW was comparable to (or even higher than that of) NF12, twice their age (Table 1).

Body weight (Table 1) and fat content (Fig. 1) of CR12 rats were significantly smaller than those of age-matched NF controls. These values were similar to those of much younger NF4 animals. While fat mass was significantly reduced, no decline of muscle mass was observed compared to NF12 (Fig. 1 and Table 2): epididymal fat: 0.52 ± 0.04 vs. 0.26 ± 0.03 g/100 g BW ($p = 0.002$); retroperitoneal fat: 0.50 ± 0.03 vs. 0.14 ± 0.03 g/100 g BW ($p < 0.001$) (NF12 vs. CR12, respectively).

Upon 48-h fasting weight loss of NF age-groups ranged from 7% to 10% of initial BW except for a 14% BW fall in the 2 month old juvenile group. Weight loss of CR12 rats reached about 10%, while HF animals lost merely 4–6%. In NF rats, the subsequent cumulative 3-h energy

Table 1
Body weight values (BW) before a 48-h fasting and cumulative energy intake (FI) during the consequent 3-h re-feeding of rats belonging to different age-groups and nutritional states.

Group (age and nutritional state)	BW (g) before fasting	3 h cumulative FI (kJ)
NF2 control	208.9 ± 5.6	78.4 ± 6.8
NF2 CCK	209.6 ± 1.6	72.3 ± 6.2
NF4 control	396.2 ± 12.2	134.1 ± 5.8
NF4 CCK	395.7 ± 11.4	99.0 ± 6.9^{c}
NF6 control	467.5 ± 14.6	137.5 ± 17.2
NF6 CCK	466.8 ± 12.6	89.7 ± 7.6^{c}
HF6 control	565.0 ± 13.9*	215.6 ± 24.3^{a}
HF6 CCK	546.6 ± 15.5*	181.9 ± 31.1
NF12 control	534.7 ± 10.4	86.9 ± 3.2
NF12 CCK	529.1 ± 12.9	78.1 ± 5.6
HF12 control	710.1 ± 24.6$^{\#}$	153.4 ± 13.9^{b}
HF12 CCK	682.3 ± 23.5$^{\#}$	98.7 ± 13.5^{c}
CR12 control	324.4 ± 5.5*	139.7 ± 11.7^{b}
CR12 CCK	327.4 ± 5.7*	73.9 ± 9.8^{c}
NF18 control	536.3 ± 17.2	102.7 ± 10.7
NF18 CCK	518.8 ± 7.2	71.5 ± 5.8^{c}
NF24 control	514.9 ± 17.5	102.7 ± 7.7
NF24 CCK	511.8 ± 30.2	64.9 ± 6.6^{c}

Values are expressed as the mean ± S.E.M. for six-eight rats in each group.
NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted.
Numbers following the above abbreviations of animal groups indicate the age of the rats in months.
BW values of control vs. CCK-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 other groups ($p < 0.001$), * HF6 or CR12 vs. age-matched NF (HF6 vs. NF6 $p < 0.01$, CR12 vs. NF12 $p < 0.001$).
Regarding 3-h cumulative FI values of control groups the following statistically significant differences were denoted in the table: "a" HF6 vs. all other groups ($p < 0.05$), "b" HF12 or CR12 vs. age-matched NF (HF12 vs. NF12 $p < 0.001$, CR12 vs. NF12 $p < 0.01$).
CCK treatment reduced 3-h cumulative FI significantly compared to controls of the same age and nutritional state in the following groups: "c" NF4 ($p = 0.001$), NF6 ($p = 0.015$), NF18 ($p = 0.015$), NF24 ($p = 0.006$), HF12 ($p = 0.015$), CR12 ($p = 0.001$).

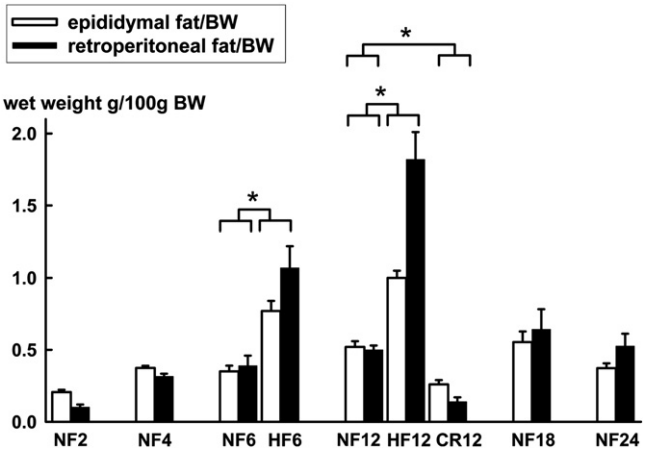

Fig. 1. Indicators of fat mass in rats of different age-groups and nutritional states given for 100 g body weight (BW). Asterisks indicate significant differences between values of rats of the same age and different nutritional states. Detailed statistical analysis of the data is described in the Results section. NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted. Numbers following the abbreviations of animal groups indicate the age of the rats in months.

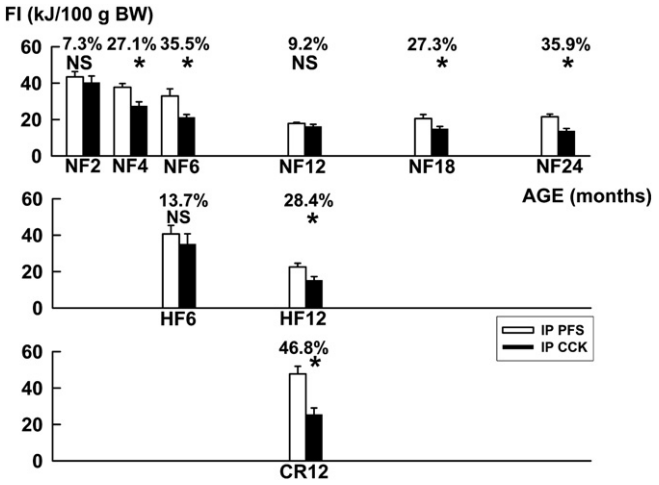

Fig. 2. Reduction in the 48-h fasting-induced cumulative 3-h food intake (FI) expressed in kJ/100 g body weight (BW, after fasting, before re-feeding) in different age-groups and nutritional states of rats following intraperitoneal (IP) cholecystokinin (CCK) treatment. Values (columns) are expressed as the mean ± S.E.M. for six-eight rats in each group. The rate of reduction is denoted above the pairs of columns in percentage of the corresponding control value. Asterisks indicate significant differences between re-feeding values of IP CCK-treated (dark columns) and control [pathogen-free saline (PFS)-treated] rats of the same age and nutritional state (light columns): NF4 $p = 0.003$, NF6 $p = 0.009$, NF18 $p = 0.037$, NF24 $p = 0.004$, HF12 $p = 0.026$, CR12 $p = 0.001$. NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted, NS: non-significant. NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted. Numbers following the above abbreviations of animal groups indicate the age of the rats in months.

intake (during re-feeding) expressed in kJ/100 g BW showed an age-dependent decline from NF2 to NF12 and remained at the same level thereafter (Fig. 2). Re-feeding expressed in kJ was the largest in the HF animals, and values of HF6 exceeded those of HF12 (Table 1). When calculated for 100 g BW, 3-h energy intake of HF6 was circa twice as high as that of HF12 (40.7 ± 4.7 vs. 22.5 ± 2.0 kJ/100 g BW, Fig. 2). Regarding CR12 rats, their cumulative 3-h energy intake in kJ-s following 48-h fasting was also very high: it exceeded significantly the value of NF12, but did not differ from that of HF12 of a much higher BW (Table 1). When expressed in kJ/100 g BW re-feeding energy intake of CR12 was the largest of all rats (47.8 ± 4.2 kJ/100 g BW, Fig. 2).

3.1. CCK-effects on food intake

In young adult rats the IP administered CCK caused significant suppression of 3-h cumulative food intake during re-feeding after 48-h fasting. In order to demonstrate the different rates of suppression Fig. 2 shows these data in kJ/100 g BW. Compared to the suppression seen in the young adult group, the most pronounced effect was observed at the age of 6 months. However, CCK was ineffective in juvenile animals and in middle-aged ones (NF12). Interestingly, at later ages (NF18, NF24) the suppression of re-feeding energy intake became again pronounced and statistically significant. Regarding the anorexic efficacy of CCK in different age groups, the highest relative dose normalized to BW (Supplement 1) failed to elicit significant effects in NF2, proceeding to exert reduction in food intake despite lower relative CCK doses in NF4 and NF6. Although middle-aged rats were characterized by a low relative dose with a lack of CCK-anorexia, the same low relative dose proved to be efficient from NF18.

Alterations in body composition influenced this pattern of CCK efficacy. Obese HF6 rats (with low relative dose, see Supplement 1) were resistant to the anorexic effect of CCK (Fig. 2) already at the age of 6 months, but the anorexic effect became again significant in HF12 animals (lowest relative dose). In contrast, during re-feeding CR12 rats consumed much more food than age-matched controls (they appeared to be hungrier than rats of the NF12 group), but CCK almost halved the consumption (Fig. 2), unlike in NF12 animals.

The ICV injected CCK (Fig. 3) was also without significant effect on food intake in juvenile rats, but caused extreme anorexia in NF4 and NF6 animals (peak suppression in NF6). The effect was attenuated but still significant in the middle-aged NF12 group, and – in contrast to the IP administration – it further decreased and became non-significant in the old NF24 animals. These results suggested an age-related monotonous decrease in the anorexic effect of centrally applied CCK.

Table 2
Indicator of muscle mass in rats of different age-groups and nutritional states.

Group (age and nutritional state)	Tibialis anterior muscle (g/100 g body weight)
NF2	0.18 ± 0.01
NF4	0.17 ± 0.01
NF6	0.20 ± 0.01
HF6	0.19 ± 0.01
NF12	0.19 ± 0.01
HF12	0.18 ± 0.01
CR12	0.20 ± 0.01
NF18	0.17 ± 0.01
NF24	0.13 ± 0.01*

Asterisk indicates significant decline in muscle mass indicator of NF24 vs. all other groups ($p < 0.001$).
NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted.
Numbers following the above abbreviations of animal groups indicate the age of the rats in months.

3.2. Effects of CCK on metabolic rate and thermoregulation

For thermoregulatory analysis of CCK, different ambient temperatures were applied: thermoneutrality (25–28 °C) allows the activation of vasodilation (heat loss), and a cool environment (20 °C) that elicits an increase in metabolic rate, permits the appearance/study of hypometabolic effects.

In line with the data of the literature (Kapás et al., 1987), young adult NF4 rats responded with hypothermia to IP injection of a pharmacological dose of CCK, due either to skin vasodilation (at a thermoneutral ambient temperature, Fig. 4A) or to a decrease in metabolic rate (at a cool ambient temperature, Fig. 4B). For technical reasons, dependence of the hypothermic response on age or nutritional state was not analyzed in the present study.

In young adult NF4 rats the metabolic effects of ICV injected CCK appeared to be coordinated (Fig. 4C). Centrally applied CCK caused an immediate significant rise in oxygen consumption with a decreasing

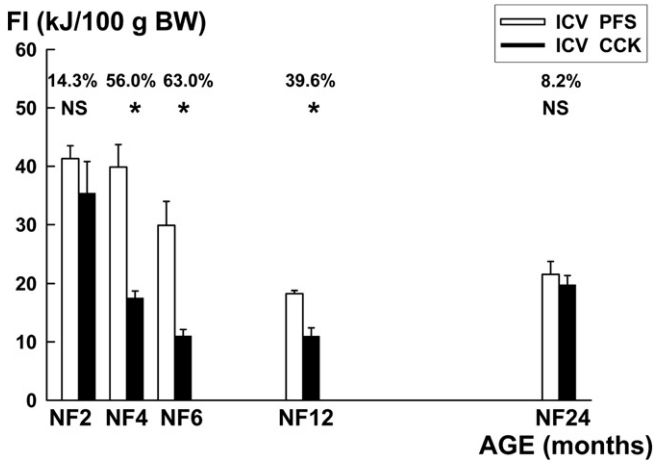

Fig. 3. Reduction in the 48-h fasting-induced cumulative 3-h food intake (FI) expressed in kJ/100 g body weight (BW after fasting, before re-feeding) in different age-groups of rats following intracerebroventricular (ICV) cholecystokinin (CCK) treatment. Values are expressed as the mean ± S.E.M. for six-eight rats in each group. The rate of reduction is denoted above the pairs of columns in percentage of the corresponding control value. Asterisks indicate significant differences between re-feeding of ICV CCK-treated (dark columns) and control [pathogen-free saline (PFS)-treated] rats of the same age: NF4 $p < 0.001$, NF6 $p < 0.001$, NF12 $p < 0.001$. NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted. Numbers following the above abbreviations of animal groups indicate the age of the rats in months.

tendency in RQ from 0.80 ± 0.01 to 0.76 ± 0.02 (suggesting a somewhat enhanced fat utilization) that did not reach statistical significance ($p = 0.061$, Fig. 5). At the lower end of the thermoneutral zone (25 °C) CCK caused no change in tail vasomotor tone and T_s, i.e., the skin vasoconstriction persisted. This response corresponds to the febrile reaction seen in response to lipopolysaccharide or prostaglandin E, and the anorexic effect fits the pattern of sickness behavior adjoining fever.

Similarly as the anorexic response, the metabolic response to ICV CCK was age-dependent (Fig. 6). The hypermetabolic/hyperthermic response that was characteristic for the NF4 rats became smaller, though still significant in NF6 and NF12 animals compared with the PFS-treated controls, but with further aging the response became even smaller and neither statistically nor biologically significant in the NF18 and NF24 old age-groups. A negative linear correlation was shown between age and the CCK-induced change in Tc of individual rats (Fig. 7). Correlation coefficient of the linear regression was $r = -0.648$. Thus, the hypermetabolic effect exhibited a monotonous decline with aging, similarly as seen in the case of the age-related decline of the anorexic effect of ICV CCK.

4. Discussion

In our study the high-fat diet induced significant weight gain and an accumulation of fat as shown by the body composition indicators. Body weight and fat mass indicators of the obese 6-month old rats exceeded those of normally fed older middle-aged 12-month old animals suggesting a premature onset of “middle-aged” obesity induced by a

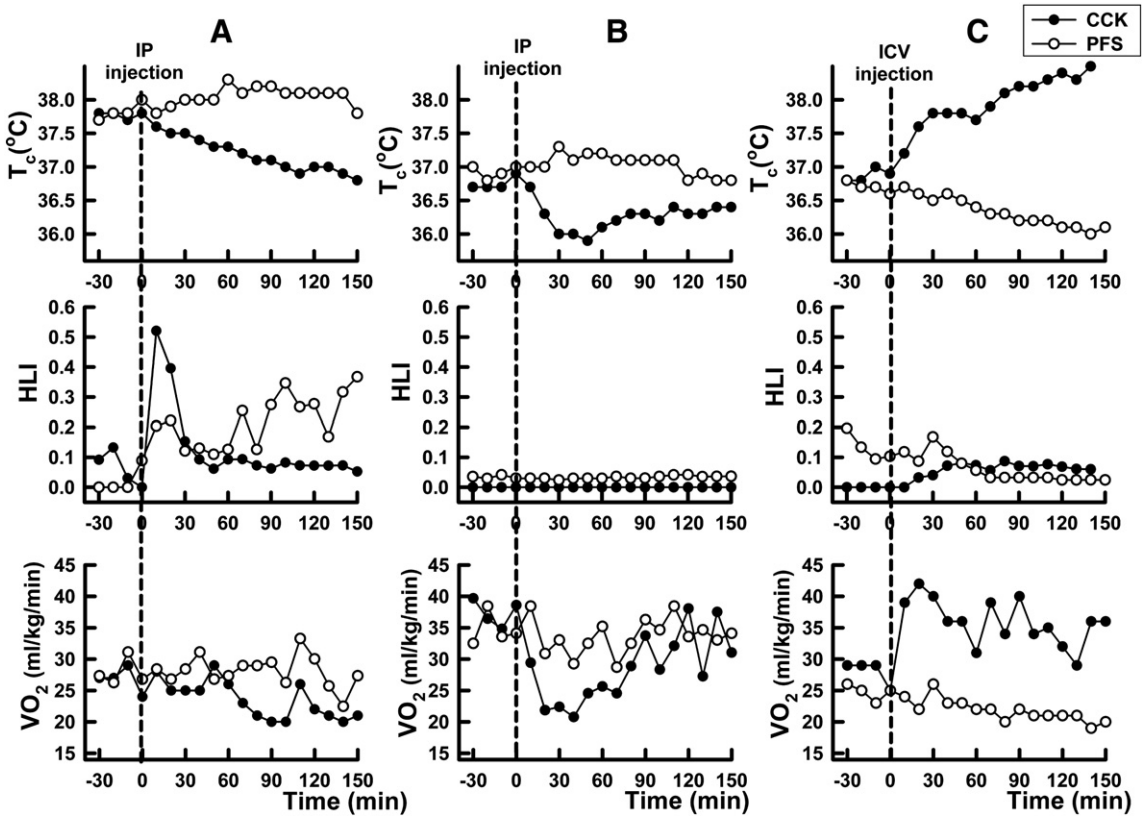

Fig. 4. Effects of intraperitoneal (IP) or intracerebroventricular (ICV) cholecystokinin (CCK) administration on metabolic rate and thermoregulation. The curves represent individual recordings of core temperature (Tc), heat loss index (HLI) and oxygen consumption (VO_2) in normally fed 4 month old rats. Full symbols represent changes following CCK-treatment, empty symbols represent controls [effects of pathogen-free saline (PFS) injection]. Panel A: CCK was injected IP (100 μg) at an ambient temperature of 28 °C. Panel B: CCK was injected IP (100 μg) at an ambient temperature of 20 °C. Panel C: CCK was applied ICV (500 ng) at an ambient temperature of 25 °C. The curves represent individual recordings of core temperature (Tc), heat loss index (HLI) and oxygen consumption (VO_2) in normally fed 4 months old rats. Full symbols represent changes following CCK-treatment, empty symbols represent controls [effects of pathogen-free saline (PFS) injection]. Panel A: CCK was injected IP (100 μg) at an ambient temperature of 28 °C. Panel B: CCK was injected IP (100 μg) at an ambient temperature of 20 °C. Panel C: CCK was applied ICV (500 ng) at an ambient temperature of 25 °C.

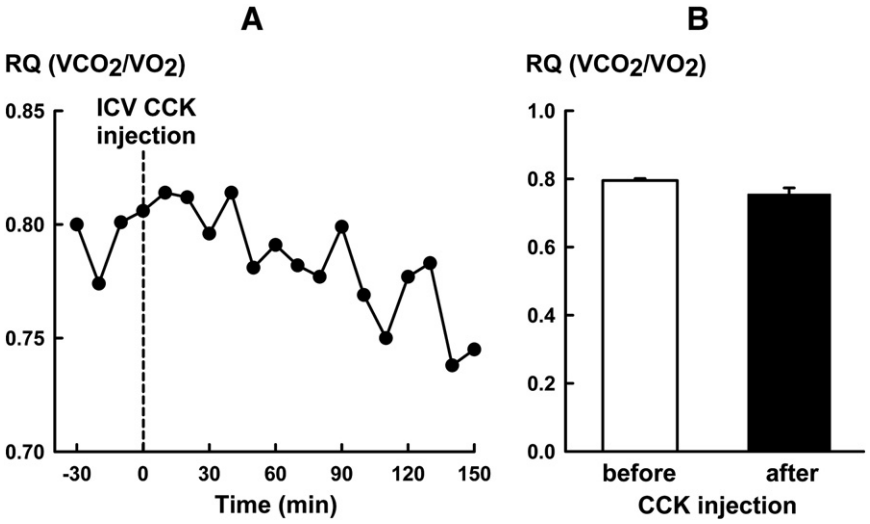

Fig. 5. Effect of intracerebroventricular (ICV) cholecystokinin (CCK) administration on respiratory quotient (RQ). Panel A: The curve represents an individual recording of RQ upon CCK injection in a normally fed 4 month old rat. Panel B: RQ values expressed as mean ± S.E.M. for a group of normally fed 4 month old rats before and after (at 120 min) an ICV CCK injection.

high-fat diet. On the other hand, these parameters of calorie-restricted representatives of the 12-month age-group were significantly smaller than those of their normally fed controls. Moreover, these parameters were reduced below those of much younger normally fed 4-month old animals. This suggests an efficient prevention of "middle-aged" obesity by the applied level of calorie-restriction.

Our results regarding the peripheral administration of CCK show that aging did not cause a gradual continuous decline in the efficacy of the peptide, rather age-related phasic changes were demonstrated for the anorexigenic CCK effect.

Whether given IP or ICV, in young adult NF4 and young middle-aged NF6 rats CCK significantly suppressed food intake (although the ICV applied 500 ng dose exerted stronger anorexigenic response in young adult rats than the IP injected 5 μg). Interestingly, both types of administration were ineffective in juvenile NF2 animals, suggesting the presence of an extremely strong orexigenic tone at this age. In juvenile rats a similar "resistance" was demonstrated for the anorexic effect of alpha-melanocyte stimulating hormone (alpha-MSH) — this was also explained by a high orexigenic tone, that is specific for this age of fast growth (Pétervári et al., 2010).

The anorexic effect of IP CCK observed in young adult rats is in line with the data of the literature (Balaskó et al., 2012; Smith and Gibbs, 1998). Signals representing information from stretch of the stomach and from the nutrient composition of its content are conveyed by fibers of the abdominal afferent vagus (Berthoud, 2004; Blessing, 1997; South and Ritter, 1988) to the NTS, brainstem, and further structures of the brain. The hindbrain alone is sufficient for the development of such CCK-anorexia: peripheral CCK causes satiety even in decerebrate animals (Grill and Smith, 1988), but not in animals with NTS lesion (Edwards et al., 1986). Some of the vagal afferent fibers (C-type) contain CCK1R-s and they are capsaicin sensitive, while other (A-type) fibers are insensitive. In rats, systemic capsaicin desensitization prevented the satiety induced by IP CCK (South and Ritter, 1988). However, decerebrate animals can only adapt to the short-term feeding state but not to long-term changes in nutrition (starvation, overfeeding). If CCK can indeed influence the long-term changes of energy balance, or such changes of nutritional state (starvation, obesity) can interfere with the effects of peripheral CCK, then other additional point(s) of action must

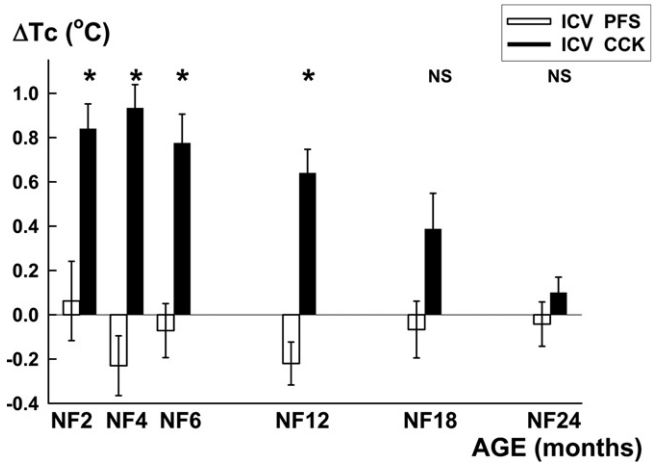

Fig. 6. Cholecystokinin (CCK)-induced hyperthermia in different age-groups of rats. Values are expressed as the mean ± S.E.M. for six-ten rats in each group. Initial core temperature values were similar in all groups (ranging from 37.4 ± 0.2 to 37.7 ± 0.2 °C). Full columns represent changes in core temperature (ΔTc) at 120 min following an intracerebroventricular (ICV) CCK injection (500 ng), empty columns indicate similar values of controls following ICV pathogen-free saline (PFS) injections. Asterisks indicate significant differences between ΔTc of ICV CCK-treated and control rats of the same age: NF2 $p = 0.009$ NF4 $p < 0.001$, NF6 $p = 0.002$, NF12 $p < 0.001$. NS: non-significant. NF: normally fed, HF: high-fat diet-induced obese, CR: calorie-restricted. Numbers following the above abbreviations of animal groups indicate the age of the rats in months.

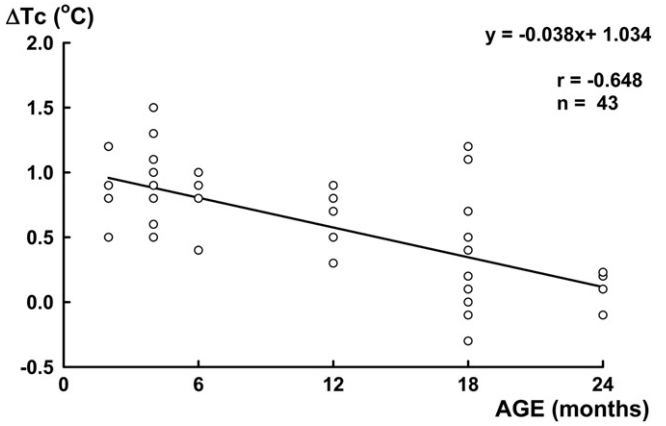

Fig. 7. Dependence of core temperature changes (ΔTc) induced by intracerebroventricular cholecystokinin (CCK) on age as shown by regression analysis. Empty symbols depict individual values of CCK-induced change in Tc of rats belonging to different age-groups ($n = 43$). One symbol may represent several identical values.

be assumed. In fact, circulating CCK may also act at the arcuate nucleus by enhancing the transport of (anorexic) leptin through the blood–brain-barrier (Cano et al., 2008), or possibly it acts directly at other structures of the brain. The anorexic effect of IP CCK in young adults appears to be related to the actual feeding state rather than the more chronic nutritional state, still, in rats the lack of CCK1R-s is connected with obesity [Otsuka Long Evans Tokushima (OLETF) rats], suggesting a possible long-term role of CCK1R activity in energy balance. These rats eat more and become obese, probably due to lack of satiety and to a high hypothalamic neuropeptide Y tone (Bi et al., 2004). Satiety deficit has also been demonstrated for CCK1R knockout mice (Kopin et al., 1999). In discrete brain areas presence of CCK1R-s has also been demonstrated (Hirosue et al., 1993) although in the brain CCK2R-s represent the dominant and abundant receptor type.

Brain CCK2R-s are generally accepted to have a role in anxiety behavior (Wang et al., 2005), but such receptors in the dorsomedial, paraventricular and ventromedial hypothalamic nuclei might also be mediators of anorexia. Following food intake CCK is released in the hypothalamus (Schick et al., 1990), probably due to signals from the stretch of the stomach which signals are conveyed by afferent vagal activity. Exogenous CCK given ICV or to various hypothalamic nuclei suppressed food intake in a number of species (Blevins et al., 2000). A similar role for endogenous CCK was demonstrated by postponing satiety via CCK2R antagonist treatment (Dourish et al., 1989). Other studies demonstrated that CCK2R knockout mice are hyperphagic and obese (Clerc et al., 2007) — their hypothalamic NPY expression was also high (Chen et al., 2006). Centrally applied CCK also induced fever-like elevation of body temperature (Szelényi et al., 1994), and capsaicin desensitization of the abdominal vagus, i.e., elimination of CCK-sensing fibers (Pétervári et al., 2005) or pretreatment with CCK1R antagonist devazepide (Pétervári et al., 2004) prevented the gastric stretch-induced postprandial hypermetabolism and hyperthermia.

We hypothesized that after the young adult age either the peripheral or the central CCK effects may vary with further aging. Age-dependence has already been demonstrated for the effects of a number of peptides involved in the regulation of food intake, energy balance, thermoregulation and body weight. For example, ICV alpha-MSH has a very strong anorexic and body weight decreasing action in young adult and again in old animals, but not in the middle-aged ones (Pétervári et al., 2010). Such alterations in activity may contribute to the explanation of the two basic age-related anomalies of energy balance, i.e., the age-related obesity and the late-appearing anorexia of aging that often leads to senile cachexia and sarcopenia — both anomalies having far-reaching health effects. In contrast, for some other peptides, e.g., neuropeptide Y, ghrelin, orexin (Akimoto and Miyasaka, 2010) and leptin (Scarpace and Tümer, 2001) another pattern of age-related change, a continuous attenuation of the effects has been demonstrated suggesting a stepwise deterioration with age for the regulatory role of the peptide. We assumed that similarly as the role(s) of other peptides, age-related variations of the CCK-dependent regulatory effects possibly contribute to the alterations of energy balance during aging. However, the age-related changes in CCK-efficacy may show either of these patterns. The present data suggest that, depending on the point of action, both patterns are possible for CCK.

Although IP injected CCK suppressed the ingestive behavior in young adult (NF4, NF6) rats, by the age of 12 months this effect of CCK was practically lost. Later on, however, in old animals (NF18, NF24) the anorexic responsiveness to IP administered CCK increased again. The application of one single intraperitoneal dose of CCK (5 μg) in our study (instead of varying the dose in proportion to body weight of the animals) may constitute certain limitations of interpretation of our data. However, when regarding the CCK dose normalized to 100 g body weight, it appears that the highest relative dose in juvenile animals remained inefficient, while the lowest relative doses in old age-groups or middle-aged diet-induced obese rats reduced food intake significantly. In addition, within the young adult group the dose of 1 μg (unpublished data of Balasko et al.) was also able to induce similar and significant suppression of food intake in a similar setting as the 5 μg dose. Moreover, body weights of all NF adult age-groups were rather similar to one another, while showing significantly different responses to an identical dose of CCK. These latter findings also support our conclusion that CCK-resistance in our middle-aged groups is based on lack of responsiveness and not on an insufficient dose.

Enhanced responsiveness to CCK in the old age-group may be surprising in view of age-related leptin resistance as there is a well-documented interdependence between effects of these catabolic peptides of mainly peripheral origin (de Lartigue et al., 2012). Although leptin signaling in vagal afferent neurons is required for appropriate satiating effects of CCK, moreover high-fat diet-induced leptin resistance reduced this satiating effect (at low doses) in young adult rats (de Lartigue et al., 2012), it has not been completely abolished. A higher dose of CCK was shown to inhibit food intake (de Lartigue et al., 2012). As CCK level increases in old age-groups (as discussed later) where some leptin resistance but not complete abolishment of leptin effects is seen, this higher CCK level may be sufficient to induce anorexia. Melanocortin agonist alpha-MSH (Pétervári et al., 2010) that acts downstream of leptin in the hypothalamus shows similar enhancement of anorexigenic efficacy in old age-groups despite leptin-resistance.

The above demonstrated changes in the efficacy to CCK during the course of aging may contribute to insufficient satiety, overeating and obesity in middle-aged rats (age-related obesity) as well as to enhanced satiety and aging anorexia in old animals. As a first approach, the satiety-inducing effect of CCK seems to suggest that it influences the short-term rather than the long-term regulation of food intake. Still, it has been repeatedly reported (Smith et al., 1985) that not so much the number, rather the duration of feeding bouts (determining meal size) is decreased by the peptide what is apparently not fully compensated by feeding frequency. This allows for long-term shifts in energy balance as cumulative effects of changing CCK activity or efficacy. This is likely to be the explanation of obesity in OLETF rats.

It may be of particular importance that – according to most human data – the fasting plasma levels of CCK are higher in the elderly than in young individuals (Di Francesco et al., 2005; MacIntosh et al., 2001; Serra-Prat et al., 2009). This results in a suppressed level of hunger that is not altered very much by the relatively small postprandial CCK-release in old persons (Serra-Prat et al., 2009). A period of caloric restriction is poorly compensated in elderly men (Winkels et al., 2011). Animal experiments similarly show higher CCK levels in old animals: in synaptosomes of brain samples from old rats the CCK-content was higher than in young ones, although the CCK-release in the brain samples upon stimulation was smaller (Ohta et al., 1995).

There are limited and controversial data concerning CCK production/effect (and effects of other neuropeptides) in the high-fat diet-induced obese rat models even in the young adult age-group. Such dietary interventions were shown to lead to elevation of plasma CCK-concentration in rats (Li et al., 2011). Nevertheless, various effects of exogenous CCK are not necessarily simultaneously enhanced (How et al., 2011; Little et al., 2008). Other reports described suppression of gastrointestinal CCK gene- and protein expression (as well as those of peptide YY and glucagon-like peptide-1) (Duca et al., 2013) and reduced satiety in response to CCK and bombesine (Covasa and Ritter, 1998; Torregrossa and Smith, 2003). No relevant information regarding age-related alterations in CCK level or activity are available in high-fat diet-induced obese rodent models.

The effects of long-term calorie-restriction have not been investigated on peripheral CCK expression or activity either in young adult rats or during the course of aging. According to our previous observations calorie-restriction appears to enhance some aspects of neuropeptide effects (Soos et al., 2010, 2011).

In the present studies CCK-responses were decreased in dietary obese rats already at the age of 6 months (HF6), unlike the pronounced anorexic CCK effects in normally fed rats of the same age (NF6). Contrary to this, in CR rats of probably low plasma CCK levels CCK-resistance did

not develop even at the age of 12 months (CR12), when normally fed middle-aged (NF12) rats were "resistant" to CCK-anorexia. In NF rats a rebound of CCK-responsiveness was observed with aging after middle-age (i.e., in NF18–NF24 groups), in HF rats the rebound was present already at the age of 12 months. Apparently, calorie-restriction seemed to postpone, obesity to speed up the age-related changes in CCK-responsiveness.

It may be concluded that peripheral CCK-actions seem to be important in the overall energy balance by determining food intake and consequently the nutritional state. These actions change with phases of aging and they also depend on body composition.

The ICV injected CCK suppressed the ingestive behavior in young adult rats, but – unlike in the case of IP administration – this effect of the peptide became gradually weaker with the aging process and by the age of 24 months (NF24) there was practically no effect. Apparently, not only the anorexic, but also the hypermetabolic and hyperthermic effects of ICV CCK vanished with increasing age. This pattern of change in neuropeptide effects is characteristic for some peptides like NPY, ghrelin, orexin, etc. (Akimoto and Miyasaka, 2010). Considering that a decrease in metabolic rate is characteristic for old age (McGandy et al., 1966), the lack of effect of centrally applied CCK suggests that the central CCK activity may have but little importance in determining metabolic rate, at least in old animals, while the lack of anorexic effect in old rats suggests that the age-related anorexia is probably also independent of central CCK activity.

Altogether, cerebral CCK2R-s are likely to have some catabolic role in energy balance of young adult animals: the CCK2R-dependent postprandial anorexia and hypermetabolism possibly play a role in the metabolic adaptation to calorie intake, to maintain energy equilibrium. Studies on the control of food intake in older men have shown that, unlike in their young counterparts, an excessive calorie containing diet of the same length was not readily compensated following the dietary period — this is part of the phenomenon known as "dysorexia" of the elderly (Roberts et al., 1994).

Central CCK (CCK2R-s) may also participate in fever and sickness behavior (Székely et al., 1994; Weiland et al., 2007). Aging is associated with diminished fever response (Buchanan et al., 2003).

Central CCK-actions seem to be important in the overall energy balance by determining fever-like metabolic response with additional anorexic effect. According to our data these effects decline with aging. Our present findings raise the hypothesis that age-related decline in the central hyperthermic and anorexic effects of CCK may contribute to the age-related diminishment of fever, alterations in sickness behavior and insufficiency of metabolic adaptation to feeding.

Although CCK of peripheral origin also acts in the brain, on its CCK1R-s at various nuclei, the points of action of this CCK and those of centrally applied/released CCK (acting on CCK2R-s of the brain) cannot be identical as shown by the opposite metabolic/thermal effects and by the fact that the age-related changes in their efficacy are different.

5. Concluding remarks and perspectives

5.1. Conclusions

Both the peripheral and the central CCK-effects (anorexic as well as metabolic effects) are age-dependent. The peripheral effects change with age and may contribute to the age-related phasic changes in overall energy balance and consequent changes in body weight, i.e., to the age-related obesity in middle-aged and the aging anorexia in old subjects. The central effects may change in a way that the metabolic compensation of calorie intake (postprandial hypermetabolism) becomes attenuated or is lost completely in old age. Diet-induced obesity appears to accelerate, calorie-restriction to slow down these age-related processes.

5.2. Perspectives

Our results raise the hypothesis that peripheral and central receptor mechanisms of CCK play distinctly differential roles in the regulation of energy balance. Short-term regulation of food intake and the characteristic shifts in the long-term regulation of energy balance in the course of life (depending on the nutritional state) appear to be connected mainly with peripheral receptors, while the activity of central receptors may present a metabolic compensation for calorie intake and defense against energy accumulation in young age-groups. In old rats the loss of metabolic responsiveness to centrally applied CCK appears to be reasonable, since these animals tend to lose weight anyhow, and a high metabolic response to calorie intake would speed up this unfavorable process (this is exactly what may be connected with the increased sensitivity to peripheral CCK). It may be of interest to see whether the loss of metabolic responsiveness is similar in obese old rats with abundant energy reserves some of which may be lost without severe consequences. Specific antagonists of peripheral and central CCK receptors would be useful in the analysis of differential CCK functions during the course of aging.

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Disclosure statement

All authors disclaim any form of conflicts of interest.

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