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Age-related changes in the cocain- and amphetamin regulated transcript peptide expression in feeding-related brain areas of rats

Doctoral (Ph.D.) Thesis

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INTRODUCTION

Food intake and weight gain are precisely regulated mechanisms that show age-related changes. The positive energy balance of young animals supports anabolic mechanisms, but with age the catabolic processes become predominating. In middle aged rodents age-related obesity has been observed, while in old animals reduction of food intake and the loss of body weight results in a state of anorexia (Blanton et al., 1998, 2001; Petervari et al., 2009, 2010, 2011). The reason for the decreased food-intake is multi-factorial relating to both peripheral and central mechanisms. Many central mediators including peptides synthesized by neurons of the central nervous system control appetite.

Alteration of expression and effectiveness of several neuropeptides including orexigenic and anorexigenic peptides were shown to correlate with changes of food intake and metabolism characteristic for different age-groups (Scarpace et al., 2000; Teter, 2009). One of these neuropeptides which is abundant in the hypothalamus and other areas related to the control of food-intake is the cocaine- and amphetamine-regulated transcript (CART) peptide which has a strong anorexic effect (Koylu et al., 1997, 1998). Mutual interaction between CART peptide and neuropeptide-Y (NPY) was shown and central administration of CART peptide potently suppressed feeding and blocked the increase in food intake induced by NPY (Kristensen et al., 1998).

In many animal models of obesitiy and diabetes genetically modified mouse strains are used. However, spontanous mutations can result in obesity in rat strains as well. An expample is the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, in which the functional cholecystokinin-1 (CCK-1) receptor is missing due to a deletion in the gene of the CCK-1 receptor (Takiguchi et al., 1997; Yamada et al., 2012).

Anatomical and functional evidence demonstrates multiple levels of interaction between CART and CCK. Broberger et al. (1999) showed that CART peptide is colocalized with CCK-1 receptors in the central projections of vagal afferent neurons within the nucleus of the solitary tract (NTS). In the cell bodies of vagal afferent neurons, CCK stimulated CART peptide expression and secretion (de Lartigue et al., 2007). A co-operative action

between CART peptide and CCK was shown in the NTS, as well as in the hypothalamic paraventrivular (PVN) and dorsomedial (DMH) nuclei (Maletinska et al., 2008).

Recently has been shown that CART peptide immunoreactivity was significantly reduced in the rostral part of the nucleus accumbens and in the rostro-medial part of the nucleus of the solitary tract in adult CCK-1 receptor deficient obese diabetic Otsuka Long Evans Tokushima Fatty (OLETF) rats compared to Long Evans Tokushima Otsuka (LETO) lean controls (Abraham et al., 2009). It is not clear, however, whether altered CART expression is caused primarily by the deficiency in CCK-1 signaling or whether is related to the obese and diabetic phenotype of the OLETF strain which develops at a later age. Furthermore, it can be presumed that changes in CART epxression observed between young, non-obese non-diabetic and older obese diabetic animals were developed by the age. Therefore in our investigation, the distribution and the intensity of CART peptide immunoreactivity in the nucleus accumbens (NACC) of aged Long-Evans rats were compared to that of lean, healthy younger adult animals.

AIMS OF THE STUDY

Previous studies have shown that the expression of CART peptide in several brain regions (the rostral area of the NACC, the rostro-medial part of the NTS, and the basolateral amygdala) was significantly reduced in CCK-1 receptor deficient hyperphagic, obese, diabetic OLETF rats compared to the control animals (Abraham et al, 2009). Accordingly, the question arose whether the gene mutation and diminished CCK-1 receptor signaling through the CCK-1 receptor directly affects the CART peptide expression, or the reduced CART peptide immunreactivity is the consequence of the hyperphagia, obesity or the metabolic changes induced by the diabetes mellitus .

1) We wanted to examine how the absence of CCK-1 receptor affects the CART peptide expression in young, non-obese, non- diabetic OLETF animals. Therefore, the CART peptide immunoreactivity were compared to younger non- obese, non-diabetic LETO controls of the same age in the areas of the central nervous system responsible for regulating the food-intake. Our results were compared to the changes observed int older (35-40 weeks), diabetic, and

hyperphagic OLETF animals. Longitudinal follow-up was designed to search for a causal link between the altered signaling in CART and the development of obesity.

2) It is unknown whether the changes in the expression of the CART peptide in the NACC plays a role in the development of anorexia in old age. For this reason we studied the distribution and intensity of the CART peptide immunoreactivity in the NACC of elderly, healthy Long-Evans rats. The results were compared to the results obtained in young, middle aged, healthy, male and female rats.

MATERIALS AND METHODS

Animals

1. CART peptide expression in OLETF rats

In our experiment young (6.5 weeks ± 1 week old, weighted 150-255 g) male OLETF rats (n=3) and their age-matched lean LETO controls (n=3) were used (generous gift of the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan). The rats were housed individually and kept on a 12:12-hour light-dark cycle receiving ad libitum tap water and pelleted rat chow (Teklad 2018).

2. Age-related changes of the CART peptide expression in the NACC

In our study, 17 Long-Evans rats were used. Both male and female adult and aged animals were included in this study, and the animals were classified in three age groups. Groups containing young adult (4 months old males, n = 4, weighing 324 ± 20.22 g; 7 months old females, n = 3, weighing 220 ± 16.01 g), middle-aged adult (15 months old females, n = 3, weighing 232 ± 9.12 g) and aging (25–32 months old males, n = 4, weighing 426.6 ± 24.94 g, and 26–30 months old females, n = 3, weighing 271.6 ± 17.44 g) rats were formed. Animals were housed individually and kept on a 12:12-h light–dark cycle receiving ad libitum tap water and pelleted rat chow. Before their sacrifice, the amount of the consumed food by the young adult and aging animals was measured for a month.

In both experiments, all protocols were conducted in accordance with the National Institute of Health Guide for the Care and Use ofLaboratory Animals (NIH Publications No. 88-2959, 2002) as well as with the EU Directive 2010/63/EU for animal experiments that was approved by the institutional committees of animal care and use in the Pennsylvania State University and Pécs University

Oral glucose tolerance test

Because the OLETF strain has been identified and used as a model of type-2 diabetes due to gradual development of pre-diabetes and ultimately diabetes mellitus over the life-span of these rats, we have tested the presence of diabetes in our subjects using oral glucose tolerance test (OGTT). However, CART peptide has been implicated in stress responses. Therefore, to avoid any potential confounds with respect to strain differences in developmental effects to stress such as the restrain and tail nicks for blood collections required for the tests, OGTT were performed in a separate cohorts of age-matched littermates (n=3 OLETF, n=3 LETO). Following a 16 hours fast, an oral glucose load (2g/kg) was delivered to each rat via latex gavage. Blood glucose was measured before and at 30, 60, 90, and 120 minutes post-glucose loading using a standard glucometer (LifeScan, One-Touch Basic). Animals were classified as diabetic if the peak level of plasma glucose was ≥300 mg/dl or 16.66 mmol/l and a peak glucose level at 120 minutes > 200 mg/dl or 11.11 mmol/l

Immunohistochemistry

Since plasma and brain levels of CART peptide exhibit a diurnal variation, rats were sacrificed at the same time in the afternoon (between 2 and 4 p.m.). Animals were terminally anaesthetized with pentobarbital (Nembutal 100 mg/kg body weight), then transcardially perfused with 0.1 M phosphate buffer (PB, pH 7.4), followed by perfusion of 4% formaldehyde in PB. The brains were removed from the skull and postfixed overnight in the same fixative used for the perfusion. Following fixation, blocks containing the NACC from its rostral portion to the caudal part of the shell region (rostro-caudal Paxinos coordinates: Bregma 0.7–2.7) were cut out from the brains. Free floating sections at 60 μm were cut using a vibratome, then collected and processed for immunocytochemistry as described earlier.

Briefly, after washing in PB, sections were pretreated with a solution of 1% hydrogen-peroxide for 30 min to block endogenous peroxidase activity, then pre-incubated in normal horse serum (1% in PB) containing 0.4% Triton X-100 for 1 h. This step was followed by incubation with the primary anti-CART (55–102) antibody (Phoenix Pharmaceuticals, Burlingame, CA) diluted in PB (1:10,000) overnight at room temperature. Binding sites were visualized with biotinylated secondary antibody (1:100, Vector Laboratories, Burlingame, CA) and the avidin-biotin peroxidase detection system (1:50, Vector Laboratories, Burlingame, CA) using 3,3'-diaminobenzidine as chromogene. The sections were mounted on glass slides, air-dried, dehydrated, cleared with xylene and covered with Eukitt (Fluka). The specificity of the primary antibody was determined by the company. However, when the primary antiserum was omitted from the procedure, no staining was detected.

Quantification of intensity of CART peptide-immunoreactivity

Areas related to feeding and reward functions including NACC, medial, central and basolateral nuclei of the amygdala, nuclei of the hypothalamus, NTS and areas related to stress such as centrally projecting Edinger-Wetsphal nucleus (EWcp) and the periventricular nucleus of the hypothalamus were digitally photographed with an Olympus BX51 microscope. To test the available methods for densitometry, intensity of immunostaining detected in the animals was determined on black-andwhite images using two different software, ImageJ (an open source image processing and analyzer program written in Java and supported by the NIH, USA) and AnalySIS software (Olympus Corporation). As a validation process we compared the results obtained with the two software to each other. As the suitability of both software was proven, CART-immunoreactivity (CART-IR) in the NACC was determined with ImageJ.

In each animal, measurements were made on the antero-posterior extension of the region of interest (ROI) in non-consecutive sections. Because CART peptide is released at the synaptic terminals, in the same section, white matter that lacks CART was considered as background and the staining intensity of it was measured to quantify background intensity. After the area to be measured was determined, both ImageJ and AnalySIS software took samples regularly, and measured the intensity of pixels, expressed them as numerical values. The values measured were then averaged, and the intensity of the ROI and that of the white

matter was determined, respectively. The relative intensity which is the real intensity of the CART peptide immunostaining, referred in Results and Discussion as "intensity of CART-immunoreactivity" or ''staining intensity'', was calculated by subtracting intensity values of the ROI from intensity values of the white matter. Since the light intensity and the magnification used greatly affect the intensity values, all measurements in each rat were done using the same light and the same objective for magnification (20X).

Statistics

After determination of intensity of immunostaining, statistical significance was assessed using Student's *t*-test for paired samples, non-parametric Mann-Whitney U-test as well as one-way analysis of variance (ANOVA) with SPSS V22.0 for Windows software. All data were expressed as means + SD, and differences were considered statistically significant if $p < 0.05$.

RESULTS

1. Young LETO and OLETF rats

Result of oral glucose tolerance test

Values of OGTT before the glucose loading at 0 min (Mean ± SEM: 90 ± 200 and 89 ± 3.05 in LETO and OLETF, respectively) and the area under curve (Mean ± SEM: 15515 ± 1357.15 and 18270 ± 14383.3 in LETO and OLETF, respectively) were not significantly different between groups and blood glucose levels at any time-point of the OGTT did not meet criteria for either diabetes or pre-diabetes.

CART-peptide expression in the brain

In addition to neuronal cell bodies and dendrites, CART-peptide is located in both axons and axon terminals (Smith et al., 1997). Therefore, immunohistochemistry reveals

CART expression in axon bundles as well as in individual fibers. In our study, we examined CART expressing neuronal somata and fibers in particular brain areas, and expression observed in young non-obese non-diabetic OLETF rats was compared to that found in age-matched LETO controls.

CART-peptide immunoreactivity in the forebrain

In the forebrain of both LETO and young, non-obese non-diabetic OLETF animals, the distribution of CART-IR was identical to that reported in previous studies (Abraham et al., 2009, Janzso et al., 2010; Koylu et al., 1997, 1998; Seress et al., 2004).

Distribution of CART-IR neurons in the olfactory bulb and piriform cortex were similar in LETO and in OLETF rats. Strong CART-IR neurons and fibers were found in both the rostral and caudal part of NACC with no visible difference in both young LETO and OLETF rats. Caudate-putamen, globus pallidus, and claustrum exhibited CART-IR neurons and axons without difference between OLETF and LETO animals. Both the medial and the lateral septum contained CART-IR fiber network similar to that described earlier with no substantial difference between the strains.

Strong immunoreactive neurons and axons were observed in the paraventricular, periventricular and ARC nuclei of the hypothalamus both in the LETO and OLETF rats. As previously published (Fekete et al., 2000; Koylu et al., 1997), we have found CART-IR neurons as well as fibers in both the the magno- and the parvicellular subdivisions of the paraventricular nucleus. In the parvicellular region, the anterior, periventricular, medial and ventral subdivisions contain CART-IR neurons and fibers without visible difference between LETO and OLETF strains. In the median eminence very intensive CART-IR could be observed in both strains. The perifornical nucleus of both LETO and OLETF rats contained large, strongly CARTimmunopositive neurons. CART-IR neurons and axons were present in the DMH of both the OLETF and LETO animals.

In the amygdaloid complex, distribution of CART-positive elements and staining intensity of the different nuclei were similar in OLETF and LETO rats. The medial, central,

posterior nuclei of amygdala contained CART-positive fibers whereas immunostained neurons as well as fibers were found in the basolateral, central and cortical nuclei.

In the archicortical and neocortical areas CART-IR neurons and axons could be found similarly as previously described and published (Abraham et al., 2007, 2009; Seress et al., 2004).

CART-peptide immunoreactivity in the midbrain

Large numbers of strongly CART-positive neurons were observed in the EWcp of both strains. The periaqueductal gray contained large amounts, while the VTA contained less CART-IR fibers in both young OLETFs and LETOs. The distribution of cells and fibers showing CART-IR was also similar in these regions in young OLETF rats compared to their age-matched controls.

CART-peptide immunoreactivity in the hindbrain

Both the dorsal and the ventral pontine parabrachial nuclei contained CART-IR fibers, although larger numbers were present in the dorsal than in the ventral nucleus in both the young OLTEF and LETO rats. Kolliker-Fuse nucleus contained CART-IR neurons in both strains. No difference was observed in the distribution of CART-immunopositive elements and the intensity of CART-IR between the two strains. The NTS of the medulla oblongata contained CART-IR neurons and large number of immunopositive fibers. Dense CART-IR fiber network could be seen in the medial and less dense in the lateral NTS, both at the rostral and caudal extension of the nucleus.

Quantification of CART immunoreactivity in selected forebrain, midbrain and hindbrain areas

Measurements of intensity of CART-immunostaining were conducted on the young non-obese non-diabetic OLETF rats and in their age-matched LETO controls in regions,

where our previous study revealed significant difference between older obese and diabetic OLETF and lean LETO animals (Abraham et al., 2009). In the present work, intensity of CART-IR was measured in the rostral part of the NACC, in the medial, central and basolateral nuclei of the amygdala and in the rostral part of the medial and lateral NTS. In addition, intensity of immunostaining was quantified in the periventricular zone of the hypothalamus that is involved in the regulation of stress responses. CART-IR was measured in the EWcp, another area related to stress responses, and according to recent reports, to food-intake and reward functions (Giardino et al., 2011; Weitermier and Ryabinin, 2005).

We did not observe significant differences between intensity of CART-IR in the NACC of young non-diabetic, non-obese OLETF rats and in their age-matched LETO controls. Although in the rostral part of the NACC intensity of CART-IR was slightly higher in the OLETF (78.07 ± 17.54) rats than in LETO (49.48 ± 1.7) controls, difference of CARTimmunostaining between the two strains did not reach statistical significance (p=0.14).

Similar CART-IR intensity was found in OLETF and LETO rats in the medial (61.08 ± 14.01 in LETO, 66.79 ± 12.62 in OLETF) central (77.45 ± 17.77 in LETO, 78.55 ± 14.84 in OLETF) and the basolateral (44.22 ± 10.1 in LETO, 50.78 ± 9.6 in OLETF) nuclei of the amygdala. CART-IR in the rostral part of the medial and lateral NTS was slightly stronger in OLETF (medial: 97.41 ± 8.57; lateral: 59.06 ± 3.81) compared to LETO controls (medial: 66.73 ± 9.00; lateral: 39.21 ± 5.26), but without significant difference between the two strains (medial: p=0.11; lateral: p=0.12). In the periventricular zone of the hypothalamus, CART-IR was similar in the LETO strain to that found in the OLETF (OLETF: 113.24 ± 34.58; LETO: 124.6 ± 26.15, p=0.296). However, staining intensity measured in the EWcp was higher in OLETF (93.27 ± 21.69) than in LETO control (70.96 ± 27.15), although, the difference still did not reach the level of statistical significance (p=0.056).

2. Young and Old LETO rats

Food consumption by young and aged animals

When the weight of daily consumed food of the young adult and aged Long-Evans rats were compared no significant difference was observed. Young male animals eat 20.89 ± 1.69

g lab chow per day, while the weight of food consumed by old male animals was 21.35 ± 3.88 g. Generally, less lab chow was consumed by the females than the males, and the young females eat more (18.73 ± 6.02 g) than the aged females (16.36 ± 1.52 g) but the difference was not significant. However, when the amount of daily consumed food was calculated to 100 g body-weight, significant ($p < 0.005$) age-related difference could be found in the male group as well as in the female group. Explicitly, young male animals eat more (6.44 ± 0.52 g/100 g body weight) than aged male rats (5.00 ± 0.90 g/100 g body weight). Similarly, younger females consumed more food per day (8.51 ± 2.81 g/100 g body weight) than aged females (6.02 ± 0.52 g/100 g body weight).

CART-peptide immunoreactivity in the nucleus accumbens

In the forebrain including the NACC of young adult, middle-aged and elderly Long-Evans male as well as female rats, the distribution of CART peptide immunoreactivity was identical to that observed previously in control rats. Accordingly, strong CART peptide-immunoreactive neurons and fibers were found in both the rostral and caudal part of the NACC in all groups of animals. Comparing the distribution and the intensity of CART peptide immunoreactivity under light microscope, no visible age-related or gender-related difference could be observed.

Quantification of CART immunoreactivity in the nucleus accumbens

In order to collect trustable data on CART peptide immunoreactivity for young adult, middle-aged and elderly animals, the density of the immunoreaction was measured on black and white photos of the NACC with computerized densitometry. To validate the methods for the quantification, we used two different software, the ImageJ which is a free access software and the AnalySIS (Olympus Corporation). There were no significant differences in the intensity results when the two image processing and analyzing software were compared. When the measurements of intensity of CART immunostaining were performed on the young adult and aged male rats, no significant age-related differences were observed. The intensity of CART immunoreactivity in the NACC of the 4 months old (21.6 ± 7.02) and 25–32 months old (22.2 ± 3.81) rats measured with the ImageJ software was similar. With this software, the

highest intensity value was 33.76 in the young and 24.38 in the old animals, with the lowest value of 16.60 and 17.67 in the young and old rats, respectively. Similarly to the results of the ImageJ program, the AnalySIS software did not show difference between the CART immunoreaction in the young (57.0 ± 5.80) and in the aged (62.3 ± 4.17) animals. The highest intensity value was 61.23 in the young and 63.19 in the old ones, with a minimum value of 36.86 in the young and 53.27 in the aged rats. When the CART peptide immunoreactivity was analyzed no age related correlation could be seen among the individuals of the group of old animals with both software. In addition, we concluded that both methods are appropriate to determine CART immunodensity in the NACC.

Next, we compared the intensity of CART immunoreaction in the NACC of male (25–32 months old) and female (26–30 months old) elderly rats using ImageJ software. We have found no significant difference between the intensity of NACC in aging male (22.2 ± 3.81) and female rats (19.4 ± 1.73) indicating that there is no gender- related difference in the CART expression in the aged rats. We also compared CART immune-intensity in young male (21.6 ± 7.02) and female (20.0 ± 6.32) adult rats, and found that no significant gender-related difference can be observed in the NACC. Comparing CART expression in young (20.0 ± 6.32) middle aged (27.02 ± 7.29) and elderly (19.4 ± 1.73) females, no significant age-related difference could be observed.

Because of the small sample size, we have taken into consideration that our mean data are skewed, therefore we performed non-parametric Mann-Whitney U-test which is less sensitive to extreme values. In addition, we have performed one-way ANOVA to compare the means of the CART-immunodensity values. Both statistic tests and a post-hoc statistical power calculation for Student *t*-test resulted non-significant differences between the examined animal groups, which indicates that CART peptide expression is stable in adults, and does not change during aging.

DISCUSSION

CART peptide expression in OLETF rats

In our study we investigated whether reduced brain CART signaling in obese OLETF rats is the direct consequence of CCK-1 receptor gene mutation, or that of obese and diabetic phenotype of the OLEFT rat. The results show that the lack of CCK-1 receptor does not substantially impair the peptide expression of the CART gene in the rat brain during development and postnatal maturation. Specifically, we have found a) that the distribution of CART peptide-IR neurons and their axons in the young non-obese non-diabetic OLETF rats were identical to that found in age-matched LETO lean controls; b) that the distribution of CART-immunopositive elements in the brain was identical in young OLETFs and in control rats; and finally c) no significant difference was observed in the intensity of CART-IR in areas functionally related to feeding between young non-obese OLETF and age-matched LETO controls.

In a previous study, significantly lower intensity of CART-IR was reported in the rostral part of NACC, in the basolateral amygdala, and in the rostro-medial NTS of obese and diabetic (35-40 weeks old) OLETF rats than in their age-matched lean LETO controls (Abraham et al., 2009). In our present work, the intensity of CART-IR measured by computer-aided densitometry did not change significantly in the rostral part of NACC, in the medial, central and basolateral nuclei of the amygdala, and in the rostral part of the NTS in young non-obese non-diabetic OLETF rats and in young LETO controls. This indicates that the decrease of CART-IR found in the obese diabetic rats cannot be interpreted as a result of the missing CCK-1 signaling in these regions. Even in the NTS where CART peptide is colocalized with CCK-1 receptors (Broberger et al., 1999), no significant difference could be observed between the CART expression in young non-obese non-diabetic OLETFs and in age-matched controls. This suggests that regulation of CART expression is not downstream of the CCK-1 signaling. The reduced CART peptide expression in the NTS, in the basolateral amygdala and in the NACC of the OLETFs, that can be observed when obesity and type-2 diabetes were already developed (Abraham et al., 2009), could be only explained by other alterations. These alterations might be related to the age of the animal, to the hyperphagia or obesity characteristic to OLETF, or to the metabolic state characteristic to diabetes.

OLETF pups differ from LETO controls regarding their nourishment much earlier than the decrease of CART-IR intensity could be observed. After birth, OLETF rats consume more milk in individual suckling bouts than LETO controls (Schroeder et al., 2007). Despite the difference in milk consumption, body weight of OLETF rats is identical to that of the LETO controls at the age of 6 weeks (Moran et al., 2008). The age of 6 weeks corresponds to the age of our cohorts (6.5 ± 1 week), in which no significant difference but a slight tendency towards an increased CART expression in the examined brain areas related to feeding in OLETFs was observed comparing to age-matched LETOs. This indicates that hyperphagia does not directly modify CART expression in the NACC, in the basolateral amygdala and in the NTS. Furthermore, the slightly, but not significantly higher CART expression in these area can be due to compensatory mechanism as an answer to the larger food consumption. Fast and dynamic change of CART expression and decreased CART-signaling was shown when food-intake was reduced in rodents indicating that CART-producing cells are involved in energy homeostasis (Dandekar et al., 2012; Higuchi et al., 2008; Robson et al., 2002). However, such compensatory mechanism as a response to hyperphagia has never been shown.

The difference in CART expression found in young non-obese and non-diabetic 6 weeks old OLETF rats compared to older 35-40 weeks old diabetic obese animals can be related to obesity and the metabolic change characteristic for diabetes mellitus. Although reduced CART expression causes obesity (Ascinar et al., 2001; Boone et al., 2008; Del Guidice et al., 2001; Guerardel et al., 2005), in another models of obesity induced by CART independent factors reduced CART expression was found as well (Kristensen et al., 1998; Li et al., 2008; Schulz et al., 2012; Tian et al., 2004). This indicates that reduction of CART expression in certain cases can be the cause, but under another circumstances the consequence of obesity. Since in our model, hyperphagia is already present when CART expression is not decreased, we propose that the reduction of CART in obese and diabetic CCK-1 receptor defective OLETF rats is not the cause but the consequence of obesity.

Independently of the hyperphagia and obesity, we can presume that the difference found in the CART peptide expression in the above mentioned brain regions of young non-obese OLETF and of older, obese, diabetic OLETF rats is developed parallel with the age.

CART peptide in the nucleus accumbens during aging

The ages of the old rats varied between 25 and 32 months, which correspond to the senescent stage in humans. At this age food-intake declines and aging related anorexia appears in dramatic weight loss. However, in contrast to the long period of time when strong decrease of food consumption can be seen in humans, this time period is restricted to only 3 weeks before death in rats. Earlier than 3 weeks before death the daily food intake does not differ from that of younger rats. When calculating of the consumed food for 100 grams body weight, aged animals take up significantly less food than young rats, although the old rats were not yet in the stage of the anorexia. In contrast to the difference in food-intake, the expression of CART peptide was not changed in the NACC of both male and female aged rats. However, we have to emphasize that in most of the recently published studies, that have shown age-related changes of food-consumption and altered of expression of peptides linked to food intake, rats between 25-30 months of age were used.

Hypothalamic expression of numerous orexigenic neuropeptides such as NPY, AgRP and orexins was changed during aging, however, expression of the anorexigenic neuropeptide POMC was similar in young and old animals. In the arcuate nucleus of aging animals, decrease of NPY expression was detected, which coincided by an increase of CART expression. However, similar change of CART peptide expression could not be detected in the NACC. Although CART-immunoreactivity differed slightly in the NACC along with normal aging, the changes occurred in old animals were not statistically significant when compared to younger or middle-aged adult rats.

As we have demonstrated, CART-immunoreactivity in the NACC was due to CART-immunoreactive neurons and neuronal processes including terminals of local and long projecting axons. CART axon terminals are mostly originate from local axon collaterals of neurons of the NACC, whereas small proportion of axons are coming from CART-immunoreactive cell bodies of the perifornical area of the lateral hypothalamus as well as of the arcuate and amygdala nuclei. Among these regions, alteration of CART expression during aging has been shown only in the arcuate nucleus. CART-immunopositive neuronal somata in the NACC are densely innervated by dopaminergic axon terminals mostly coming from the VTA, another brain area that regulates feeding behavior. Concerning the dopamine signal in the NACC of elderly animals, decreased binding to dopaminergic receptors and lower basal

extracellular dopamine release were measured indicating age-related change in the VTA. Since injection of D3 dopamine receptor agonists lowers levels of CART mRNA in the NACC, decreased dopamine signaling in the aged animals would reasonably increase CART expression. However, based on CART-dopamine interaction, a homeostatic function of CART peptide in the NACC was suggested, which means that if dopamine signaling in the NACC increases, CART peptide is released and tends to oppose dopamine action. According to this suggestion, the decreased dopamine signaling would result in the lack of the release of CART peptide from the neurons of the NACC, which indicate unchanged expression of CART peptide by NACC neurons. Considering that most axon terminals in NACC belong to local axon-collaterals of its CART-immunoreactive neurons, the suggested homeostatic function of CART peptide in the NACC is a reasonable explanation to our results.

We have to note, however, that the unaltered CART expression does not inevitably mean unaltered effect of the CART peptide in the NACC. Thus, the precise determination of the role of CART peptide in the senescent anorexia needs further studies.

NEW FINDINGS

The most important fndings of the thesis are the followings:

1. We found that, young, non-diabetic OLETF rats revealed unaltered distribution of CART-peptide expressing neurons and axons throughout the brain when compared to age-matched LETO rats. In young, CCK-1 receptor deficient OLETF rats - who does not suffer from obesity compared to aged animals – we did not find CART-IR reduction in the nucleus tractus solitarii, nor in the basolateral amygdala, and nor in the nucleus accumbens compared to control LETO rats.

2. CART-immunodensity is similar in the old rats and in the younger animals without significant difference between age-groups. In addition, no gender-difference was observed when CART-immunoreactivity in the nucleus accumbens of male and female animals were compared.

3. CART peptide expression is not directly regulated by signaling pathways originating from the CCK-1 receptor.

PUBLICATIONS

Articles and Book chapters related to the Thesis

- Armbruszt S., Figler M., Abraham H. (2015) Stability of CART peptide expression in the nucleus accumbens in aging. *Acta Biologica Hungarica*. 66(1):1-13.

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- Armbruszt S. (2015) A humán táplálkozás mechanizmusa, táplálékaink szerepe a betegség megelőzésben. Dietetika a háziorvosi gyakorlatban (Figler M., Kubányi J. szerk.). SpringMed Kiadó, Budapest. 21-44.

- Armbruszt S. (2013) A táplálékfelvétel központi idegrendszeri szabályozása. *Új Diéta*. 4:26-27.

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