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Cocaine- and amphetamine-regulated transcript (CART) peptide immunoreactivity in feeding- and reward-related brain areas of young OLETF rats

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Abstract

Cocaine- and amphetamine regulated transcript (CART) peptide is expressed in brain areas involved in the control of appetite, drug reward and homeostatic regulation and it has an overall anorexigenic effect. Recently, we have 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. 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. Therefore, in the present study, CART-immunoreaction in feeding-related areas of the brain was compared in young, age-matched (6-7 weeks old) non-obese, non-diabetic OLETF rats and in LETO controls. 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 contrast to previous results observed in the obese diabetic adult rats, intensity of CART immunoreaction did not differ in the areas related to control of food-intake and reward in the young OLETFs compared to young LETO rats. Our findings suggest that factors secondary to obesity and/or diabetes rather than

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Author contributions HA performed immunohistochemistry; SA performed immunohistochemistry and quantification; FM and AH supervised, AH and HA conceived and designed the study; HA, SA, KT, AH wrote the paper.

Conflict of interest statement There are no conflicts of interest.

Ethical statement All protocols used in the present study have been carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 88-2959, 2002) that was approved by The Pennsylvania State University Institutional Animal Care and Use Committee as well as with the EU Directive 2010/63/EU for animal experiments.

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impaired CCK-1 receptor signaling may contribute to altered CART expression in the OLETF strain.

Keywords

central food intake regulation; cholecystokinin receptor; obesity; type-2 diabetes; reward system

1. Introduction

The satiety effects of the brain-gut peptide cholecystokinin (CCK) are mediated mainly through CCK-1 receptors. In addition to other detected, but have not yet specifically determined genetic changes, the Otsuka Long Evans Tokushima Fatty (OLETF) rats lack functional CCK-1 receptors due to spontaneous deletion of the CCK-1 receptor gene (Takiguchi et al., 1997; Yamada et al., 2012). As a consequence, OLETF rats have deficits in the control of meal size, they display hyperphagia and become obese, and, with aging, gradually develop type-2 diabetes mellitus through the life span of the animal with a distinct and long prediabetic stage (Kawano et al., 1992; Moran, 2000).

During the prediabetic stage of OLETF rats, different regulatory deficits have been elucidated behind overeating. In addition to loss of short-term satiety signal due to the absence of vagal CCK-1 receptor signaling, increased orosensory stimulation associated with dopaminergic deficits were reported. At the age of 10 weeks, OLETF rats differ from Long Evans Tokushima Otsuka (LETO) controls in their gustatory function with an overall augmented sensitivity for sweet taste that progresses during prediabetes (De Jonghe et al., 2005; Hajnal et al., 2005). A functional relationship was found between alteration of synaptic dopamine regulation in the nucleus accumbens (NACC) of the OLETF rats and a differential sensitivity to dopamine receptor antagonists and agonists on preference and operant behavior for sucrose solution (Anderzhanova et al., 2007; Hajnal et al., 2007a,b).

Another deficit reported as the consequence of lack of CCK-1 receptor is the altered expression of neuropeptides in hypothalamic nuclei. When animals were fed ad libitum, decreased NPY expression was observed in the arcuate nucleus (ARC) and the dorsomedial hypothalamus (DMH) in adult OLETF rats (Bi et al., 2007; Moran and Bi, 2006). In contrast, in pair-fed rats, NPY was overexpressed in the DMH. During the first 10 weeks, when obesity develops in OLETF, expression of NPY in the ARC and DMH reveals notable age-dependent change (Schroeder et al., 2009). Similar age-related changes were detected in the mRNA expression of the anorexigenic peptide proopiomelanocortin (POMC) in the ARC when young animals start to gain fat (Schroeder et al., 2009).

A subpopulation of POMC-expressing neurons in the ARC coexpress another anorexigenic neuropeptide, the cocaine-amphetamine regulated transcript (CART) peptide (Elias et al., 1998). In addition to the ARC, CART peptide is widely expressed in the areas of the rat brain involved in reward, visceral sensory processing and homeostatic regulation of stress responses as well as regulation of appetite (Douglass and Daoud 1996; Koylu et al., 1997, 1998; Kozicz 2003; Rogge et al., 2008; Seress et al., 2004; Xu et al., 2010). Subsequent studies on humans and transgenic animals provide evidence that mutations of the CART gene are linked to eating disorders and/or obesity (Boone et al., 2008; Del Guidice et al., 2001: Guérardel et al., 2005). Expression of CART peptide is closely associated with the actions of other important regulators of food-intake such as NPY or leptin signaling, both impaired in OLETF. In vitro administration of NPY on hypothalamic explants increases expression of CART peptide in a dose-dependent manner (Dhillo et al., 2002). Central administration of CART peptide inhibited feeding in rats stimulated by NPY (Kristensen et

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al., 1998). This mutual interaction can be explained by the close appositions of NPY-immunoreactive nerve terminals on CART-peptide immunoreactive cell bodies in DMH and in the ARC (Broberger, 1999). In animal models of obesity when leptin signaling is not present, CART expression is decreased in ARC (Brogerger, 1999; Kristensen et al., 1998). Peripheral or intranasal administration of leptin into obese rats increases CART expression in the ARC and other nuclei of the hypothalamus, including the DMH (Kristensen et al., 1998; Schulz 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 nucleus and DMH (Maletinska et al., 2008). Thus, the role of CART peptide in food-intake and its functional relationship to CCK indicate the plausibility of altered CART expression in the OLETF rats. Indeed, when compared to age-matched lean LETO controls, we have found significantly reduced CART peptide expression in the rostral portion of the NACC, in the rostral part of the basolateral nucleus of the amygdala (BLA) and in the rostral portion of the medial part of the NTS in adult, 35-40 weeks old obese, diabetic OLETF rats (Abraham et al., 2009). In other central areas related to food intake, such as the ventral tegmental area, the parabrachial nucleus as well as nuclei of the amygdala and the hypothalamus, we discovered a tendency for a decrease in CART peptide expression in obese and diabetic OLETFs compared to lean controls, but the difference in these area did not reach the level of statistical significance.

Taken together, the above data show that CART peptide immunoreactivity is reduced in various brain areas in the adult CCK-1 receptor deficient obese OLETF rats compared to LETO lean controls. However, the questions, 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 remains to be answered.

Therefore, this study aims to assess 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. In order to clarify this, in the present study, we examined CART-immunoreactivity (CART-IR) in feeding-related areas of the CNS in young (6.5 ± 1 weeks old) OLETF rats. At this age, OLETF rats consume more than age-matched lean LETO controls, but there is no significant difference in weight, and they are still non-obese and non-diabetic (Moran, 2008). We hypothesize that if CART expression in the young OLETFs is reduced in the same manner as in the older, obese and diabetic rats, direct regulation of CART downstream to CCK signaling can be inferred. If, however, CART peptide expression is increased in the juvenile OLETFs, then regulation of CART expression is independent of CCK signaling, and the early compensatory counteracting mechanism of CART occurs in response to the hyperphagia. If CART peptide expression in young non-obese OLETFs is unchanged compared to LETO rats, then it suggests that CART expression is not directly regulated by CCK-signaling and the reduced CART-IR observed previously in adult, obese and diabetic OLETF rats (Abraham et al., 2009) is the consequence of obesity, accumulation of fat or metabolic changes due to diabetes mellitus.

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2. Material and methods

2.1 Animals

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 to AH). 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). All protocols used in the present study were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 88-2959, 2002) that was approved by The Pennsylvania State University Institutional Animal Care and Use Committee, as well as with the EU Directive 2010/63/EU for animal experiments.

2.2 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 (Gozen et al., 2007; Hunter et al., 2007; Xu et al., 2010). 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 (Kawano et al., 1992).

2.3 Immunohistochemistry

Since plasma and brain levels of CART peptides exhibit a diurnal variation (Vicentic et al., 2004), rats were sacrificed at the same time in the afternoon (between 2-4 PM). Animals were terminally anaesthetized with pentobarbital (Nembutal 100mg/kg body weight), then transcardially perfused with 0.1M phosphate buffer (PB, pH 7.4), followed by perfusion of 5% formaldehyde in PB. The brains were removed from the skull and postfixed in the same fixative used for the perfusion overnight. Following fixation, brains were cut from the rostral portion of the NACC to the medulla oblongata at 60μm using a vibratome, and sectioning resulted 100-120 sections per animal. Free floating sections were collected and processed for immunocytochemistry. Sections of one animal were processed together, but, because of the large numbers of sections, processing of all sections of all animals were impossible. However, we used our standard procedure for immunohistochemistry to ensure that all animals were processed under comparable conditions. Briefly, after washing in PB, sections were pretreated with a solution of 1% hydrogen-peroxide for 30 minutes to block endogenous peroxidase activity, then pre-incubated in normal horse serum (1% in PB) containing 0.4% Triton X-100 for 1 hour. This step was followed by incubation with the primary anti-CART (55-102) antibody (Phoenix Pharmaceuticals) diluted in PB (1:10000) overnight at room temperature with continuous shaking. 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 slides, air-dried, dehydrated, cleared with xylene and covered with Permount.

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2.4 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. (Kozicz et al., 2011). Intensity of immunostaining was determined on black-and-white images using AnalySIS software, measuring the gray value of each area listed above.

In each animal, measurements were made on the antero-posterior extension of each measured area, in non-consecutive sections. Depending on the size of the region of interest, 3-34 measurements were performed in each nuclei. Because CART peptide is released at the synaptic terminals (Smith et al., 1997), white matter tracts that lacks CART peptide located on the same section was considered as background and the staining intensity of them was measured to quantify background intensity. To do that, the cursor was moved in zigzag line on the screen over the black and white digital photo of the nucleus of interest, from the medial to the lateral border of the particular CART-immunostained brain nucleus. The zigzag line covered the measured area as tightly as possible. Along this line, the AnaLYSIS software took samples regularly, and measured the intensity of pixels of the section, expressed it as numerical values and we referred it according the manual of the software as "point intensity". During one single measurement staining intensity of 7,000 – 36,000 points of the background and 2,000 – 56,000 points of the region of interest were measured. The values of the 7,000 – 36,000 points measured in the background were then averaged, and the results of the calculation gave the absolute intensity of the background, e.g. the white matter. Similarly, the average of the 2,000 – 56,000 point-values of the region of interest was calculated as absolute intensity of the examined region. The relative intensity which is the real intensity of the CART-immunoreaction of the examined nucleus, referred in the Results and in the Discussion as "CART-IR intensity" or "staining intensity", was calculated by subtracting absolute intensity of the region of interest from absolute intensity of the background. After the measurement of one complete nucleus of one animal and the above mentioned calculation, we have got 3-34 intensity values depending on the size of the particular nucleus. Thus, measuring of the CART-IR intensity in one particular nucleus in all the 3 animals that belong to one experimental group, resulted 18-50 intensity values. The number of photomicrographs that was used for the obtaining of the intensity values of each brain nucleus referred in the Results section and demonstrated in Figure 6. varied (in the NACC 18 and 21; in the PVN 44, and 27, in the nuclei of the amygdala 28 and 20, in the medial and lateral NTS 50 and 29 and in the EWcp 18 and 36 in the OLETF and LETO, respectively).

Since the light intensity and the magnification used greatly affect the intensity values, all measurements in both LETO and OLETF rats were done using the same light and the same objective for magnification.

2.5 Statistics

After determination of intensity of immunostaining, statistical significance was assessed using Student's t-test for paired samples computed with SPSS 15.0 for Windows software. All data were expressed as means + SEM, and differences were considered statistically significant if $p<0.05$.

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3. Results

3.1 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 (Kawano et al., 1992).

3.2 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.

3.2.1 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 (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 (Fig. 1). 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 (Fig. 2). Strong immunoreactive neurons and axons were observed in the paraventricular, periventricular and ARC nuclei of the hypothalamus both in the LETO and OLETF rats (Fig. 3). 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 CART-immunopositive neurons (Fig. 3 E, F). CART-IR neurons and axons were present in the DMH of both the OLETF and LETO animals (Fig. 3 E, F).

In the amygdaloid complex, distribution of CART-positive elements and staining intensity of the different nuclei were similar in OLETF and LETO rats (Fig. 4 A, B). 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).

3.2.2 CART-peptide immunoreactivity in the midbrain—Large numbers of strongly CART-positive neurons were observed in the EWcp of both strains (Fig. 4C and D). 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.

3.2.3 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 (Fig. 4E and F). Kolliker-Fuse nucleus contained CART-IR neurons in both strains (Fig. 4E and F). 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 (Fig. 5). 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.

3.2.4 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 (Fig. 6). 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 CART-immunostaining 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).

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4. Discussion

This study 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 Long-Evans 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 our 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.

The possibility that aging might be the cause of the decreased CART expression in older OLETF animals can be ruled out because in 24-month-old aged control rats CART-IR in the NACC was significantly higher than in 3 months old rats (our unpublished observation). Similar to this, increased CART mRNA expression was observed in the hypothalamic nuclei in aged rats compared to young animals (Sohn et al., 2002).

Hyperphagia can also be a possible candidate that modify CART expression. However, 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, 2008). The age of 6 weeks corresponds to the age of our cohorts, 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

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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; Guérardel 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.

Several factors are known to be related to obesity and type-2 diabetes mellitus. Among them glucocorticoids play a central role, and they can modulate CART expression in brain areas. CART peptide in the blood displays a diurnal rhythm parallel with glucocorticoids and diurnal change of CART expression was shown also in brain areas such as the NACC, the amygdala and the hypothalamus (Jaworski et al., 2003; Vicentic et al., 2004, 2005; Vrang et al., 2003). Performing the perfusion of the animals at the same hour of the day, we have reduced the effect of the diurnal rhythm of CART expression, therefore we propose that the change of glucocorticoids and the diurnal rhythm of the CART expression do not play a role in our study. In addition in fasted rats, the daily rhythm of CART peptide in the hypothalamus demonstrated a dependence on food intake (Bertile et al., 2003; Savontaus et al., 2002). Food-dependent expression and rhythmicity of CART peptide were also reported in mesolimbic brain regions including the amygdala and NACC (Vicentic et al., 2005). It is well known that corticosteroids play important roles in the regulation of energy homeostasis and the development of obesity (Duclos et al., 2005; Perello et al., 2004). In addition, CCK stimulates glucocorticoid sceretion and activation of CCK-1 receptor induces elevation of plasma corticosterone (Katsuura et al., 1992; Sander and Porter, 1988). In harmony with this, serum and urinary corticosterone level and weight of the adrenal gland were significantly lower and plasma ACTH level was significantly higher in OLETF rats than measured in LETO controls (Noguchi et al., 2007). Interestingly, these differences were present in animals older than 16 weeks, and could not be noticed at the age of 5-8 weeks, when rats are non-obese and non-diabetic like our cohorts. Since CART rhythm parallels with the rhythm of corticosteroids, and CART expression induced by feeding seems to be modulated by glucocorticoid (Germano et al., 2007, Liu et al., 2011), the similarity in the pattern of corticosterone level and CART expression is plausible. Whether CART expression is regulated by corticosterone directly or indirectly remains unclear.

Another candidate that can be responsible for the regulation of CART expression is leptin, since high circulating concentration of leptin and leptin-resistence coincide usually with obesity (Considine et al., 1996). Leptin regulates expression of CART peptide in the hypothalamus and disrupted leptin signaling, due to obesity, results in dramatic reduction of CART mRNA, while leptin treatment restores CART expression in the ARC (Kristensen et al., 1998). Indeed, CART gene expression is under strong regulation by leptin as the CART promoter contains a STAT site which is activated by leptin (Dominguez et al., 2002; Elias et al., 1998). In the obese OLETF rats, when animals are prediabetic at the age of 8-14 weeks, plasma leptin level is two to three times higher in OLETFs than in LETO controls, but it does not differ significantly at age of 5 weeks (Niimi et al., 1999).

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Interestingly, both serum leptin level and adrenal function reveal age dependent changes in OLETF rats, and strong correlation was shown between serum leptin and serum corticosterone and ACTH level throughout the life-span of CCK-1 receptor deficient animals (Noguchi et al. 2007). Since both factors, leptin and corticosterones, may modulate CART expression, both can be responsible for the reduction of CART peptide expression that was observed in 35-40 weeks old obese and diabetic OLETF rats in our previous study (Abraham et al., 2009). At the age of six weeks, when OLETF rats are non-obese and non-diabetic, both serum leptin and corticosterone levels are normal. This can explain the unaltered CART-peptide expression detected in the examined brain areas of young non-obese and non-diabetic OLETF rats.

5. Conclusions

Our data show that regulation of brain CART expression is not downstream of CCK-1 signaling, and the reduction of CART expression in the adult, obese and diabetic CCK-1 deficient OLETF rat is not the cause but rather the consequence of obese and/or diabetic phenotype.

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Highlights

- Cocaine- and amphetamine regulated transcipt (CART) peptide has an anorexic effect.
- The satiety effect of cholecystokinin (CCK) is mediated mainly via CCK-1 receptors.
- The expression of CART peptide was studied in young CCK-1 receptor deficient rats.
- CART expression seen in CCK-1 receptor deficient rats was similar to the controls.
- CART expression is not directly regulated by CCK through CCK-1 receptor.

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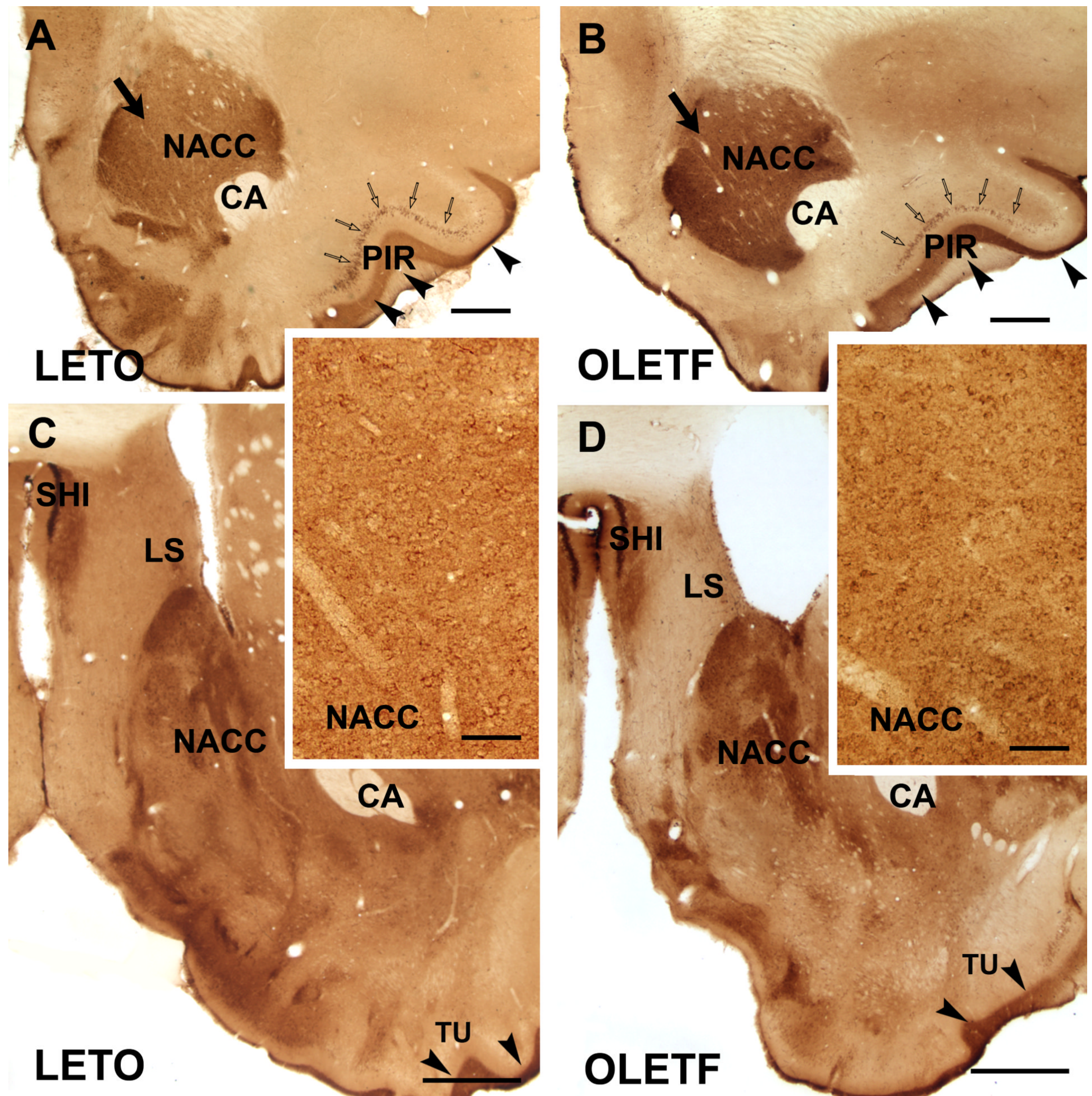

Figure 1.

CART-IR neuronal elements in the nucleus accumbens in a LETO control (A, C) and young non-obese OLETF rat (B, D). A: In the LETO, dorsomedially to the anterior commissure (CA) the rostral portion of nucleus accumbens (NACC) reveals a dense network of CART-IR fibers (arrow). In addition, CART-immunopositive neurons (open arrows) and densely packed immunoreactive axons (arrowheads) are localized in the piriform cortex (PIR). B: In OLETF, the rostral portion of NACC (arrow), the piriform cortex that contains CART peptide-immunopositive neurons (open arrows) and a dense axonal plexus (arrowheads) reveals similar immunodensity to that found in the LETO (A). C and D: No substantial difference is seen between the LETO (C) and OLETF strains (D) in the caudal part of NACC which expresses moderate density of immunoreactive fibers and neurons. A dense network of immunoreactive fibers (arrowheads) can be seen in the olfactory tubercule (TU) in LETO (C) as well as in OLETF (D) strains. In the lateral septum (LS) and in the

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septohippocampal nucleus (SHI) moderate density of immunoreactive fibers is detected in both LETO (C) and OLETF (D) rats.. Insets reveals CART peptide immunoreactivity in the rostral part of the nucleus accumbens (NACC) with higher magnification. Scale bars = 500 μm in A and B, 600 μm in C-D and 50 μm for the insets.

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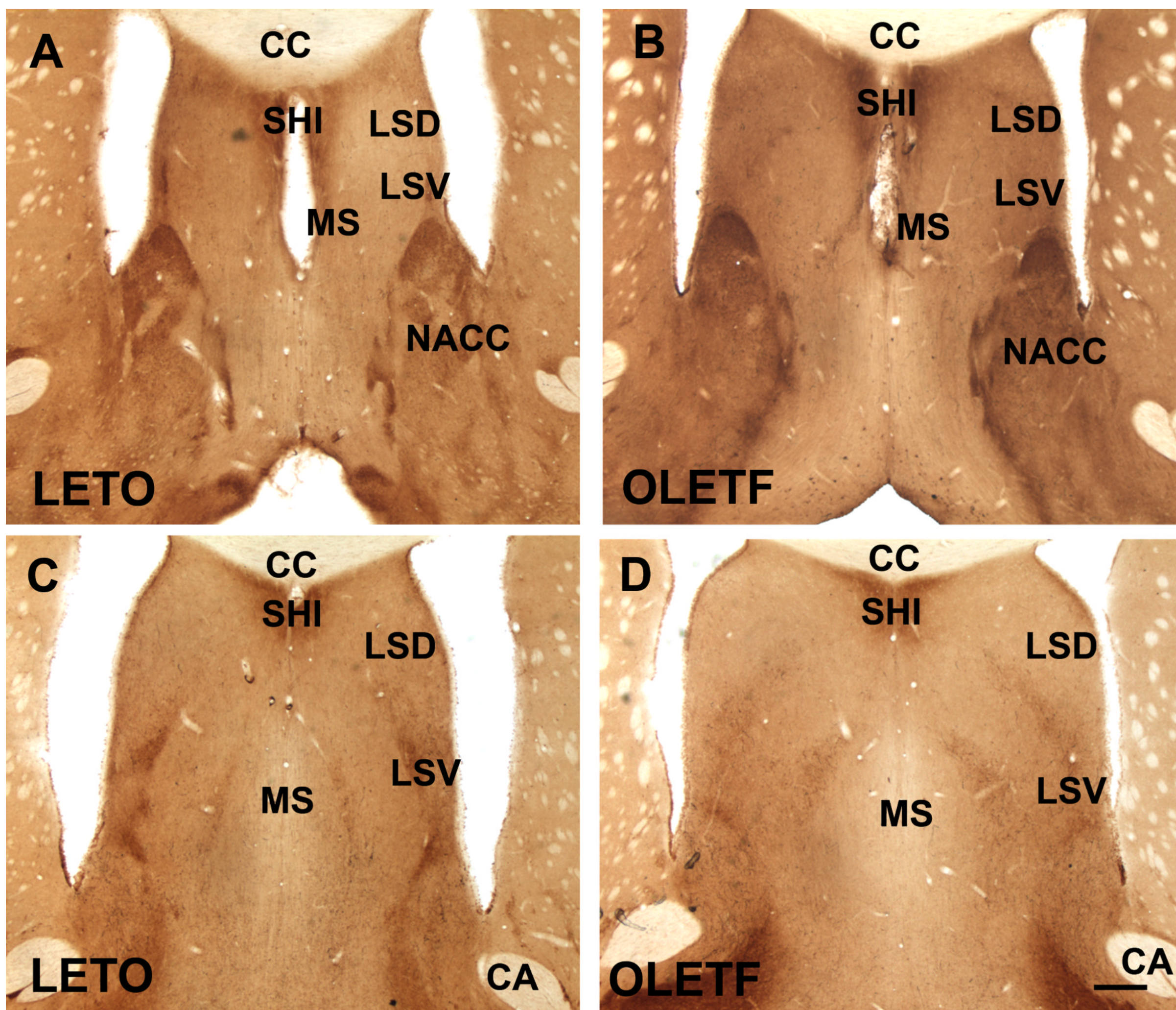

Figure 2.
Photomicorgraphs showing CART peptide-IR in the septum of a young LETO (A and C) and of a young non-obese OLETF (B and D) rat. A and B: The rostral part of the septum contains a dense CART-positive fiber-network in the septohippocampal nucleus (SHI), while moderate density of CART-IR fibers is visible in the dorsal (LSD) and ventral parts of the lateral septum (LSV) in both LETO (A) and OLETF (B). The medial septum (MS) contains low density of CART-IR elements. In contrast, NACC at the septal level exhibits moderately dense CART-IR axons in both LETO (A) and OLETF (B) rats. C and D: Similar to the rostral part of the septum, the caudal septohippocampal nucleus (SHI) expresses dense CART peptide-IR elements, the dorsal (LSD) and ventral parts (LSV) of the lateral septum contain moderately dense networks of immunostained fibers in both LETO (C) and OLETF (D) rats. CART peptide-immunodensity is low in the medial septum (MS) due to the loose network of fibers. Additional abbreviation: CC, corpus callosum, CA, anterior commissure. Scale bar = 250 μm in A and B, 500 μm in C and D.

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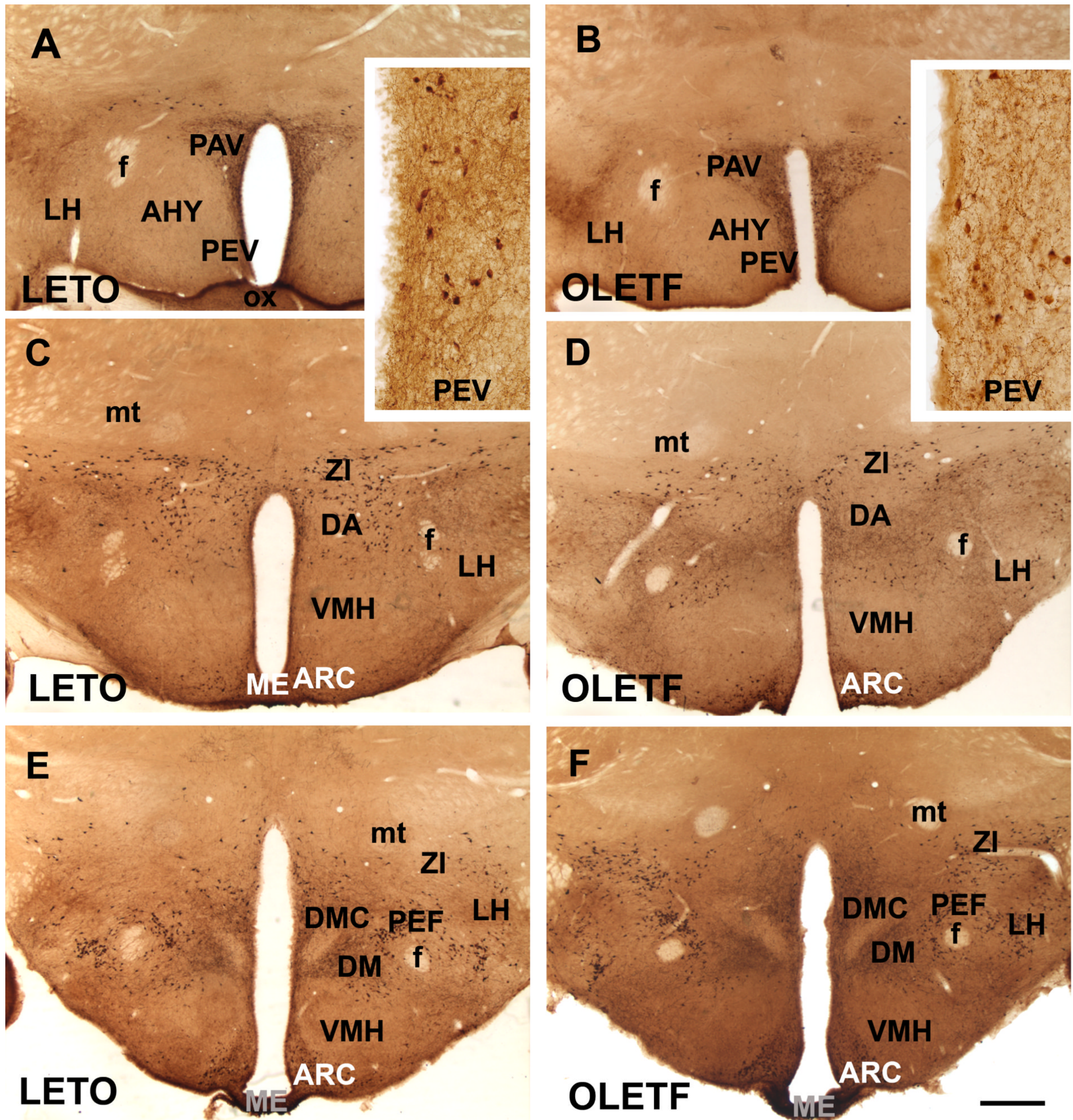

Figure 3.
Photomicrographs demonstrating CART peptide immunoreaction in the hypothalamus of a control LETO (A, C and E) and a non-obese young OLETF (B, D and F) rat. A and B: Rostrally, the paraventricular (PAV) and the periventricular (PEV) nuclei of the hypothalamus are densely populated by CART-IR cells and fibers, whereas the anterior hypothalamic nuclei (AHY) as well as the lateral hypothalamus (LH) exhibit moderately dense networks of immunoreactive fibers in both LETO (A) and OLETF (B) rats. Higher magnification of the periventricular hypothalamus (PEV) containing CART-IR neurons and fibers is shown in the insets. C and D: Similarly to the LETO (C), in the OLETF rat (D) the zona incerta (ZI) expresses a large number of CART-IR neurons, whereas the arcuate (ARC) nucleus contains, in addition to the CART-positive neurons, dense immunoreactive axonal network. The lateral hypothalamic area (LH) contains a less dense network of

immunoreactive fibers, and the ventromedial hypothalamic nucleus (VMH), as well as the dorsal hypothalamic area (DA) displays very low level of immunoreactivity in both LETO (C) and OLETF rats (D). E and F: In both LETO (E) and OLETF (F) rats, the zona incerta (ZI) and the perifornical nucleus (PEF) exhibit numerous strongly stained neurons, whereas the periventricular (PEV), the arcuate (ARC) and the dorsomedial nuclei (DM) contain moderately dense fiber plexus and numerous CART-IR neurons. The pars compacta of the dorsomedial hypothalamic nucleus (DMC), the ventromedial hypothalamic nucleus (VMH) and the lateral hypothalamic area (LH) exhibit low densities of immunoreactive axons in both LETO (E) and OLETF (F) strains. In both strains, very strong immunoreaction is detected in the median eminence (ME). Additional abbreviations: mt, mammillothalamic fascicle; f, fornix. Scale bar = 500 μm for A-F and 100 μm for the insets.

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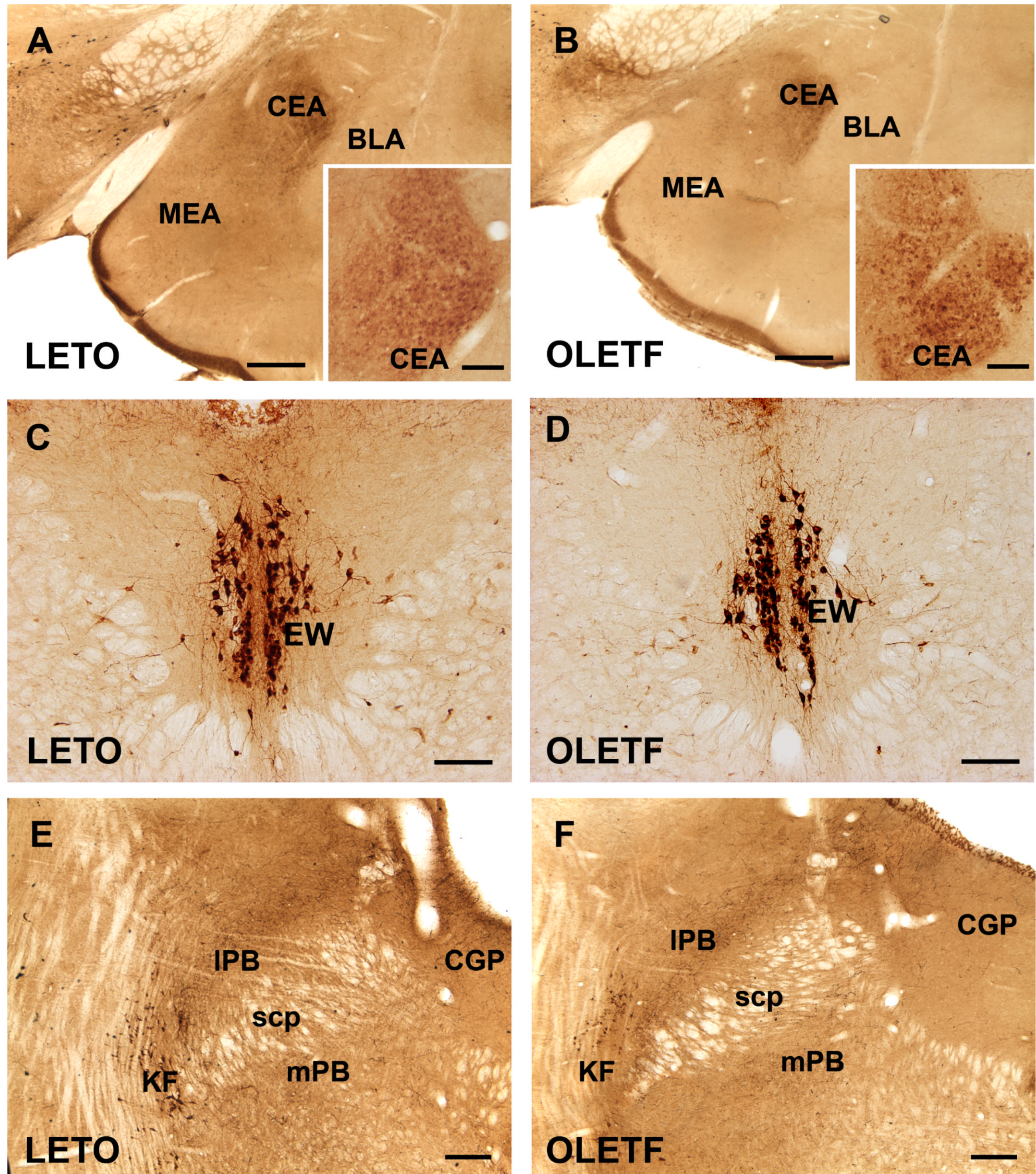

Figure 4.

CART peptide immunoreaction in nuclei of the amygdala (A and B), in the centrally projecting nucleus of Edinger-Westphal (C, D) and in the parabrachial nucleus (E, F) of a young LETO (A, C, E) and a non-obese young OLETF rat (B, D, F). A and B: Large number of CART peptide-IR neurons and fibers are present in the central nuclei of the amygdala (CEA), but fiber density is moderate in the medial nucleus (MEA) both in LETO (A) and OLETF rats (B). In the rostral part of the basolateral amygdala nuclei (BLA) CART-IR fibers are the least dense in both the OLETF and the LETO rats. Insets reveal CART-IR neurons in the central nuclei of the amygdala (CEA) with higher magnification. C and D: Strongly CART-IR large cells in the nucleus Edinger-Westphal (EW) in the OLETF

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(D) and in LETO controls (C). E and F: In the pons, the lateral parabrachial nucleus (lPB) contains strongly immunoreactive dense fiber plexus whereas in the medial parabrachial nucleus (mPB) and in the central gray (CGP) CART-IR axonal density is weaker in both LETO (E) and OLETF (F) rats. CART peptide-IR neurons are localized in the Kolliker-Fuse nucleus (KF) in both strains. Additional abbreviation: scp, superior cerebellar peduncle. Scale bars = 200 μm for A, B, E, F and 100 μm for C, D and for the insets.

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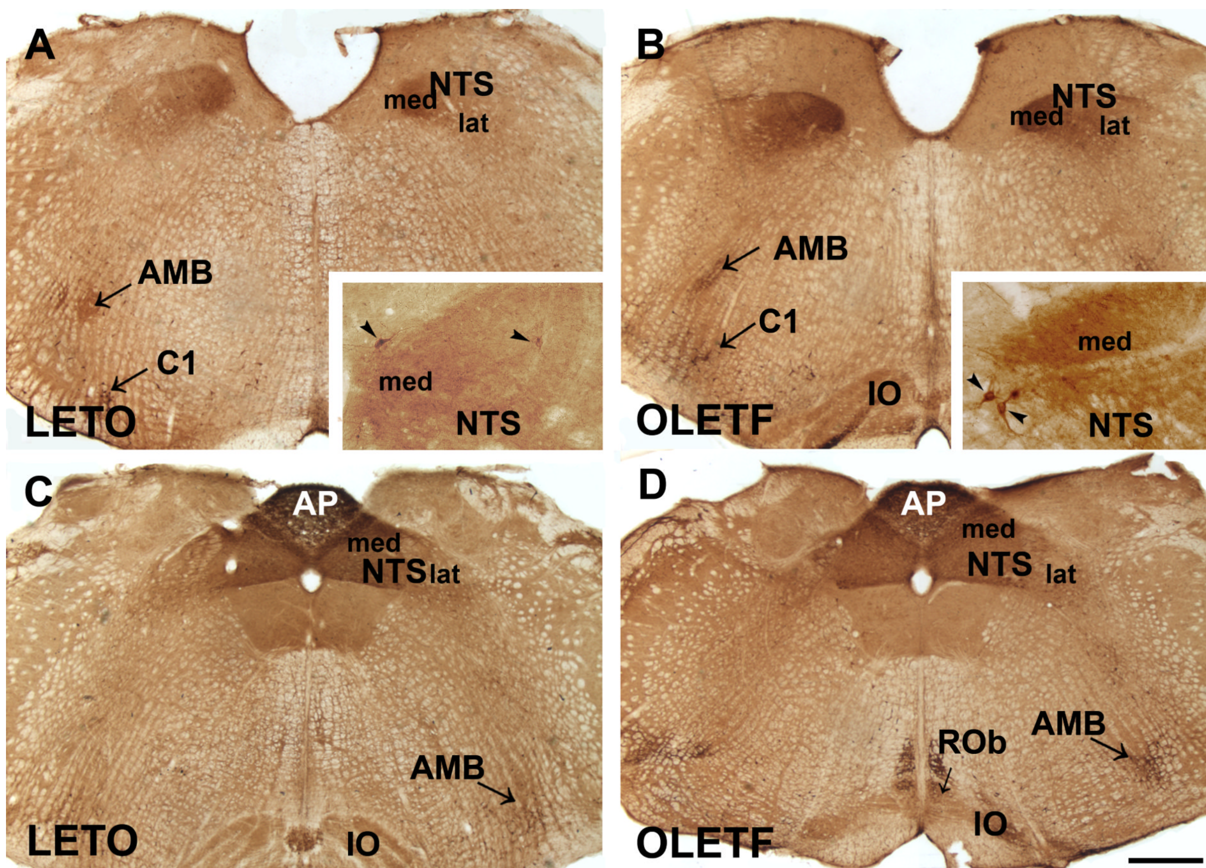

Figure 5.

CART peptide-IR neurons and axonal networks in the medulla oblongata of a young LETO (A, C) and an OLETF (B, D) rat. A and B: The medial part of the nucleus of the solitary tract (NTS, med) contains dense CART-IR fiber networks, whereas the lateral part (NTS, lat) exhibits lower density of immunoreactive axons than the lateral one in both LETO (A) and OLETF (B) rats. In the nucleus ambiguous (AMB) and in the inferior olive (IO) CART-IR fibers are detected, and CART peptide-containing cells are visible in region of C1 in both animals. CART-IR neurons (arrowheads) and the dense fiber network are shown in the insets of the medial nucleus of the solitary tract (NTS, med) with higher magnification. C and D: Similarly to the rostral level of the medulla oblongata, the medial part (med) of the nucleus of solitary tract (NTS) expresses a dense network of CART-IR fibers, while the lateral part (lat) contains moderate densitiy of CART-immunopositive axons in both strains. In addition, the area postrema (AP) exhibits strong CART peptide-IR. The nuclei raphe obscurus (ROb), ambiguous (AMB) and the inferior olive (IO) express also weak CART-IR in both LETO (C) and OLETF (D) rats. Scale bar = 500 μm for A-D and 80 μm for the insets.

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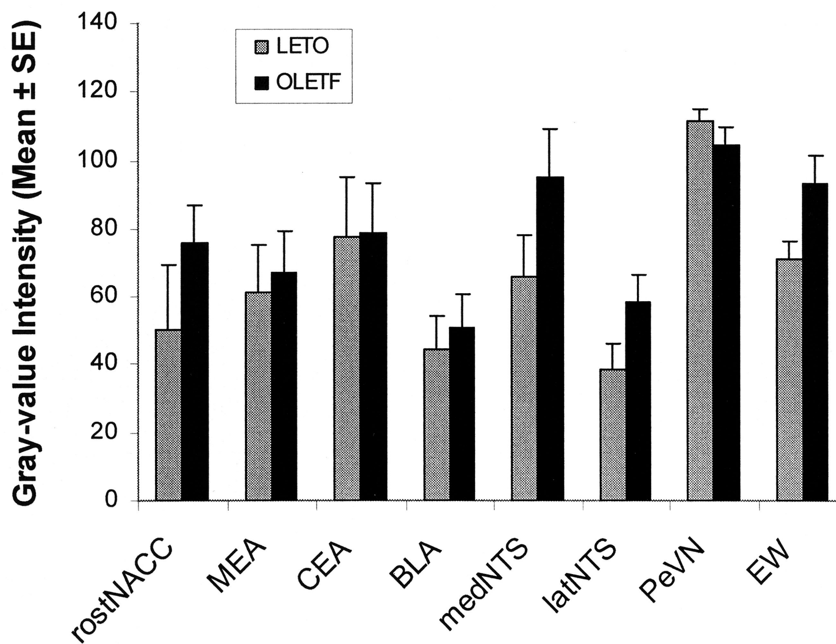

Figure 6.
Summary of data obtained by gray-value intensity measurements for CART-IR in various brain areas. Black bars indicate values obtained in young non-obese non-diabetic OLETF rats, gray bars represent data from age-matched LETO controls. One bar represents intensity values obtained from various number of photomicrographs. The number of photos used for the measurement are the followings: nucleus accumbens 18 (OLETF) and 21 (LETO), the periventricular nucleus of the hypothalamus 44 (OLETF), 27 (LETO), the nuclei of the amygdala 28 (OLETF), 20 (LETO), the medial and lateral parts of the nucleus of solitary tract 50 (OLETF), 29 (LETO) and the centrally projecting nucleus of the Edinger-Westphal 18 (OLETF), 36 (LETO). Error bars indicate standard error (SE). Abbreviations: NACC caudal nucleus accumbens, MEA medial nucleus of the amygdala, CEA central nucleus of the amygdala, BLA basolateral nucleus/ complex of the amygdala, med-NTS rostral portion of the medial part of the nucleus tractus solitarii; lat-NTS rostral part of the lateral part of the nucleus tractus solitarii, PeVN periventricular nucleus of the hypothalamus, EW nucleus Edinger-Westphal.