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Involvement of neuropeptide CART in the central effects of insulin on feeding and body weight

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ABSTRACT

While insulin secreted from pancreas plays a pivotal role in the control of glucose homeostasis, it also interacts with hypothalamic sites and negatively influences the energy balance. The present study was undertaken to reveal the functional interaction between cocaine- and amphetamine-regulated transcript (CART), a well-known anorexic peptide, and insulin within the framework of hypothalamus in the regulation of feeding behavior and body weight. Insulin was administered daily by intracerebroventricular (icv) route, alone or in combination with CART (icv) for a period of seven days. Immediately thereafter, preweighed food was offered to the animals at the commencement of the dark phase. The food intake and body weight were measured daily just prior to next injection. Furthermore, brains of insulin-treated rats were processed for the immunohistochemical analysis of CART-containing elements in the hypothalamus. Treatment with insulin (6 mU, icv) for a period of 7 days caused a significant decrease in food intake and body weight as compared to control. Concomitant administration of CART (0.5 μg, icv) potentiated insulin-induced anorexia and weight loss. Insulin administration resulted in a significant increase in CART immunoreactivity in the hypothalamic arcuate, paraventricular, dorsomedial and ventromedial nuclei. We suggest that increased CART contents in the hypothalamus may be causally linked with anorexia and weight loss induced by insulin.

1. Introduction

Hypothalamus integrates various peripheral signals to regulate important central pathways that control food intake and energy expenditure. Peripheral insulin is transported into the brain through circumventricular regions that lack blood-brain-barrier (BBB) and through receptor-mediated BBB transcytosis, and binds with hypothalamic insulin receptors (Ajoy and Chou, 2018; Baranowska-Bik and Bik, 2017). Insulin is also known to be synthesized centrally by neuronal and astrocyte glial cells (Baranowska-Bik and Bik, 2017; Mehran et al., 2012; Molnár et al., 2014) including hypothalamic neurons (Madadi et al., 2008; Young, 1986). In laboratory animals, direct administration of insulin into the brain caused anorexia and body weight loss (Air et al., 2002; Brief and Davis, 1984; Clegg et al., 2005; Ikeda et al., 1986; Niswender et al., 2003; Plata-Salaman and Oomura, 1986; Vasselli et al., 2017), whereas insulin antibody produced opposite effects (McGowan et al., 1992; Strubbe and Mein, 1977). Insulin receptor knockdown or central injection of antisense oligodeoxynucleotide directed against insulin receptor precursor protein resulted in hyperphagia, increased adiposity and weight gain (Begg and Woods, 2012; Obici et al., 2002). In humans, intranasal administration of insulin not only increased insulin levels in cerebrospinal fluid (CSF) without affecting its plasma levels (Born et al., 2002), but also reduced food intake and body weight (Hallschmid et al., 2004a, 2004b). Although these studies indicate a role of central insulin system in the negative regulation of feeding and energy balance, the underlying neuronal mechanisms remain unclear.

Hypothalamic neuropeptide, cocaine- and amphetamine-regulated transcript (CART) is one of the important components of the feeding-regulatory systems (Zhu et al., 2018; Crespo et al., 2014). CART is highly expressed in the paraventricular (PVN), arcuate (ARC), dorsomedial (DMH), ventromedial (VMH) and lateral (LH) nuclei of the hypothalamus and negatively influences the food intake and body weight (Kristensen et al., 1998; Subhedar et al., 2014). Insulin receptors are ubiquitously present in the aforementioned CART expressing areas (Havrankova et al., 1978a, 1978b, 1981; Pardini et al., 2006), and therefore may affect CART expression in the hypothalamus. In fact, intracranial injection of insulin significantly increased CART mRNA

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expression in the hypothalamus of chicks and produced anorexia (Honda et al., 2007). CART immunoreactivity was increased in the entopeduncular nucleus of catfish following intracranial insulin treatment (Subhedar et al., 2011). It is noteworthy that CART plays anorectic role in catfish (Kobayashi et al., 2008) and goldfish (Volkoff and Peter, 2000). While above studies reflect insulin-CART interaction at the level of the hypothalamus, the precise outcome of interaction is lacking.

The present study was undertaken to clarify the role of hypothalamic CART system in the effects of insulin on food intake and body weight. Insulin was administered in the rats via icv route for 7 days, alone or in combination with CART or CART antibody, and food intake and body weight were recorded (g) daily. Furthermore, the profile of CART following insulin treatment was evaluated in hypothalamic nuclei like PVN, ARC, DMH, VMH and LH using immunohistochemistry. The hypothalamus was chosen, since it contains an abundance of CART (Kristensen et al., 1998; Subhedar et al., 2014) and insulin receptors (Havrankova et al., 1978b; Pardini et al., 2006), and plays an important role in the regulation of feeding and body weight (Konturek et al., 2005; Subhedar et al., 2014).

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats weighing around 250 g were used. The animals were housed in acrylic cages (24 × 17 × 12 cm) under constant room temperature (25 ± 2 °C), relative humidity (50 ± 5%), and maintained on 12 h dark/light cycle (lights on at 12:00 am). Food and water were available ad libitum. All the experimental procedures were approved and carried out under strict compliance with Institutional Animal Ethics Committee, constituted for the purpose of control and supervision of experimental animals by the Ministry of Environment and Forests, Government of India, New Delhi, India.

2.2. Drug solutions

CART peptide (54–102) and monoclonal antibodies against CART (54–102) were generously gifted by Dr. Jes Thorn Clausen, Department of Protein Chemistry, Novo Nordisk A/S, Bagsvaerd, Denmark and Dr. Lars Thim, Department of Assay and Cell Technology, Novo Nordisk A/S, Bagsvaerd, Denmark. CART was dissolved in 0.2 M phosphate buffer and before administration, diluted to final concentration in artificial cerebrospinal fluid (aCSF) having composition:140 mM NaCl, 3.35 mM KCl, 0.3 mM NaH_2PO_4,1.2 mM Na_2HPO_4,1.26 mM $CaCl_2$ and 1.15 mM $MgCl_2$ (pH 70.4) containing 0.5% bovine serum albumin (Bhisikar et al., 2009; Bhorkar et al., 2014). The commercially available insulin (Recosulin-R® 100 I.U., Shreya Sciences Pvt. Ltd., Aurangabad, India) was used.

2.3. Intracerebroventricular (icv) cannulation

The rats were subjected to the stereotaxic surgery as described in our previous studies (Nakhate et al., 2009, 2018a; Rangani et al., 2012). Briefly, the rats were anaesthesized with intraperitoneal (ip) injection of thiopentone sodium (45 mg/kg). A midsagittal dorsal incision was made on the scalp to expose the skull, and a 24 gauge stainless steel guide cannula, fabricated in-house (Kokare et al., 2011; Nakhate et al., 2016), directed at right lateral cerebral ventricle was implanted using the stereotaxic coordinates, −0.8 mm posterior, +1.3 mm lateral to midline and 3.5 mm ventral with respect to bregma (Paxinos and Watson, 1998). To prevent blockage, a dummy cannula was placed into the guide cannula. Guide cannula was anchored to the skull with three stainless steel screws and dental acrylic cement. After hardening of acrylic, the animal was removed from stereotaxic frame. Care was taken to minimize the post-surgical pain and systemic or local infection (Taksande et al., 2011; Upadhya et al., 2012). After wound closure, animals were housed individually and allowed to recover for a period of seven days. Animals with any neurological/motor deficits were excluded from the study (Dandekar et al., 2011; Meena et al., 2009). In the present study, 2 animals were excluded. Icv injection (5 µl volume) was given manually, over a period of 1 min, using microliter syringe (Hamilton, Nevada) connected by polyethylene tubing to a 31 gauge internal cannula (fabricated in-house; Kokare et al., 2011) that projected 1 mm below the guide cannula.

2.4. Measurement of the effect of icv insulin treatment on cumulative food intake

Since rodents consume maximum food during the dark phase (Bruckdorfer et al., 1974; Kimura et al., 1970), the treatments were given at the onset of the dark phase. Separate groups of rats (n = 8 per group) were injected with aCSF (5 µl per rat, icv) or insulin (2, 6 and 10 mU per rat, icv) 10 min prior to the onset of dark phase. Immediately thereafter, the pre-weighed food pellets were offered to the rats, and cumulative food intake was recorded (g) at 2, 6 and 24 h post-injection time-points.

2.5. Measurement of the effect of CART, administered concomitantly, on the insulin-triggered anorexia and weight loss

While the effective dose of insulin (6 mU per rat, icv) was selected from the dose response study, the subeffective dose CART (0.5 µg per rat, icv) was taken from our earlier studies (Nakhate et al., 2016, 2018b). Different sets of rats (n = 6–8 per group) were administered daily at the onset of the dark phase for 7 days with (1) aCSF (5 µl per rat, icv) + aCSF, (2) CART (0.5 µg per rat, icv) + aCSF, (3) aCSF + insulin (6 mU per rat, icv) or (4) CART (0.5 µg per rat, icv) + insulin (6 mU per rat, icv). The injections were given at 30-min intervals. Ten min after second injection, the pre-measured food pellets were offered to the animals. The food intake and body weights were recorded (g) daily before the next injection time-points.

2.6. Measurement of the effect of CART antibody, administered concomitantly, on the insulin-triggered anorexia and weight loss

The subeffective dose of CART antibody (5 µl per rat, icv; 1:1500 dilution) was taken from dose dependent study (Nakhate et al., 2010, 2011). CART antibodies were used as information is not available about either CART receptors or specific CART antagonist. Different sets of rats (n = 6–8 per group) were administered daily for 7 days with (1) aCSF (5 µl per rat, icv) + aCSF, (2) CART antibody (5 µl per rat, icv; 1:1500 dilution) + aCSF, (3) aCSF + insulin (6 mU per rat, icv) or (4) CART antibody (5 µl per rat, icv; 1:1500 dilution) + insulin (6 mU per rat, icv). The injections were given 10 min prior to the lights out, and immediately thereafter the pre-measured food pellets were given to the animals. The food intake and body weight were recorded (g) daily before the next injection time-points.

2.7. Cannula placement verification

At the end of the experiments, dilute India ink (5 µl) was injected by icv route, and the animals were euthanized with an overdose of thiopentone sodium (75 mg/kg, ip). The brains were dissected out, cut in coronal plane to verify the placement of guide cannula and diffusion of ink in the ventricle (Desai et al., 2014; Kamdi et al., 2009). The data from 4 rats with incorrect cannula placement were excluded from the study.

2.8. Measurement of the effect of insulin on the hypothalamic CART immunoreactivity

CART is synthesized in ARC and PVN neurons and transported to the ARC, PVN, DMH, VMH and LH (Fekete et al., 2004; Elias et al., 1998, 1999; Wynne et al., 2005). Therefore, we evaluated CART immunoreactivity in neurons of the ARC and PVN and fibers in the ARC, PVN, DMH, VMH and LH. Brains of rats treated with aCSF (5 µl per rat, icv) and insulin (6 mU per rat, icv) daily for the duration of 7 days were processed for the immunohistochemical labeling with CART antibody (n = 6 per group) using streptavidin-biotin-peroxidase method (Nakhate et al., 2013, 2018b; Upadhya et al., 2011). Briefly, one hour following last dose of insulin, rats were deeply anaesthetized with thiopentone sodium (65 mg/kg, ip), perfused transcardially with heparinized phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, post-fixed for 6 h, cryoprotected in 30% sucrose for 48 h, embedded into 15% polyvinylpyrrolidone (Sigma), and serially sectioned in the coronal plane at 30 µm thickness using a cryostat (−28 °C) and collected in PBS. The sections were incubated in CART antibodies, diluted in PBS (1:5000) containing 2% normal horse serum (block non-specific binding), 0.3% Triton X-100 (enhance penetration of antisera), 0.08% sodium azide (preservative) and 0.2% Kodak PhotoFlo solution for 48 h at 4 °C. The addition of PhotoFlo to all the immunoreagents, together with a relatively long incubation time, results in a balanced compromise between the demands of preservation of the ultrastructure and penetration of the antisera. After rinsing in PBS, sections were incubated in biotinylated goat anti-mouse IgG (1:100; Vector) for 2 h followed by in ExtrAvidin-peroxidase conjugate (1:100; Sigma) for 1 h at room temperature. For visualization of the immunoreaction product, the sections were incubated for 3 min in a solution containing 0.03% hydrogen peroxide and 3-amino-9-ethyl-carbazole (Sigma). Reddish-brown precipitate indicated the presence of antigen in the sections. The sections were washed and mounted in glycerol-gelatin.

Five sections passing through the PVN, ARC, DMH, VMH and LH of each rat were subjected to morphometric analysis (Dandekar et al., 2012; Nakhate et al., 2010, 2018b). Therefore, ten readings (five per hemisphere) were obtained for each nucleus from each animal. Since we incorporated six rats per group, mean of sixty readings from each group was obtained for the morphometric analysis of CART immunoreactive product. The area occupied by CART-immunoreactive cells/fibers in the PVN, ARC, DMH, VMH and LH of both the groups of rats were evaluated using microscopic images of the sections. The images (480×) were captured using Leica Leitz-LaborLux S microscope and analyzed with Leica QWin Standard software (version 3.1.0). The image of brain section was digitized and the background of non-immunoreactive area was considered as threshold. The area occupied by immunostained cells and fibers, above the threshold, was filled with overlaid color. The area (*per* 50,000 µm^2) of the color overlay within the evaluated field was automatically obtained using Leica-QWin Standard software. While evaluating the area of the CART-immunoreactive cells, the area covered by immunoreactive fibers was erased, and therefore, not considered for the measurement and vice versa. The measurements from predetermined fields of the each hypothalamic nucleus on both sides of each brain were taken. To ensure reliable comparisons among different groups and maintain stringency in tissue preparation and staining conditions, the brain sections from various groups were processed at the same time under identical conditions. Omission of primary antibody and replacement with bovine serum albumin produced no immunoreaction. In the preadsorption controls, application of 1 ml diluted antibody pre-incubated with CART at 10^{-5} M for 24 h completely blocked the immunoreaction. The data from all the animals in each group were pooled separately and the mean ± standard error of mean (S.E.M.) was calculated.

Table 1
Effect of acute icv administration of insulin on the cumulative food intake.

Treatment (icv/rat)	Food intake (g ± SEM)		
	2 h	6 h	24 h
aCSF	3.38 ± 0.33	8.82 ± 0.51	19.14 ± 0.78
Insulin			
2 mU	2.66 ± 0.19	7.89 ± 0.45	18.05 ± 0.53
6 mU	1.99 ± 0.20*	6.31 ± 0.28*	14.76 ± 0.79*
10 mU	0.97 ± 0.27**	3.11 ± 0.37**	13.36 ± 0.71**

Separate groups of rats (n = 8 per group) were administered with aCSF (5 µl per rat, icv) or insulin (2–10 mU per rat, icv) 10 min prior to the onset of dark phase. The cumulative food intake (g) was measured at 2, 6 and 24 h post-injection time-points. The values represent as mean ± SEM for each group at respective time-points. The data were analyzed by one-way ANOVA followed by post-hoc Bonferroni's multiple comparisons test.

* $p < .01$ vs aCSF.

** $p < .001$ vs aCSF.

2.9. Data analyses

The data are presented as mean ± SEM. The data were analyzed with repeated measures one-way ANOVA for acute studies and two-way ANOVA for combination studies followed by Bonferroni's multiple comparison test. Moreover, immunohistochemical data were analyzed by unpaired *t*-test. Differences were considered significant at $p < .05$.

3. Results

3.1. Effects of icv insulin treatment on the cumulative food intake

Insulin administration (6–10 mU per rat, icv) produced a dose dependent reduction in food intake below aCSF treated control rats at 2 h ($F_{(3,31)} = 18.90$ ($p < .0001$)), 6 h ($F_{(3,31)} = 54.42$ ($p < .0001$)) and 24 h ($F_{(3,31)} = 14.50$ ($p < .0001$)) time-points. As compared with control rats, insulin at the dose of 6 mU decreased food intake ($p < .01$) by 41%, 29% and 23%, while at 10 mU dose, the reduction ($p < .001$) was 71%, 65% and 30% at 2, 6 and 24 h respectively. However, at lower dose (2 mU per rat, icv), insulin did not show much significant anorectic effect ($p > .05$) (Table 1).

3.2. Effect of CART, administered concomitantly, on the insulin-induced anorexia and body weight loss

Treatment with insulin (6 mU per rat per day, icv) for 7 days produced a significant inhibition in food consumption ($p < .01$) throughout the period of administration as compared with that in the aCSF treated rats (factor 'insulin treatment' $F_{(1,104)} = 78.15$ ($p < .0001$); factor 'duration in days' $F_{(7,104)} = 1.94$ ($p = .06$) and the interaction 'insulin treatment x duration in days' $F_{(7,104)} = 2.4$ ($p = .02$)). On day 7, the food intake by insulin treated rats was 20% less than the control animals. Although injection of CART (0.5 µg per rat per day, icv) did not affect the food intake ($p > .05$) per se, the co-injection of CART, at the same dose, potentiated the anorectic action of insulin as indicated by a significant difference ($p < .05$) in food intake between insulin and CART + insulin treatment groups (factor 'CART + insulin treatment' $F_{(1,104)} = 71.07$ ($p < .0001$); factor 'duration in days' $F_{(7,104)} = 16.52$ ($p < .0001$) and the interaction 'CART + insulin treatment x duration in days' $F_{(7,104)} = 1.33$ ($p = .2$)). On day 7, the food intake of CART + insulin treated rats was 18% less than in the rats treated with insulin alone (Fig. 1a).

Similar to feeding data, administration of insulin (6 mU per rat per day, icv) for 7 days produced a significant reduction in body weight ($p < .01$) as compared with aCSF treated rats (factor 'insulin treatment' $F_{(1,104)} = 91.40$ ($p < .0001$); factor 'duration in days' $F_{(7,104)} = 1.09$ ($p = .3$) and the interaction 'insulin

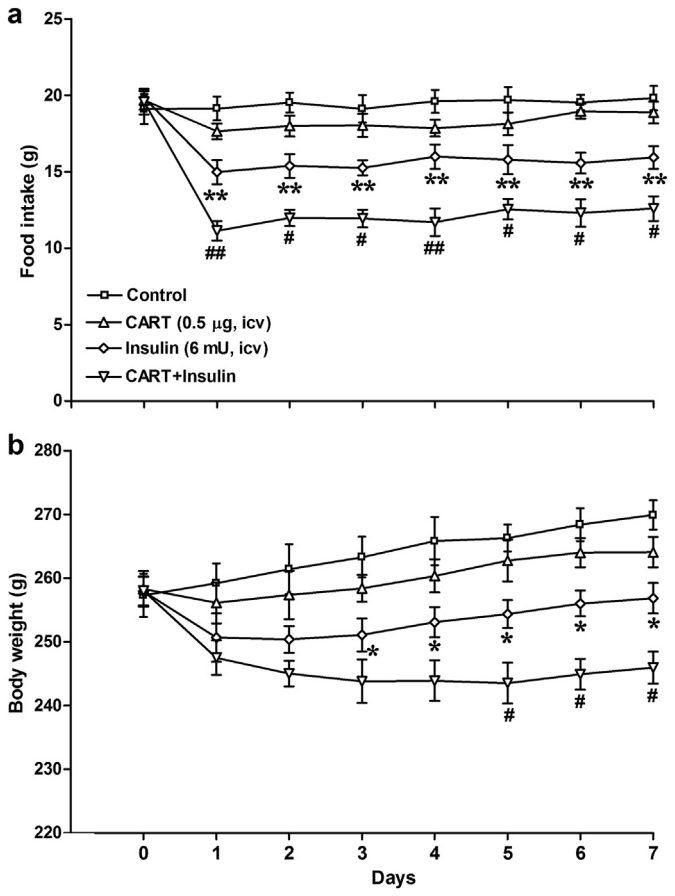

Fig. 1. Effect of CART, administered concomitantly, on insulin-induced anorexia (a) and body weight loss (b). Different groups of rats were administered daily, 10 min prior to the onset of the dark phase, with (1) aCSF + aCSF (control), (2) CART (0.5 μg per rat, icv) + aCSF, (3) aCSF + insulin (6 mU per rat, icv) or (4) CART (0.5 μg per rat, icv) + insulin (6 mU per rat, icv) for a period of 7 days. Food intake and body weight were recorded (g) after an interval of 24 h, just prior to the next injection time-point. Each line and bar represents mean ± SEM (n = 6–8 per group). The data were analyzed by two-way ANOVA followed by a post-hoc Bonferroni's multiple comparisons test. $^{*}p < .05$, $^{**}p < .01$ vs control; $^{\#}p < .05$, $^{\#\#}p < .01$ vs insulin.

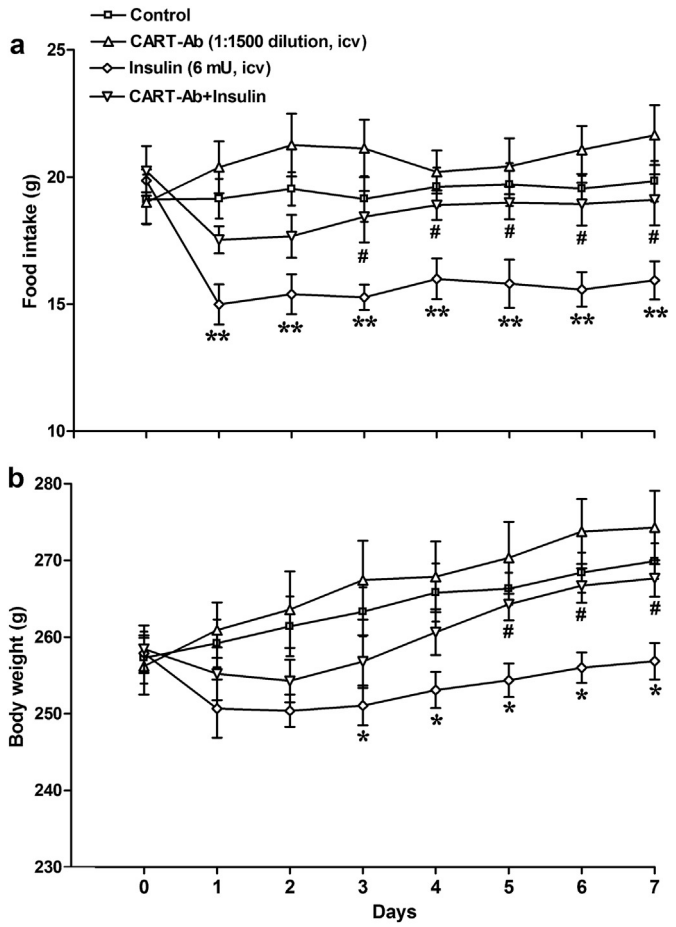

Fig. 2. Effect of CART antibody (CART-Ab), administered concomitantly, on insulin-induced anorexia (a) and body weight loss (b). Different groups of rats were administered daily, 10 min prior to the onset of the dark phase, with (1) aCSF + aCSF (control), (2) CART-Ab (1:1500 dilution per rat, icv) + aCSF, (3) aCSF + insulin (6 mU per rat, icv) or (4) CART-Ab (1:1500 dilution per rat, icv) + insulin (6 mU per rat, icv) for a period of 7 days. Food intake and body weight were recorded (g) after an interval of 24 h, just prior to the next injection time-point. Each line and bar represents mean ± SEM (n = 6–8 per group). The data were analyzed by two-way ANOVA followed by a post-hoc Bonferroni's multiple comparisons test. $^{*}p < .05$, $^{**}p < .01$ vs control; $^{\#}p < .05$ vs insulin.

treatment × duration in days' $F_{(7,104)} = 2.79$ ($p = .01$)). A significant weight loss was observed from day 3 onwards following insulin treatment ($p < .05$). On day 7, the body weight of insulin treated rats was 7% less than the control animals. Although treatment with CART (0.5 μg per rat per day, icv) per se did not affect the body weight ($p > .05$), the concomitant administration of CART at the same dose significantly potentiated ($p < .05$) the weight reducing effect of insulin from day 5 onwards (factor 'CART + insulin treatment' $F_{(1,104)} = 39.89$ ($p < .0001$); factor 'duration in days' $F_{(7,104)} = 7.49$ ($p < .0001$) and the interaction 'CART + insulin treatment × duration in days' $F_{(7,104)} = 1.39$ ($p = .2$)). On day 7, the body weight of CART + insulin treated rats was 5% less than the insulin per se treated animals (Fig. 1b).

3.3. Effect of CART antibody, administered concomitantly, on the insulin-induced anorexia and body weight loss

Administration of CART antibody at the 1:1500 dilution did not increase food intake significantly ($p > .05$), however, its co-administration at the same dilution prevented the anorectic effect of insulin. This is indicated by a significant difference ($p < .05$) in food intake between insulin and CART antibody + insulin treatment groups from day 3 onwards (factor 'CART antibody + insulin treatment' $F_{(1,104)} = 45.87$ ($p < .0001$); factor 'duration in days' $F_{(7,104)} = 4.48$ ($p = .0002$) and the interaction 'CART antibody + insulin treatment × duration in days' $F_{(7,104)} = 0.79$ ($p = .5$)). On day 7, the food intake of CART antibody + insulin treated rats was 16% more than that in the rats treated with insulin alone (Fig. 2a).

Treatment with CART antibody at 1:1500 dilution per se did not increase body weight significantly ($p > .05$), although, its co-administration with insulin prevented the weight reducing effect of insulin as indicated by a significant difference ($p < .05$) in body weight between insulin and CART antibody + insulin treatment groups from day 5 onwards (factor 'CART antibody + insulin treatment' $F_{(1,104)} = 24.95$ ($p < .0001$); factor 'duration in days' $F_{(7,104)} = 4.01$ ($p = .0007$) and the interaction 'CART antibody + insulin treatment × duration in days' $F_{(7,104)} = 0.94$ ($p = .4$)). On day 7, the body weight of CART antibody + insulin treated rats was 4% more than that in the rats treated with insulin alone (Fig. 2b).

3.4. Effects of icv insulin treatment on the CART immunoreactivity in the paraventricular (PVN), and arcuate (ARC) nuclei of hypothalamus

Fig. 3 summarizes the changes in the immunohistochemical profile

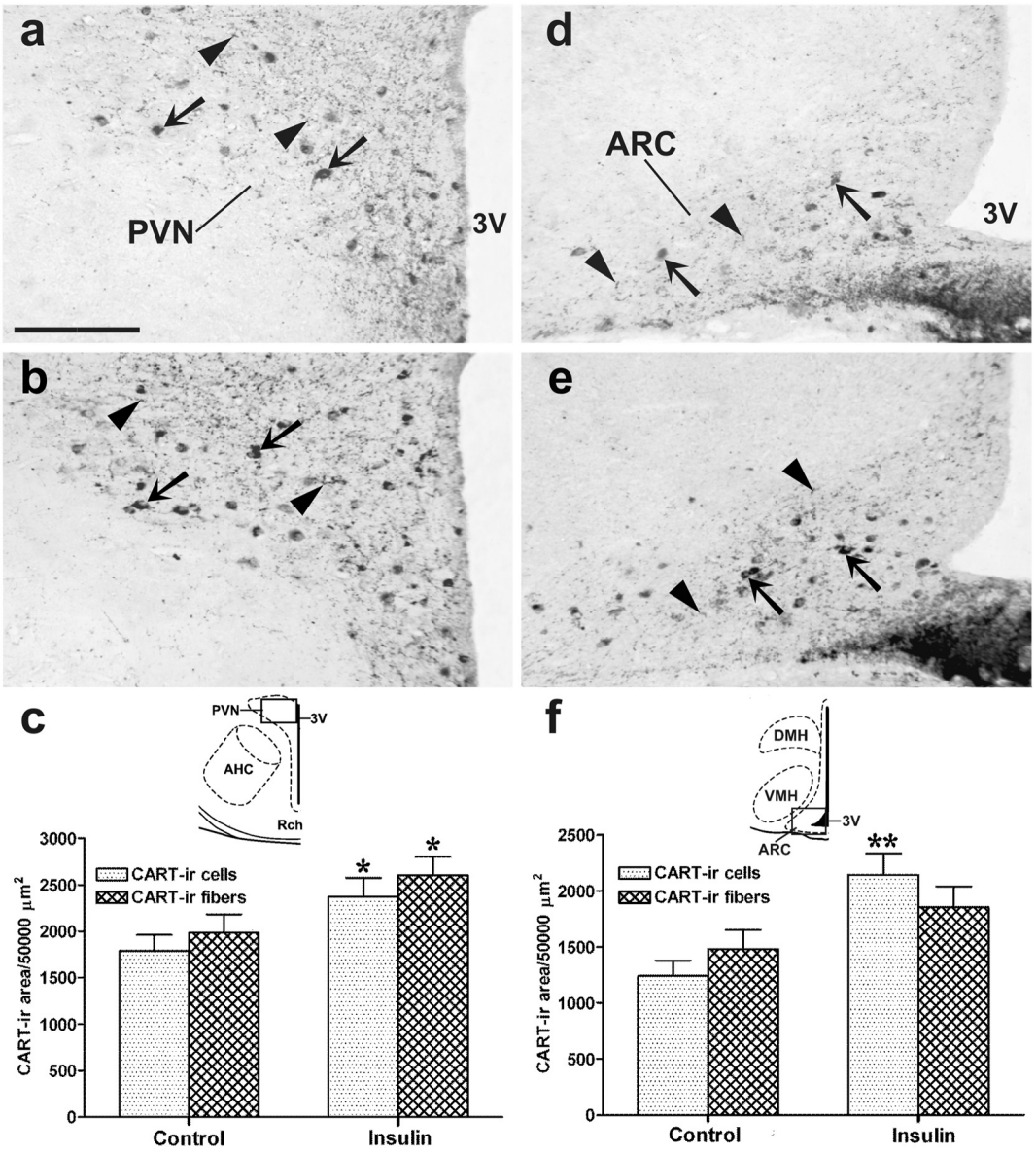

Fig. 3. Photomicrographs showing the CART-immunoreactive (ir) cells (arrows) and fibers (arrowheads) in the hypothalamic paraventricular (PVN) and arcuate (ARC) nuclei of rats following 7 days of icv treatment with aCSF (control; Figs. 3a and d respectively) or insulin (6 mU per rat per day; Figs. 3b and e respectively). Moreover, the semiquantitative morphometric analysis of CART immunoreactivity in cells and fibers of the PVN and ARC of control and insulin treated rats is represented in Figs. 3c and f respectively. The outline of the transverse section through brain indicates the regions of the PVN and ARC at the co-ordinates −1.80 mm and −3.30 mm with reference to bregma respectively (Paxinos and Watson, 1998) from which the measurements were collated (square, not to scale). 3 V, third ventricle; AHC, central part of the anterior hypothalamus; DMH, dorsomedial nucleus of hypothalamus; Rch, retrochiasmatic nucleus; VMH, ventromedial nucleus of hypothalamus. Scale bar = 200 µm. The bar values are shown as the mean ± SEM of five measurements from predetermined fields of the PVN and ARC on both the sides of each brain ($n = 6$ per group). The data were analyzed by unpaired *t*-test. $^{*}p < .05$, $^{**}p < .001$ vs control.

of the CART in the PVN and ARC of rats treated with aCSF (Figs. 3a and d respectively) and insulin (Figs. 3b and e respectively). The morphometric analysis of the CART-immunoreactive cells and fibers in the PVN and ARC is represented in Figs. 3c and f respectively. As compared with those in control animals, insulin treatment significantly increased the CART immunoreactivity in cells and fibers in the PVN by about 32% ($p < .05$). Although CART immunoreactivity in the ARC cells increased significantly (73%; $p < .001$), the fibers population did not increase to a significant level as compared to control ($p > .05$).

3.5. Effects of icv insulin treatment on the CART immunoreactivity in the dorsomedial (DMH), ventromedial (VMH) and lateral (LH) nuclei of the hypothalamus

Fig. 4 summarizes the changes in the immunohistochemical profile of the CART in the DMH, VMH and LH of rats treated with aCSF (Figs. 4a, d and g respectively) and insulin (Figs. 4b, e and h respectively). The morphometric analysis of the CART-immunoreactive fibers in the DMH, VMH and LH is represented in the Figs. 4c, f and i respectively. Following 7 days of insulin treatment, a significant increase in CART-immunoreactive fibers was observed in the DMH ($p < .01$) and VMH ($p < .05$) by about 45% as compared to those in aCSF-treated animals. However, no significant difference was noticed in the CART immunoreactivity in the LH fibers across insulin treated and

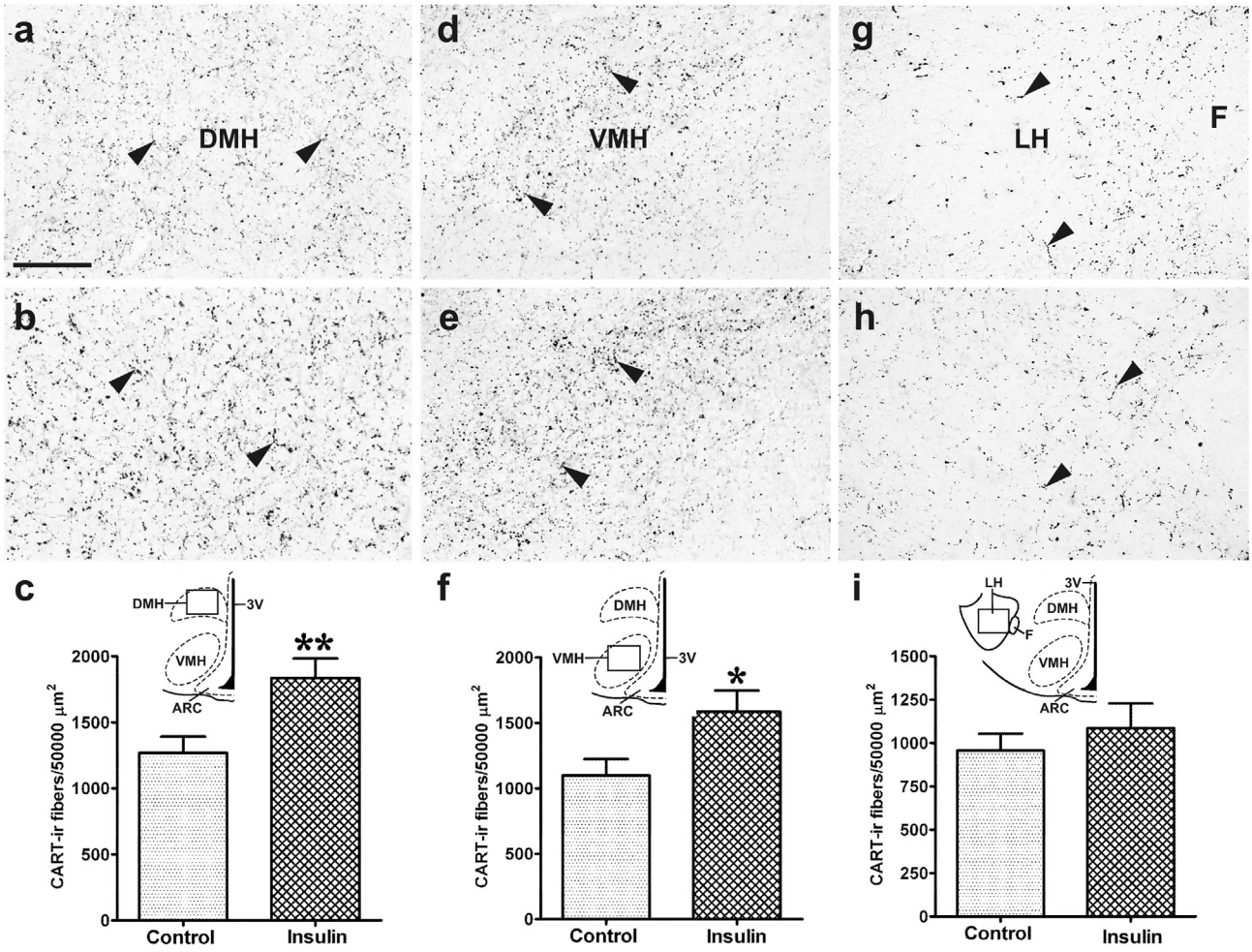

Fig. 4. Photomicrographs showing the CART-immunoreactive (ir) fibers (arrowheads) in the hypothalamic dorsomedial (DMH), ventromedial (VMH) and lateral (LH) nuclei of rats following 7 days of icv treatment with aCSF (control; Figs. 4a, d and g respectively) or insulin (6 mU per rat per day; Figs. 4b, e and h respectively). Moreover, the semiquantitative morphometric analysis of CART immunoreactivity in the fibers of DMH, VMH and LH of control and insulin treated rats is represented in Figs. 4c, f and i respectively. The outlines of the transverse sections through brain indicate the regions of the DMH or LH at co-ordinate −2.56 mm and VMH at co-ordinate −2.80 mm with reference to bregma respectively (Paxinos and Watson, 1998) from which the measurements were collated (square, not to scale). 3 V, third ventricle; ARC, arcuate nucleus of hypothalamus; F, fornix. Scale bar = 100 µm. The bar values are shown as the mean ± SEM of five measurements from predetermined fields of the DMH, VMH and LH on both the sides of each brain ($n = 6$ per group). The data were analyzed by unpaired *t*-test. $^{*}p < .05$, $^{**}p < .01$ vs control.

control groups ($p > .05$).

4. Discussion

The pancreatic hormone insulin, along with leptin, is considered as a long-term regulator of energy balance. Circulating insulin and leptin levels increased proportionately to the peripheral fat deposition. These hormones convey the energy status-related information from the periphery to the brain, and modulate satiety and energy expenditure (Konturek et al., 2005; Woods et al., 2006). Interestingly, insulin is also synthesized in the brain (Madadi et al., 2008; Mehran et al., 2012; Molnár et al., 2014) and influences the appetite controlling neuropeptides in the hypothalamus (Fekete et al., 2006; Keen-Rhinehart et al., 2009; Schwartz et al., 1992). In the present investigation, we test the hypothesis that insulin and neuropeptide CART might interact in the framework of the hypothalamus, and synergistically regulate food intake and body weight. Insulin treatment produced anorexia and weight loss, which was associated with increased hypothalamic CART immunoreactivity. While the effects of insulin on feeding and body weight were potentiated by CART, CART antibody attenuated both the effects.

In the present study, daily icv administration of insulin for a period of 7 days caused reduction in food intake and body weight in rats. The results are in agreement with previous studies. While central administration of insulin caused anorexia and weight loss (Brief and Davis, 1984; Ikeda et al., 1986; Vasselli et al., 2017; Woods et al., 1979), insulin antibodies produced opposite effects (McGowan et al., 1992; Strubbe and Mein, 1977). Insulin receptor knockdown or central injection of antisense oligodeoxynucleotide directed against insulin receptor precursor protein resulted in hyperphagia, increased adiposity and weight gain (Begg and Woods, 2012; Obici et al., 2002). In humans, intranasal administration of insulin not only increased insulin in CSF without affecting its plasma levels (Born et al., 2002), but also reduced food intake and body weight (Hallschmid et al., 2004a, 2004b). The effects of centrally administered insulin on feeding and body weight seem confined to the brain. Neither insulin levels nor its receptors in the brain were influenced by its peripheral levels (Havrankova et al., 1979; Oomura and Kita, 1981), although persistent rise in plasma insulin levels down-regulated insulin transporter receptors in BBB and thereby reduced its entry into the brain (Banks, 2004; Watson and Craft, 2006). Centrally administered insulin reduced food intake with a longer latency, and therefore ruled out the interference of any peripheral effects like hypoglycemia (Plata-Salaman and Oomura, 1986). Viewed

collectively, the data suggest anorectic and weight loss promoting effects of the central insulin system.

ARC neurons play a critical role in processing of energy status-related information. Some of the major energy status-regulating agents like neuropeptide Y (NPY), agouti-related peptide (AgRP), pro-opiomelanocortin and CART are secreted by the neurons of this nucleus. Moreover, receptors for peripheral agents like insulin and leptin, that encode the energy available in the periphery, are abundantly expressed on the neurons of ARC (Konturek et al., 2005). In the current investigation, we focused on neuropeptide CART. The peptide is synthesized in ARC neurons and transported to other areas like the PVN, DMH, VMH and LH to regulate feeding and body weight (Fekete et al., 2004; Elias et al., 1998, 1999; Wynne et al., 2005). The present immunohistochemical study revealed that insulin treatment significantly increased the CART immunoreactivity in the fibers of DMH, VMH and PVN, and in the cells of PVN and ARC. It may be noted that the DMH, VMH and PVN are innervated by the CART fibers primarily from the ARC neurons (Wynne et al., 2005). Increased CART immunoreactivity in the DMH, VMH and PVN fibers might be attributed to increased synthesis and mobilization of CART by insulin in the ARC neurons. Central injection of insulin up-regulated the CART mRNA levels in the hypothalamus of chicks (Honda et al., 2007) and CART peptide in the entopeduncular nucleus of catfish (Subhedar et al., 2011). Our data do not address the cellular mechanism by which insulin might modify the hypothalamic CART gene expression. However, it may be noted that insulin receptors are abundantly present on the neurons of the hypothalamus (Corp et al., 1986; Havrankova et al., 1978b), and hence, the action of insulin to increase CART levels are likely to be mediated via its own receptors. Since hypothalamic CART activity is inversely associated with feeding behavior (Nakhate et al., 2011; Stanley et al., 2001; Tian et al., 2005; Wang et al., 2000), increased CART signaling by insulin might be reflected as anorexia and weight loss. Both the effects of insulin were potentiated by pre-treatment with CART. Therefore, we suggest that the anorectic action of insulin may be mediated by the hypothalamic CART system.

It is generally acknowledged that peripheral insulin enters the brain (Banks, 2004; Woods et al., 2006) through insulin receptor-facilitated transcytosis in BBB (Banks et al., 1997; Baura et al., 1993) or via the circumventricular regions that lack BBB (Baranowska-Bik and Bik, 2017). Insulin receptors present in the ARC mediate the effects of peripheral insulin on feeding and energy balance (Hewson et al., 2002). It is also worth noting that insulin increased the expression of CART in the ARC (Kleinridders et al., 2014). Therefore, possibility exists that CART may be involved in mediating the effects of insulin, either of central or peripheral origin, on satiety and energy expenditure.

In the present study, CART immunoreactivity in the LH did not change following icv insulin treatment. It may be noted that, intra-LH infusions of insulin did not influence the food intake in rats (McGowan et al., 1990). The LH has relatively less insulin binding sites as compared to other hypothalamic regions (Corp et al., 1986). This might be a reason for the non-responsiveness of LH CART system to the insulin treatment. We suggest that CART in the LH may not be involved in conveying the inhibitory influence of insulin on the food intake and body weight.

As discussed above, increased CART activity seems to contribute to the anorectic and weight reducing effects of insulin. However, Fekete et al. (2006) reported that reduced CART mRNA expression in the ARC of rats following 3 days fasting remained unaffected by concomitant icv infusion of insulin. In this context, some alternative possibilities may be suggested. Fasting produces a condition of negative energy balance that reduced CART expression as a normal homeostatic response (Adam et al., 2002; Bertile et al., 2003; Nakhate et al., 2010). During a negative energy state, rats did not respond to exogenously administered CART (Nakhate et al., 2010). Therefore, in the fasted rats, injection of insulin might not affect the reduced CART expression. It is also possible that as a consequence of negative energy state, CART release may be prevented by axonal projections from ARC neurons, which secondarily might inhibit the CART expression in neuronal cell bodies. This line of argument is supported by the studies demonstrating that genomic activity in neuronal cell bodies is coupled to synaptic neurotransmitter release (Black et al., 1986).

Besides CART, insulin also interacts with other hypothalamic neuropeptides. Orexigenic peptides in the hypothalamus like NPY and AgRP are regulated by insulin via insulin receptors. Expression of NPY and AgRP was repressed by insulin in hypothalamic cloned cell lines (mHypoE-46) through a MAPK MEK/ERK-dependent pathway (Mayer and Belsham, 2009). Lack of insulin signaling in NPY expressing neurons has been reported to increase energy stores (Loh et al., 2017). Intra-PVN administration of long-acting insulin detemir reversed ghrelin-induced food intake and other metabolic effects (Vasselli et al., 2017).

It is noteworthy that insulin acts centrally to increase thermogenesis (Menéndez and Atrens, 1991; Rothwell and Stock, 1988). Similarly, central administration of CART not only stimulated the lipid mobilization from adipose tissues to circulation (Wortley et al., 2004), but also induced the expression of uncoupling proteins in adipose tissues and skeletal muscles (Wang et al., 2000). Therefore, the possibility also exists that CART and insulin may act in synergism to reduce the body mass.

In conclusion, the results of the present study suggest that CART in the hypothalamus may serve as an important neuroanatomical substrate that processes the insulin triggered inhibitory responses on feeding and body weight.

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References

Adam, C.L., Archer, Z.A., Findlay, P.A., Thomas, L., Marie, M., 2002. Hypothalamic gene expression in sheep for cocaine- and amphetamine-regulated transcript, pro-opiomelanocortin, neuropeptide Y, agouti-related peptide and leptin receptor and responses to negative energy balance. Neuroendocrinology 75 (4), 250–256.

Air, E.L., Benoit, S.C., Blake Smith, K.A., Clegg, D.J., Woods, S.C., 2002. Acute third ventricular administration of insulin decreases food intake in two paradigms. Pharmacol. Biochem. Behav. 72 (1–2), 423–429.

Ajoy, R., Chou, S.Y., 2018. Studying the hypothalamic insulin signal to peripheral glucose intolerance with a continuous drug infusion system into the mouse brain. J. Vis. Exp. (131).

Banks, W.A., 2004. The source of cerebral insulin. Eur. J. Pharmacol. 490 (1–3), 5–12.

Banks, W.A., Jaspan, J.B., Kastin, A.J., 1997. Selective, physiological transport of insulin across the blood-brain barrier: novel demonstration by species-specific radioimmunoassays. Peptides 18 (8), 1257–1262.

Baranowska-Bik, A., Bik, W., 2017. Insulin and brain aging. Prz. Menopauzalny 16 (2), 44–46.

Baura, G.D., Foster, D.M., Porte, D.Jr., Kahn, S.E., Bergman, R.N., Cobelli, C., Schwartz, M.W., 1993. Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. J. Clin. Invest. 92 (4), 1824–1830.

Begg, D.P., Woods, S.C., 2012. The central insulin system and energy balance. In: Handb. Exp. Pharmacol. vol. 209. pp. 111–129.

Bertile, F., Oudart, H., Criscuolo, F., Maho, Y.L., Raclot, T., 2003. Hypothalamic gene expression in long-term fasted rats: relationship with body fat. Biochem. Biophys. Res. Commun. 303 (4), 1106–1113.

Bhisikar, S.M., Kokare, D.M., Nakhate, K.T., Chopde, C.T., Subhedar, N.K., 2009. Tolerance to ethanol sedation and withdrawal hyper-excitability is mediated by neuropeptide Y Y1 and Y5 receptors. Life Sci. 85 (21 – 22), 765–772.

Bhorkar, A.A., Dandekar, M.P., Nakhate, K.T., Subhedar, N.K., Kokare, D.M., 2014. Involvement of the central melanocortin system in the effects of caffeine on anxiety-like behavior in mice. Life Sci. 95 (2), 72–80.

Black, I.B., Adler, J.E., LaGamma, E.F., 1986. Impulse activity differentially regulates colocalized transmitters by altering messenger RNA levels. In: Hokfelt, T., Fuxe, K., Pernow, B. (Eds.), Progress in Brain Research. vol. 68. Elsevier Science Publishers, New York, pp. 121–127.

Born, J., Lange, T., Kern, W., McGregor, G.P., Bickel, U., Fehm, H.L., 2002. Sniffing neuropeptides: a transnasal approach to the human brain. Nat. Neurosci. 5 (6), 514–516.

Brief, D.J., Davis, J.D., 1984. Reduction of food intake and body weight by chronic

intraventricular insulin infusion. Brain Res. Bull. 12 (5), 571–575.
Bruckdorfer, K.R., Kang, S.S., Khan, I.H., Bourne, A.R., Yudkin, J., 1974. Diurnal changes in the concentrations of plasma lipids, sugars, insulin and corticosterone in rats fed diets containing various carbohydrates. Horm. Metab. Res. 6 (2), 99–106.
Clegg, D.J., Benoit, S.C., Reed, J.A., Woods, S.C., Dunn-Meynell, A., Levin, B.E., 2005. Reduced anorexic effects of insulin in obesity-prone rats fed a moderate-fat diet. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288 (4), R981–R986.
Corp, E.S., Woods, S.C., Porte Jr., D., Dorsa, D.M., Figlewicz, D.P., Baskln, D.G., 1986. Localization of 125I-insulin binding sites in the rat hypothalamus by quantitative autoradiography. Neurosci. Lett. 70 (1), 17–22.
Crespo, C.S., Cachero, A.P., Jiménez, L.P., Barrios, V., Ferreiro, E.A., 2014. Peptides and food intake. Front. Endocrinol. 5, 58.
Dandekar, M.P., Nakhate, K.T., Kokare, D.M., Subhedar, N.K., 2011. Effect of nicotine on feeding and body weight in rats: involvement of cocaine- and amphetamine-regulated transcript peptide. Behav. Brain Res. 219 (1), 31–38.
Dandekar, M.P., Nakhate, K.T., Kokare, D.M., Subhedar, N.K., 2012. Involvement of CART in estradiol induced anorexia. Physiol. Behav. 105 (2), 460–469.
Desai, S.J., Borkar, C.D., Nakhate, K.T., Subhedar, N.K., Kokare, D.M., 2014. Neuropeptide Y attenuates anxiety- and depression-like effects of cholecystokinin-4 in mice. Neuroscience 277, 818–830.
Elias, C.F., Lee, C., Kelly, J., Aschkenasi, C., Ahima, R.S., Couceyro, P.R., Kuhar, M.J., Saper, C.B., Elmquist, J.K., 1998. Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron 21 (6), 1375–1385.
Elias, C.F., Aschkenasi, C., Lee, C., Kelly, J., Ahima, R.S., Bjorbaek, C., Flier, J.S., Saper, C.B., Elmquist, J.K., 1999. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. Neuron 23 (4), 775–786.
Fekete, C., Wittmann, G., Liposits, Z., Lechan, R.M., 2004. Origin of cocaine- and amphetamine-regulated transcript (CART)-immunoreactive innervation of the hypothalamic paraventricular nucleus. J. Comp. Neurol. 469 (3), 340–350.
Fekete, C., Singru, P.S., Sanchez, E., Sarkar, S., Christoffolete, M.A., Riberio, R.S., Rand, W.M., Emerson, C.H., Bianco, A.C., Lechan, R.M., 2006. Differential effects of central leptin, insulin, or glucose administration during fasting on the hypothalamic-pituitary-thyroid axis and feeding-related neurons in the arcuate nucleus. Endocrinology 147 (1), 520–529.
Hallschmid, M., Benedict, C., Born, J., Fehm, H.L., Kern, W., 2004a. Manipulating central nervous mechanisms of food intake and body weight regulation by intranasal administration of neuropeptides in man. Physiol. Behav. 83 (1), 55–64.
Hallschmid, M., Benedict, C., Schultes, B., Fehm, H.L., Born, J., Kern, W., 2004b. Intranasal insulin reduces body fat in men but not in women. Diabetes 53 (1), 3024–3029.
Havrankova, J., Schmechel, D., Roth, J., Brownstein, M., 1978a. Identification of insulin in rat brain. Proc. Natl. Acad. Sci. U. S. A. 75 (11), 5737–5741.
Havrankova, J., Roth, J., Browstein, M., 1978b. Insulin receptors are widely distributed in the central nervous system of the rat. Nature 272 (5656), 827–829.
Havrankova, J., Roth, J., Brownstein, M., 1979. Concentrations of insulin and of insulin receptors in the brain are independent of peripheral insulin levels: studies of obese and streptozotocin-treated rodents. J. Clin. Invest. 64 (2), 636–642.
Havrankova, J., Brownstein, M., Roth, J., 1981. Insulin and insulin receptors in the rodent brain. Diabetologia 20 (1), 268–273.
Hewson, A.K., Tung, L.Y.C., Connell, D.W., Tookman, L., Dickson, S.L., 2002. The rat arcuate nucleus integrates peripheral signals provided by leptin, insulin, and a ghrelin mimetic. Diabetes 51 (12), 3412–3419.
Honda, K., Kamisoyama, H., Saneyasu, T., Sugahara, K., Hasegawa, S., 2007. Central administration of insulin suppresses food intake in chicks. Neurosci. Lett. 423 (2), 153–157.
Ikeda, H., West, D.B., Pustek, J.J., Figlewicz, D.P., Greenwood, M.R.C., Porte, D.Jr., Woods, S.C., 1986. Intraventricular insulin reduces food intake and body weight of lean but not obese Zucker rats. Appetite 7 (4), 381–386.
Kamdi, S.P., Nakhate, K.T., Dandekar, M.P., Kokare, D.M., Subhedar, N.K., 2009. Participation of corticotropin-releasing factor type 2 receptors in the acute, chronic and withdrawal actions of nicotine associated with feeding behavior in rats. Appetite 53 (3), 354–362.
Keen-Rhinehart, E., Desai, M., Ross, M.G., 2009. Central insulin sensitivity in male and female juvenile rats. Horm. Behav. 56 (3), 275–280.
Kimura, T., Maji, T., Ashida, K., 1970. Periodicity of food intake and lipogenesis in rats subjected to two different feeding plans. J. Nutr. 100 (6), 691–697.
Kleinridders, A., Ferris, H.A., Cai, W., Kahn, C.R., 2014. Insulin action in brain regulates systemic metabolism and brain function. Diabetes 63 (7), 2232–2243.
Kobayashi, Y., Peterson, B.C., Waldbieser, G.C., 2008. Association of cocaine- and amphetamine-regulated transcript (CART) messenger RNA level, food intake, and growth in channel catfish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 151 (2), 219–225.
Kokare, D.M., Shelkar, G.P., Borkar, C.D., Nakhate, K.T., Subhedar, N.K., 2011. A simple and inexpensive method to fabricate a cannula system for intracranial injections in rats and mice. J. Pharmacol. Toxicol. Methods 64 (3), 246–250.
Konturek, P.C., Konturek, J.W., Czes'nikiewicz-Guzik, M., Brzozowski, T., Sito, E., Konturek, S.J., 2005. Neuro-hormonal control of food intake: basic mechanisms and clinical implications. J. Physiol. Pharmacol. 56 (6), 5–25.
Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., Larsen, P.J., Hastrup, S., 1998. Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393 (6680), 72–76.
Loh, K., Zhang, L., Brandon, A., Wang, Q., Begg, D., Qi, Y., Fu, M., Kulkarni, R., Teo, J., Baldock, P., Brüning, J.C., Cooney, G., Neely, G.G., Herzog, H., 2017. Insulin controls food intake and energy balance via NPY neurons. Mol. Metab. 6 (6), 574–584.
Madadi, G., Dalvi, P.S., Belsham, D.D., 2008. Regulation of brain insulin mRNA by glucose and glucagon-like peptide 1. Biochem. Biophys. Res. Commun. 376 (4), 694–699.
Mayer, C.M., Belsham, D.D., 2009. Insulin directly regulates NPY and AgRP gene expression via the MAPK MEK/ERK signal transduction pathway in mHypoE-46 hypothalamic neurons. Mol. Cell. Endocrinol. 307 (1–2), 99–108.
McGowan, M.K., Andrews, K.M., Kelly, J., Grossman, S.P., 1990. Effects of chronic intrahypothalamic infusion of insulin on food intake and diurnal meal patterning in the rat. Behav. Neurosci. 104 (2), 373–385.
McGowan, M.K., Andrews, K.M., Grossman, S.P., 1992. Chronic intrahypothalamic infusions of insulin or insulin antibodies alter body weight and food intake in the rat. Physiol. Behav. 51 (4), 753–766.
Meena, H., Nakhate, K.T., Kokare, D.M., Subhedar, N.K., 2009. GABAA receptors in nucleus accumbens shell mediate the hyperphagia and weight gain following haloperidol treatment in rats. Life Sci. 84 (5–6), 156–163.
Mehran, A.E., Templeman, N.M., Brigidi, G.S., Lim, G.E., Chu, K.Y., Hu, X., Botezelli, J.D., Asadi, A., Hoffman, B.G., Kieffer, T.J., Bamji, S.X., Clee, S.M., Johnson, J.D., 2012. Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. Cell Metab. 16 (6), 723–737.
Menéndez, J.A., Atrens, D.M., 1991. Insulin and the paraventricular hypothalamus: modulation of energy balance. Brain Res. 555 (2), 193–201.
Molnár, G., Faragó, N., Kocsis, Á.K., Rózsa, M., Lovas, S., Boldog, E., Báldi, R., Csajbók, É., Gardi, J., Puskás, L.G., Tamás, G., 2014. GABAergic neurogliaform cells represent local sources of insulin in the cerebral cortex. J. Neurosci. 34 (4), 1133–1137.
Nakhate, K.T., Dandekar, M.P., Kokare, D.M., Subhedar, N.K., 2009. Involvement of neuropeptide Y Y (1) receptors in the acute, chronic and withdrawal effects of nicotine on feeding and body weight in rats. Eur. J. Pharmacol. 609 (1–3), 78–87.
Nakhate, K.T., Kokare, D.M., Singru, P.S., Taksande, A.G., Kotwal, S.D., Subhedar, N.K., 2010. Hypothalamic cocaine- and amphetamine-regulated transcript peptide is reduced and fails to modulate feeding behavior in rats with chemically-induced mammary carcinogenesis. Pharmacol. Biochem. Behav. 97 (2), 340–349.
Nakhate, K.T., Kokare, D.M., Singru, P.S., Subhedar, N.K., 2011. Central regulation of feeding behavior during social isolation of rat: evidence for the role of endogenous CART system. Int. J. Obesity 35 (6), 773–784.
Nakhate, K.T., Subhedar, N.K., Bharne, A.P., Singru, P.S., Kokare, D.M., 2013. Involvement of cocaine- and amphetamine-regulated transcript peptide in the hyperphagic and body weight promoting effects of allopregnanolone in rats. Brain Res. 1532, 44–55.
Nakhate, K.T., Yedke, S.U., Bharne, A.P., Subhedar, N.K., Kokare, D.M., 2016. Evidence for the involvement of neuropeptide Y in the antidepressant effect of imipramine in type 2 diabetes. Brain Res. 1646, 1–11.
Nakhate, K.T., Bharne, A.P., Verma, V.S., Aru, D.N., Kokare, D.M., 2018a. Plumbagin ameliorates memory dysfunction in streptozotocin induced Alzheimer's disease via activation of Nrf2/ARE pathway and inhibition of β-secretase. Biomed. Pharmacother. 101, 379–390.
Nakhate, K.T., Subhedar, N.K., Kokare, D.M., 2018b. A role of neuropeptide CART in hyperphagia and weight gain induced by olanzapine treatment in rats. Brain Res. 1695, 45–52.
Niswender, K.D., Morrison, C.D., Clegg, D.J., Olson, R., Baskin, D.G., Myers Jr., M.G., Seeley, R.J., Schwartz, M.W., 2003. Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. Diabetes 52 (2), 227–231.
Obici, S., Feng, Z., Karkanias, G., Baskin, D.G., Rossetti, L., 2002. Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. Nat. Neurosci. 5 (6), 566–572.
Oomura, Y., Kita, H., 1981. Insulin acting as a modulator of feeding through the hypothalamus. Diabetologia 20 (1), 290–298.
Pardini, A.W., Nguyen, H.T., Figlewicz, D.P., Baskin, D.G., Williams, D.L., Kim, F., Schwartz, M.W., 2006. Distribution of insulin receptor substrate-2 in brain areas involved in energy homeostasis. Brain Res. 1112 (1), 169–178.
Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates. Academic Press, London.
Plata-Salaman, C.R., Oomura, Y., 1986. Effect of intra-third ventricular administration of insulin on food intake after food deprivation. Physiol. Behav. 37 (5), 735–739.
Rangani, R.J., Upadhya, M.A., Nakhate, K.T., Kokare, D.M., Subhedar, N.K., 2012. Nicotine evoked improvement in learning and memory is mediated through NPY Y1 receptors in rat model of Alzheimer's disease. Peptides 33 (2), 317–328.
Rothwell, N.J., Stock, M.J., 1988. Insulin and thermogenesis. Int. J. Obes. 12 (2), 93–102.
Schwartz, M.W., Sipols, A.J., Marks, J.L., Sanacora, G., White, J.D., Scheurink, A., Kahn, S.E., Baskin, D.G., Woods, S.C., Figlewicz, D.P., Porte, D. Jr., 1992. Inhibition of hypothalamic neuropeptide y gene expression by insulin. Endocrinology 130 (6), 3608–3616.
Stanley, S.A., Small, C.J., Murphy, K.G., Rayes, E., Abbott, C.R., Seal, L.J., Morgan, D.G., Sunter, D., Dakin, C.L., Kim, M.S., Hunter, R., Kuhar, M., Ghatei, M.A., Bloom, S.R., 2001. Actions of cocaine- and amphetamine-regulated transcript (CART) peptide on regulation of appetite and hypothalamo-pituitary axes *in vitro* and *in vivo* in male rats. Brain Res. 893 (1–2), 186–194.
Strubbe, J.H., Mein, C.G., 1977. Increased feeding in response to bilateral injection of insulin antibodies in the VMH. Physiol. Behav. 19 (2), 309–313.
Subhedar, N., Barsagade, V.G., Singru, P.S., Thim, L., Clausen, J.T., 2011. Cocaine- and amphetamine-regulated transcript peptide (CART) in the telencephalon of the catfish, Clarias gariepinus: distribution and response to fasting, 2-deoxy-D-glucose, glucose, insulin, and leptin treatments. J. Comp. Neurol. 519 (7), 1281–1300.
Subhedar, N.K., Nakhate, K.T., Upadhya, M.A., Kokare, D.M., 2014. CART in the brain of vertebrates: circuits, functions and evolution. Peptides 54, 108–130.
Taksande, B.G., Kotagale, N.R., Nakhate, K.T., Mali, P.D., Kokare, D.M., Hirani, K., Subhedar, N.K., Chopde, C.T., Ugale, R.R., 2011. Agmatine in the hypothalamic

paraventricular nucleus stimulates feeding in rats: involvement of neuropeptide Y. Br. J. Pharmacol. 164 (2b), 704–718.
Tian, D.R., Li, X.D., Wang, F., Niu, D.B., He, Q.H., Li, Y.S., Chang, J.K., Yang, J., Han, J.S., 2005. Up-regulation of the expression of cocaine and amphetamine-regulated transcript peptide by electroacupuncture in the arcuate nucleus of diet induced obese rats. Neurosci. Lett. 383 (1–2), 17–21.
Upadhya, M.A., Nakhate, K.T., Kokare, D.M., Singru, P.S., Subhedar, N.K., 2011. Cocaine- and amphetamine-regulated transcript peptide increases spatial learning and memory in rats. Life Sci. 88 (7–8), 322–334.
Upadhya, M.A., Nakhate, K.T., Kokare, D.M., Singh, U., Singru, P.S., Subhedar, N.K., 2012. CART peptide in the nucleus accumbens shell acts downstream to dopamine and mediates the reward and reinforcement actions of morphine. Neuropharmacology 62 (4), 1823–1833.
Vasselli, J.R., Pi-Sunyer, F.X., Wall, D.G., John, C.S., Chapman, C.D., Currie, P.J., 2017. Central effects of insulin detemir on feeding, body weight, and metabolism in rats. Am. J. Physiol. Endocrinol. Metab. 313 (5), E613–E621.
Volkoff, H., Peter, R.E., 2000. Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, Carassius auratus: actions on neuropeptide Y- and orexin A-induced feeding. Brain Res. 887 (1), 125–133.
Wang, C., Billington, C.J., Levine, A.S., Kotz, C.M., 2000. Effect of CART in the hypothalamic paraventricular nucleus on feeding and uncoupling protein gene expression. Neuroreport 11 (14), 3251–3255.
Watson, G.S., Craft, S., 2006. Insulin resistance, inflammation, and cognition in Alzheimer's disease: lessons for multiple sclerosis. J. Neurol. Sci. 245 (1–2), 21–33.
Woods, S.C., Lotter, E.C., McKay, L.D., Porte, D. Jr., 1979. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature 282 (5738), 503–505.
Woods, S.C., Lutz, T.A., Geary, N., Langhans, W., 2006. Pancreatic signals controlling food intake; insulin, glucagon and amylin. Phil. Trans. R. Soc. B. 361 (1471), 1219–1235.
Wortley, K.E., Chang, G.Q., Davydova, Z., Fried, S.K., Leibowitz, S.F., 2004. Cocaine- and amphetamine-regulated transcript in the arcuate nucleus stimulates lipid metabolism to control body fat accrual on a high-fat diet. Regul. Pept. 117 (2), 89–99.
Wynne, K., Stanley, S., McGowan, B., Bloom, S., 2005. Appetite control. J. Endocrinol. 184 (2), 291–318.
Young, W.S., 1986. Periventricular hypothalamic cells in the rat brain contain insulin mRNA. Neuropeptides 8 (4), 93–97.
Zhu, P., Zhang, Z., Huang, X., Liang, S., Khandekar, N., Song, Z., Lin, S., 2018. RANKL reduces body weight and food intake via the modulation of hypothalamic NPY/CART expression. Int. J. Med. Sci. 15 (10), 969–977.