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# Hypothalamic cocaine- and amphetamine-regulated transcript peptide is reduced and fails to modulate feeding behavior in rats with chemically-induced mammary carcinogenesis

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ABSTRACT

Cocaine- and amphetamine-regulated transcript peptide (CART) is a major anorectic agent present in the hypothalamus. We investigated the possible role of CART in mammary cancer-induced anorexia and body weight loss in rats. Mammary carcinogenesis was induced in the female Sprague-Dawley rats by intraperitoneal injection of N-methyl-N-nitrosourea (MNU). Following administration of MNU, rats progressively showed a reduction in food intake and body weight. Fourteen weeks after MNU treatment, rats were injected daily with CART or CART-antibody intracerebroventricularly for 5 days, and food intake and body weight were monitored (g) before the next injection time-point. In normal rats, while a distinct anorexia and weight loss was observed following CART administration, injection of CART-antibody produced opposite effects. However, both the agents failed to produce any significant alterations in food intake and body weight of mammary tumor-bearing animals. An immunohistochemical application of antibodies against CART to the brain sections of cancerous rats showed a reduced immunoreactivity in the hypothalamic dorsomedial, ventromedial, lateral, paraventricular and arcuate nuclei. The results suggest that, cancerous condition might down-regulate the CART system in the hypothalamus. Alternatively, reduction in hypothalamic CART activity might be a counter-regulatory strategy to reverse food under-consumption or body mass erosion.



## 1. Introduction

Cachexia represents a state of negative energy balance, characterized by anorexia and body weight loss (Lelbach et al., 2007). It is one of the devastating manifestations of cancerous states, which increases the morbidity of underlying diseases, complicates the treatment and is accountable for the 20-40% of deaths (Knapp et al., 1991; Bruera, 1997; Tisdale, 2002; Fox et al., 2009). The clinical features of cachexia include severe anorexia, body weight loss, muscle loss and metabolic alterations (Giordano and Jatoi, 2005; Argiles et al., 2006; Lelbach et al., 2007). Patients with colorectal, pancreatic, liver, gastric, lung, head/neck or breast cancer often shows the features of cachexia (Kim and Depowski, 1975; Dewys et al., 1980; Bruera, 1997; O'Gorman et al., 1999; Fox et al., 2009). However, no effective therapy is available to treat the cancer-induced cachexia (Bruera, 1997; Vigano et al., 2004).

Numerous studies suggested the possible role of both central and peripheral regulators of energy homeostasis in the animal models of cancer anorexia (McCarthy et al., 1993; Inui, 1999; Plata-Salaman, 2000; Wisse et al., 2001; Kumar et al., 2003; DeBoer et al., 2007). Cocaine- and amphetamine-regulated transcript peptide (CART) has emerged as an important hypothalamic anorectic agent (Hunter et al., 2004; Murphy, 2005; Rogge et al., 2008). In rats, intracerebroventricular (i.c.v.) injection of CART-antibody caused an increase in food intake, whereas a suppression of normal, starvation or neuropeptide Y (NPY)-induced feeding was noticed following CART administration (Kristensen et al., 1998; Lambert et al., 1998; Vrang et al., 1999). Moreover, chronic CART infusion produced a sustained inhibitory effect on food intake and body weight in normal and obese rats (Larsen et al., 2000; Rohner-Jeanrenaud et al., 2002). However, information pertaining to the involvement of hypothalamic CART in the cancer-induced cachexia is much limited. Hashimoto et al. (2007) reported a decreased CART mRNA in hypothalamic arcuate nucleus (ARC) of rats with the humoral hypercalcemia of malignancy.

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However, the involvement of other CART containing hypothalamic nuclei like the dorsomedial (DMH), ventromedial (VMH), lateral (LH), paraventricular (PVN) and periventricular (PeA), if any, has not been studied.

The present investigation was undertaken to define the role of hypothalamic CART system in anorexia and weight loss in rats with mammary gland tumors. The mammary carcinogenesis was induced in female rats by intraperitoneal (i.p.) administration of chemical carcinogen, N-methyl-N-nitrosourea (MNU), at 50 days of age (Thompson and Adlakha, 1991). Fourteen weeks after MNU administration, rats were injected daily with CART (54-102) or CART-antibody via i.c.v. route over a period of 5 days, and food intake and body weight were monitored (g). Since cachexia is known to produce malnutrition (Fouladiun et al., 2005; Bovio et al., 2008; Krzystek-Korpacka et al., 2008), inhibition of albumin gene expression (Brenner et al., 1990) and reduction in synthesis of fatty acids/triglycerides (Kumar et al., 2003), we also monitored the levels of plasma glucose, triglycerides, cholesterol, total proteins and albumin in cancerous rats. Furthermore, in mammary tumor-bearing rats, the CART system was investigated in different hypothalamic nuclei like DMH, VMH, LH, PVN, ARC and PeA using the immunohistochemistry technique. These neuroanatomical areas were chosen since they contain an abundance of the CART (Koylu et al., 1997; Vrang, 2006, Dandekar et al., 2008a), and are mainly involved in the processing of feeding-related signals (Lambert et al., 1998; Kalra et al., 1999; Konturek et al., 2005).

## 2. Materials and Methods

### 2.1. Animals

Fifty days old female Sprague-Dawley rats were obtained from the animal house of Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, MS, India. The animals were group housed in acrylic cages under constant room temperature (25 ± 2 °C), relative humidity (50 ± 5%), and maintained under a controlled 12:12 h light-dark cycle (lights on at 0000 h). Food and water were available *ad libitum*. All procedures employed in the present study 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. Induction of mammary tumorigenesis

The procedure described by Thompson and Adlakha (1991) was employed. MNU (Sigma, St. Louis, MO, USA) was dissolved in cold saline (4 °C, 0.9% NaCl), pH adjusted to 4.0 with acetic acid (0.05%) to increase its stability, and injected to 50 days old rats. MNU solution was kept on ice and protected from the light during injection to prevent breaking down and was used within 30 min after its preparation. Rats receiving i.p. MNU, at 50 mg/kg dose, did not show any sign of acute toxicity. Control animals were injected with 0.9% saline. Food intake (g/day) and body weight (g) were measured at weekly intervals to monitor the signs of cachexia. Moreover, MNU treated rats were also palpated each week to monitor the incidence of mammary tumors. No MNU-related mortality was noticed throughout the experiment. During necropsy, mammary tissues of control and MNU treated rats were fixed in Bouin's fixative and processed for routine histology employing hematoxylin and eosin staining.

### 2.3. Measurement of the effect of mammary tumorigenesis on the plasma glucose, triglycerides, cholesterol, total proteins and albumin contents

Fourteen weeks after saline or MNU treatment, the blood samples were obtained by tail vein bleeding of normal and mammary gland tumor-bearing rats ($n = 6$/group). Concentrations of glucose (mg/dl) were measured by the glucose oxidase-peroxidase method. Triglycerides and cholesterol were determined (mg/dl) by the enzymatic colorimetry method. Total serum proteins and albumin levels were measured (g/dl) by the Biuret method.

### 2.4. Measurement of the effect of mammary tumorigenesis on hypothalamic CART-immunoreactivity

The brain sections of rats with mammary tumors (14 weeks after MNU treatment) and saline injected control animals were employed for immunostaining ($n = 6$ per group) as described previously (Singru et al., 2007; Dandekar et al., 2008b). Briefly, rats were deeply anesthetized with i.p. thiopentone sodium (60 mg/kg; Abbott Pharmaceuticals, Mumbai, India), perfused transcardially with heparinized phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were post-fixed in the same fixative for overnight and then cryoprotected in 30% sucrose solution in PBS at 4 °C. After that, the brains were embedded in polyvinyl-pyrrolidone, serially sectioned in the coronal plane at 30 μm thickness using a cryostat (Leica, CM1850) at -28 °C and collected in PBS. The brain sections were washed three times (10 min each) in PBS and incubated in mouse monoclonal antibodies against CART (54-102) diluted in PBS (1:5000) containing 2% normal horse serum, 0.3% Triton X-100, 0.2% Kodak PhotoFlo solution and 0.08% sodium azide for 48 h at 4 °C on shaker. The sections were washed in PBS and incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA; 1:100) for 2 h at room temperature. The sections were then washed in PBS, incubated in ExtrAvidin-peroxidase conjugate (Sigma; 1:100) for 45 min at room temperature and again washed with PBS. For visualization of the antigen-antibody complex, the sections were incubated for 3 min in a solution containing 0.03% hydrogen peroxide that served as substrate and 3-amino-9-ethyl-carbazole (AEC; Sigma) as chromogen. Reddish-brown precipitate indicated the presence of antigen in the sections. The sections were washed with double distilled water, taken on glass slides and mounted in glycerol-gelatin.

### 2.5. Specificity of the antibodies

The monoclonal antibodies against CART (54-102) employed in the present study were generated by Thim et al. (1998), and were found to react equally well with the CART (54-102), CART (61-102), and CART (62-102) peptides. To ensure reliable comparisons among different groups and maintain stringency in tissue preparation and staining conditions, sections from the brains of 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 preadsorption controls, application of 1 ml diluted antibody pre-incubated with CART at $10^{-5}$ M for 24 h completely blocked the immunoreaction. This procedure has already been standardized in our laboratory (Singru et al., 2007; Dandekar et al., 2008b).

### 2.6. Relative quantitative analysis of CART-immunoreactivity

Brain sections showing CART-immunoreactivity in the DMH, VMH, LH, PVN, ARC and PeA of control and tumor-bearing rats were subjected to the morphometric analysis (Singru et al., 2007; Dandekar et al., 2008b). The images (X 480) were captured using Leica Leitz-LaborLux S microscope and analyzed with Leica QWin Standard software (version 3.1.0). The imaging system was adjusted to predetermined settings on the microscope so that the objective, light intensity, openings of the condenser and base diaphragm, centering of light and condenser on the specimen, and condenser height were all used at constant settings. Neural areas of interest were

first identified in all the CART-immunostained sections. The background of non-immunoreactive area in the section was digitized and considered as threshold. The area occupied by immunostained product, above the threshold, was filled with overlaid color and measured based on the pixel intensity in a given unit area, which subsequently presented as percentage of the area in the evaluated field. While evaluating immunoreactivity in the CART-containing somata in the given field, the area covered by the immunoreactive fibers was not considered. Similarly, during measurement of immunoreactivity in the CART-containing fibers, the immunoreactive cells area was not taken. Five measurements of the percentage were taken from predetermined fields for each sub-region on either side from each brain. The data from all animals in each group were pooled separately for each brain region and the mean ± standard error of mean (SEM) was calculated.

### 2.7. I.c.v. cannulation

Twelve weeks after saline or MNU administration, control or tumor-bearing rats were subjected to the stereotaxic surgery to administer artificial cerebrospinal fluid (aCSF), CART or CART-antibody into the lateral ventricle. The detailed procedure of i.c.v. cannulation, drug administration and post-surgical care has been described earlier (Rao et al., 2003; Goyal et al., 2006; Kokare et al., 2006). Briefly, animals were anaesthetized with i.p. thiopentone sodium (45 mg/kg), and a 24-guage stainless steel guide cannula (C316G/Spc; Plastics One, Roanoke, VA, USA) was implanted into the right lateral ventricle using 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). Three stainless steel screws were fitted to the skull, and the guide cannula was fixed in place using dental cement applied around the screws and cannula. A stainless steel flush-fitting dummy cannula (C316DC/Spc; Plastics One) was inserted into the guide cannula to prevent blockage. After cannulation, animals were housed individually and allowed to recover for 7 days. During this period, those rats showing any neurological or motor deficits like impairment of locomotion, grooming, social interaction or occurrence of aggressiveness, handling-induced hyper-excitability and stereotype behavior were not included in the study (Goyal et al., 2006; Meena et al., 2009). Each rat was singly housed throughout the experimentation. I.c.v. injection of 5 μl volume

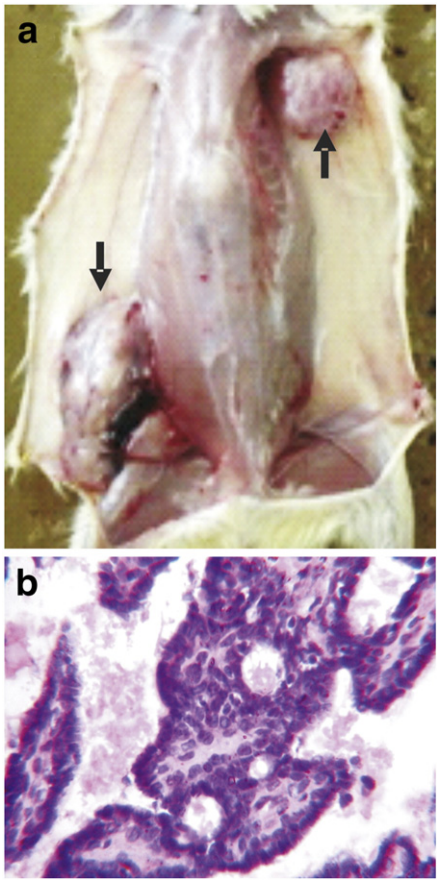



**Fig. 1.** Photograph showing N-methyl-N-nitrosourea-induced mammary gland tumors (arrows) (a), and histoarchitecture of mammary gland tumor (b) of an experimental rat.

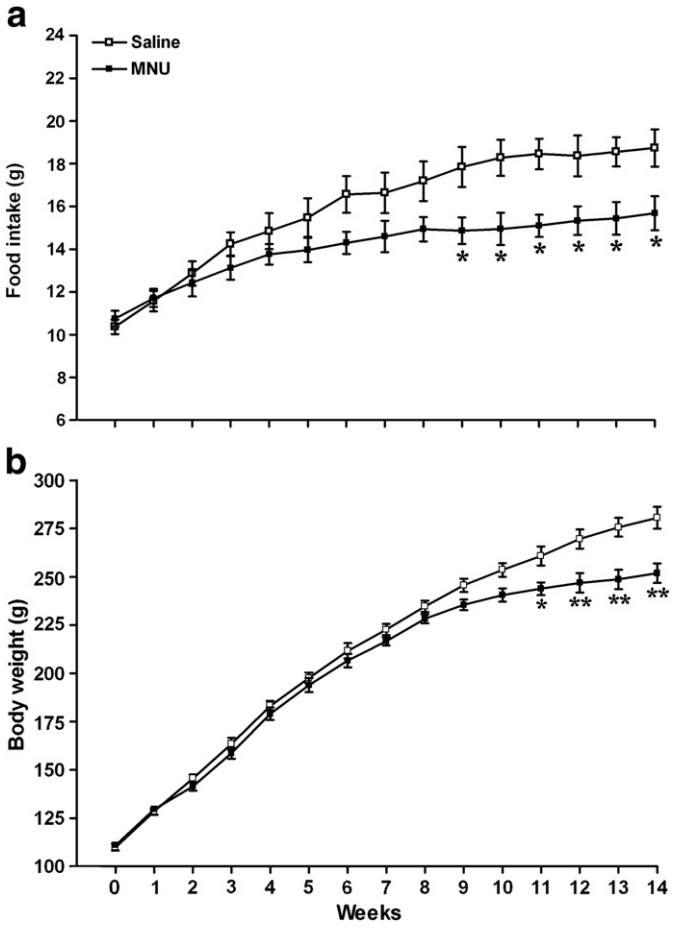



**Fig. 2.** Effect of saline and N-methyl-N-nitrosourea (MNU) treatments on food intake (a) and body weight (b). The i.p. injections of saline and MNU (50 mg/kg) were given to the different groups of 50 days old female Sprague-Dawley rats. Thereafter, changes in food intake (g/day) and body weight (g) were measured at 7 days interval for 14 weeks. Each line and bar represents mean ± SEM ($n = 8$/group). The data were analyzed by two-way ANOVA followed by a post-hoc Bonferroni's multiple comparison test. $^{*}p<0.05$, $^{**}p<0.001$ *vs* saline.

**Table 1**
Effect of mammary gland carcinogenesis on blood glucose, triglycerides, cholesterol, total proteins and albumin contents.

| Plasma constituents | Control | Tumor-bearing |
|---|---|---|
| Glucose (mg/dl) | 117.16 ± 3.17 | 110.33 ± 4.06 |
| Triglycerides (mg/dl) | 163.33 ± 6.97 | 92.50 ± 2.37* |
| Cholesterol (mg/dl) | 100.66 ± 4.03 | 76.83 ± 2.86* |
| Total Proteins (g/dl) | 14.91 ± 0.69 | 9.84 ± 0.43* |
| Albumin (g/dl) | 5.13 ± 0.24 | 2.91 ± 0.26* |

Fourteen weeks after saline or MNU treatment, the blood samples were obtained by tail vein bleeding of control and cancerous rats. Concentrations of glucose were measured by the glucose oxidase-peroxidase method. Triglycerides and cholesterol were determined by the enzymatic colorimetry method. Total serum proteins and albumin levels were measured by the Biuret method. The values are given as mean ± SEM ($n = 6$/group). The data were analyzed by unpaired *t*-test. $^{*}p<0.001$ *vs* control.

was given to each rat using microliter syringe (Hamilton, Nevada) connected by polyethylene tubing to a 31-guage internal cannula (C316I/Spc, inner diameter 0.12 mm and outer diameter 0.25 mm; Plastics One) that projected 0.5 mm below the guide cannula. Before administration, the syringe and the tubing were filled with double distilled water and a small air bubble was introduced to separate the infused solution from the double distilled water. The movement of air bubble inside the tubing also confirmed the precise flow of the solution during injection.

### 2.8. Measurement of food intake and body weight following i.c.v. administration of CART and CART-antibody in normal and tumor-bearing rats

Prior to the initiation of treatments, cannulated rats were acclimatized to the testing environment for 7 days to minimize the nonspecific stress. Daily, the animals were weighed, restrained on platform, dummy cannula was removed from guide cannula, and aCSF (5 μl) was administered slowly over the period of 1 min using internal cannula. Following microinjection, an internal cannula was kept in place for additional minute to promote diffusion and to prevent the back-flow of the fluid during removal of the injection cannula, and finally dummy cannula was replaced. The same platform was used for the administration of drugs. The injections were given 10 min prior to the onset of the dark phase, and immediately the pre-weighed food pellets were placed inside the cage hopper. The food consumed by rats was quantified by weighing leftover food in the hopper. This procedure has already been standardized in our laboratory (Kokare et al., 2006; Kamdi et al., 2009; Meena et al., 2009; Nakhate et al., 2009). Normal and tumor-bearing rats were divided into different groups and subjected to the feeding studies as described below.

Different groups of normal and mammary gland tumor-bearing rats ($n = 7$-8 per group) were administered daily, 10 min prior to the onset of the dark phase, with aCSF (5 μl/rat, i.c.v.), CART (1 μg/rat, i.c.v.) or CART-antibody (1:500 dilution, 5 μl/rat, i.c.v.) for a period of 5 days. Immediately after the treatment, food pellets were offered, and food intake and body weight were recorded (g) after an interval of 24 h, just prior to the next injection.

### 2.9. Cannula placement verification

At the end of all the experiments, placement of the guide cannula was tested for accuracy (Kokare et al., 2005, Bhisikar et al., 2009). Dilute India ink (5 μl) was injected by i.c.v. route and animals were euthanized by overdose of thiopentone sodium (65 mg/kg, i.p.). Immediately thereafter, the brains were dissected out, cut in coronal plane to verify the placement of guide cannula and distribution of ink in the ventricle. The data of animals with incorrect placement were excluded from the study.

### 2.10. Statistical analyses

The data are presented as mean ± SEM. The behavioral data were analyzed by two-way analysis of variance (ANOVA) and individual means were compared by post-hoc Bonferroni's multiple comparison test. The immunohistochemical data obtained following the morphometric analysis were processed by unpaired *t*-test. The same test was applied to analyze plasma glucose, triglycerides, cholesterol, total proteins and albumin concentrations. Differences were considered significant at $p<0.05$.

## 3. Results

### 3.1. Effect of MNU-induced carcinogenesis on food intake and body weight

While mammary tumors were first noticed following 6 weeks of MNU administration, by 8 weeks 100% rats showed the occurrence of cancer. Fig. 1 shows MNU-induced mammary gland tumors and histoarchitecture of the same of an experimental rat. Nine weeks

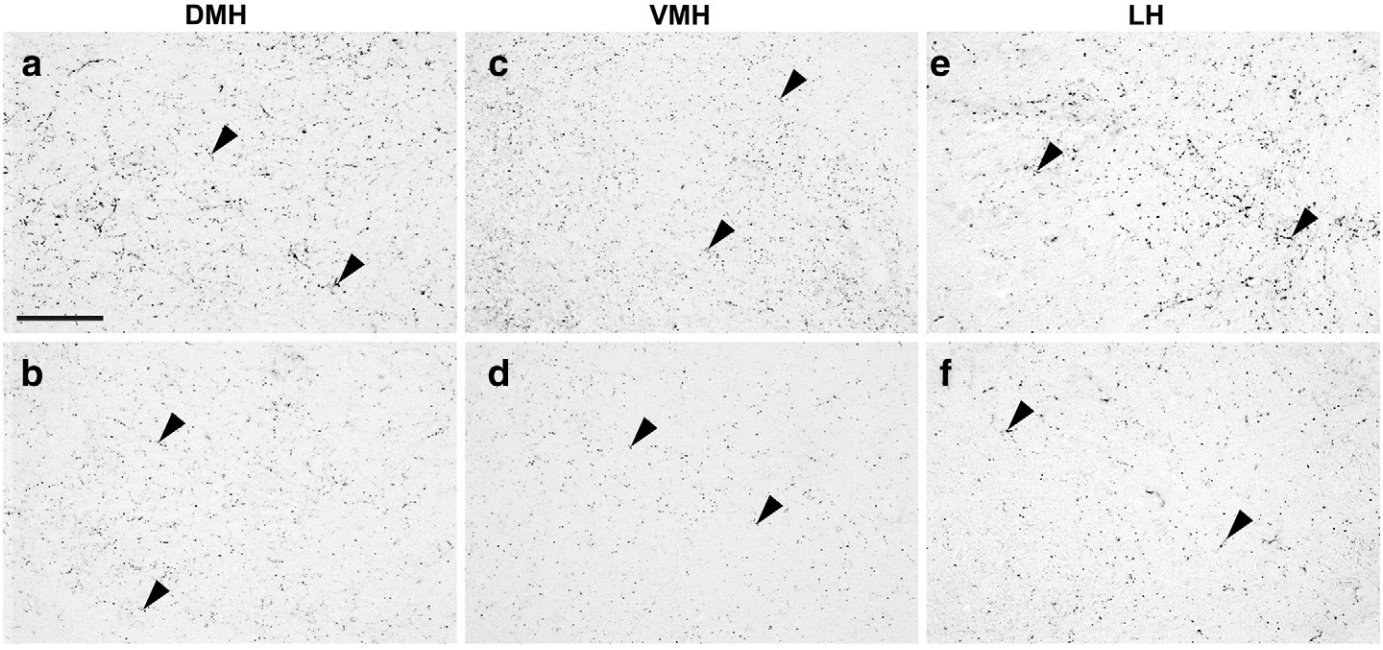
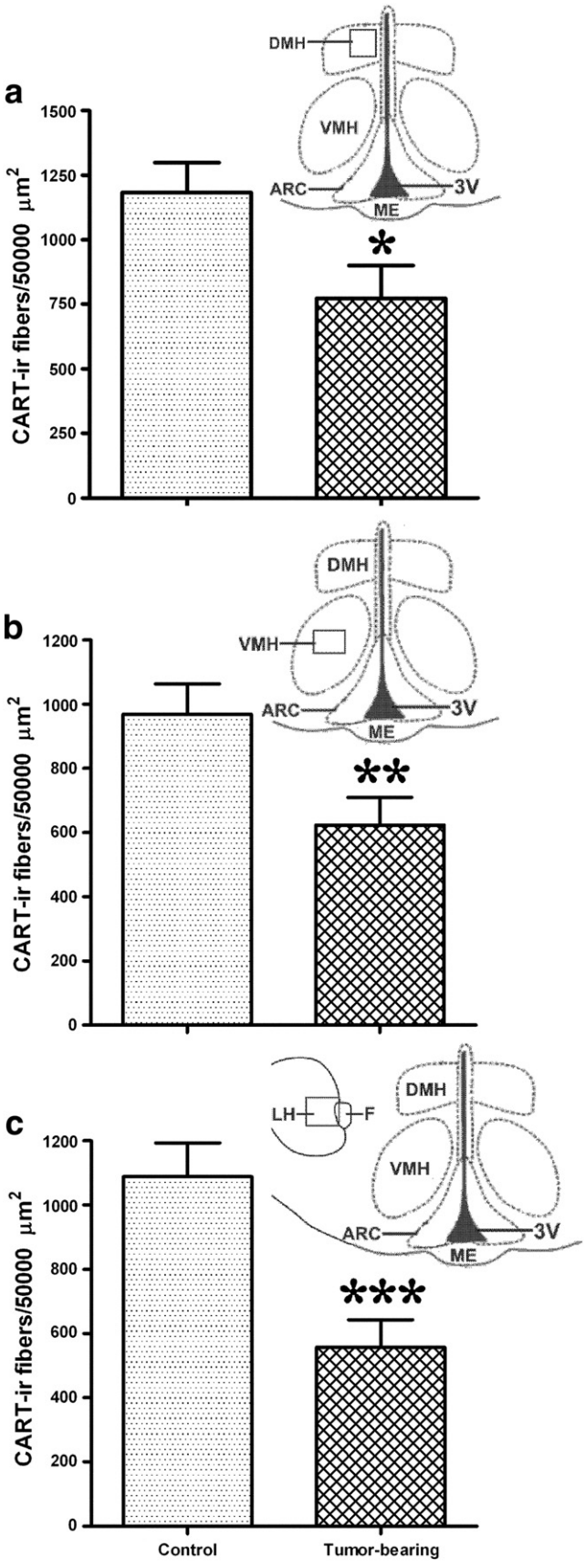



**Fig. 3.** Photomicrographs showing the CART-immunoreactive fibers (arrowheads) in the DMH, VMH and LH of control (Figs. 3a, c and e, respectively) and mammary tumor-bearing (Figs. 3b, d and f, respectively) rats. Note a significant decrease in the CART-immunoreactive fibers in the DMH, VMH and LH of tumor-bearing rats as compared to those of normal controls. DMH, dorsomedial nucleus of hypothalamus; LH, lateral hypothalamus; VMH, ventromedial nucleus of hypothalamus. Scale bar = 100 μm.

after MNU administration, rats showed a significant decrease ($p<0.05$) in food intake as compared to that of saline treated rats [factor MNU treatment $F(1,210)=56.83$, $p<0.0001$; factor 'duration in weeks' $F(14,210)=17.62$, $p<0.0001$; and interaction 'treatment × weeks' $F(14,210)=1.62$, $p=0.07$] (Fig. 2a). At the end of 14 weeks, food intake of MNU treated cancerous rats was about 16% less than that of control rats.

The effects observed on feeding were directly reflected on body weight (Fig. 2b). As compared to saline control, body weight of MNU injected animals was significantly reduced ($p<0.05$) after 11 weeks of the treatment [factor 'MNU treatment' $F(1,210)=62.11$, $p<0.0001$; factor 'duration in weeks' $F(14,210)=433.9$, $p<0.0001$; and interaction 'treatment x weeks' $F(14,210)=3.78$, $p<0.0001$]. Compared with control rats, body weight of tumor-bearing animals was about 11% less at the end of 14 weeks.




### 3.2. Effect of MNU-induced carcinogenesis on plasma glucose, triglycerides, cholesterol, total proteins and albumin contents

Table 1 depicts the effect of MNU-induced mammary carcinogenesis on plasma glucose, triglycerides, cholesterol, total proteins and albumin levels. A significant reduction in the triglycerides by 43% ($t=9.61$, $df=10$, $p<0.0001$), cholesterol by 25% ($t=4.81$, $df=10$, $p<0.0001$), total proteins by 35% ($t=6.24$, $df=10$, $p<0.0001$) and albumin by 44% ($t=6.11$, $df=10$, $p<0.0001$) was observed in the plasma of tumor-bearing animals as compared to that in normal rats. However, the plasma glucose levels did not differ significantly ($t=1.32$, $df=10$, $p=0.2$).

### 3.3. Effect of mammary tumorigenesis on hypothalamic CART-immunoreactivity

The DMH, VMH and LH of mammary tumor-bearing rats showed a significant reduction in CART-immunoreactive fibers by 35% ($t=2.364$, $df=118$, $p=0.01$), 36% ($t=2.669$, $df=118$, $p=0.008$) and 49% ($t=3.908$, $df=118$, $p=0.0002$) respectively as compared to those in normal animals (Figs. 3a-f; 4a-c). CART-immunoreactive cells of the PVN and ARC of cancerous rats also showed a reduction of 50% ($t=5.432$, $df=118$, $p<0.0001$) and 38% ($t=2.802$, $df=118$, $p=0.005$) respectively. Similarly, fibers of the PVN and ARC showed a reduction by 26% ($t=2.286$, $df=118$, $p=0.02$) and 27% ($t=2.001$, $df=118$, $p=0.04$) respectively, as compared to those in control animals (Figs. 5a-d; 6a,b). However, no difference was observed in the CART-immunoreactivity in the PeA cells ($t=0.22$, $df=118$, $p=0.8$) and fibers ($t=0.205$, $df=118$, $p=0.8$) across cancerous and control groups (Figs. 5e-f; 6c).

### 3.4. Effect of CART or CART-antibody on food intake of normal and tumor-bearing rats

In normal rats, administration of CART (1 μg/rat/day, i.c.v.) for 5 days resulted in a decrease in food intake [factor 'CART treatment' $F(1,78)=123.8$, $p<0.0001$; factor 'duration in days' $F(5,78)=4.39$, $p=0.001$; and interaction 'treatment x days' $F(5,78)=5.83$, $p=0.0001$] (Fig. 7a). A significant anorexia ($p<0.001$) was observed from day 1 of the treatment, and CART treated animals ingested about 29% less food on the last day of treatment. Injection of CART-antibody (1:500/rat/day,

**Fig. 4.** Representation of the semiquantitative morphometric analysis of CART-immunoreactivity in fibers of the DMH, VMH and LH (Figs. 4a, b and c respectively) of control and mammary gland tumor-bearing rats. 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). 3V, third ventricle; ARC, arcuate nucleus of hypothalamus; DMH, dorsomedial nucleus of hypothalamus; F, fornix; LH, lateral hypothalamus; ir, immunoreactive; ME, median eminence; VMH, ventromedial nucleus of hypothalamus. 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 ($n=6$/group). The data were analyzed by unpaired $t$-test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs* control rats.

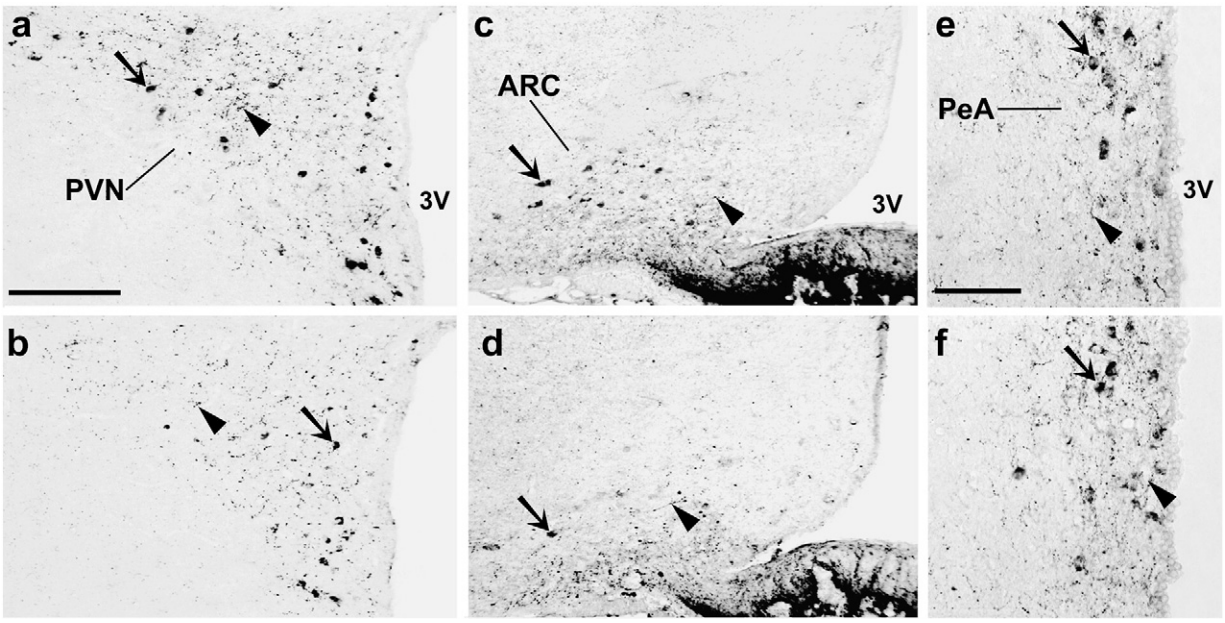



**Fig. 5.** Photomicrographs showing the CART-immunoreactive cells (arrows) and fibers (arrowheads) in the PVN, ARC and PeA of control (Figs. 5a, c and e, respectively) and mammary tumor-bearing (Figs. 5b, d and f, respectively) rats. Note a significant decrease in the CART-immunoreactive cells and fibers in the PVN and ARC of tumor-bearing rats as compared to those of normal controls. However, no difference was observed in the CART-immunoreactivity in the PeA cells and fibers across both the groups. 3V, third ventricle; ARC, arcuate nucleus of hypothalamus; PeA, periventricular nucleus of the hypothalamus; PVN, paraventricular nucleus of hypothalamus. Scale bar = 200 μm in Fig. 5a (applies to a-d) and 100 μm in Fig. 5e (applies to e and f).

i.c.v.), on the other hand, stimulated food consumption [factor 'CART-antibody treatment' $F(1,78) = 84.47$, $p < 0.0001$; factor 'duration in days' $F(5,78) = 5.56$, $p = 0.0002$; and interaction 'treatment x days' $F(5,78) = 3.54$, $p = 0.006$] (Fig. 7a). While a significant effect was observed from day 1 of the treatment ($p < 0.001$), CART-antibody injected animals consumed about 27% more food on the last day of treatment.

However, in tumor-bearing rats, no alteration in food intake was observed ($p > 0.05$) following the injection of CART [factor 'CART treatment' $F(1,84) = 20.75$, $p < 0.0001$; factor 'duration in days' $F(5,84) = 0.34$, $p = 0.8$; and interaction 'treatment x days' $F(5,84) = 0.5$, $p = 0.7$] or CART-antibody [factor 'CART-antibody treatment' $F(1,78) = 13.46$, $p = 0.0004$; factor 'duration in days' $F(5,78) = 1.9$, $p = 0.1$; and interaction 'treatment × days' $F(5,78) = 0.64$, $p = 0.6$] throughout the duration of 5 days (Fig. 7b).

### 3.5. Effect of CART or CART-antibody on body weight of normal and tumor-bearing rats

As observed in the above experiment, in normal rats, treatment with CART (1 μg/rat/day, i.c.v.) for 5 days resulted in a reduction in body weight [factor 'CART treatment' $F(1,78) = 36.20$, $p < 0.0001$; factor 'duration in days' $F(5,78) = 0.72$, $p = 0.6$; and interaction 'treatment x days' $F(5,78) = 2.11$, $p = 0.06$] (Fig. 8a). A significant weight loss ($p < 0.05$) was noticed from day 3 onwards, and it was 6% less than that of aCSF treated rats on the last day of treatment. In contrast, injection of CART-antibody to normal rats showed an increase in body weight [factor 'CART-antibody treatment' $F(1,78) = 18.05$, $p < 0.0001$; factor 'duration in days' $F(5,78) = 3.46$, $p = 0.007$; and interaction 'treatment x days' $F(5,78) = 1.31$, $p = 0.26$] (Fig. 8a). While a significant weight gain ($p < 0.05$) was noticed from day 4 onwards, it was about 6% more than aCSF treated rats on the last day of treatment.

However, in tumor-bearing rats, no significant change ($p > 0.05$) in body weight was noticed following the injection of CART [factor 'CART treatment' $F(1,84) = 6.08$, $p = 0.01$; factor 'duration in days' $F(5,84) = 0.13$, $p = 0.9$; and interaction 'treatment x days' $F(5,84) = 0.26$, $p = 0.9$] or CART-antibody [factor 'CART-antibody treatment' $F(1,78) = 2.88$, $p = 0.09$; factor 'duration in days' $F(5,78) = 0.67$, $p = 0.6$; and interaction 'treatment x days' $F(5,78) = 0.16$, $p = 0.9$] throughout the period of 5 days (Fig. 8b).

## 4. Discussion

Breast cancer, the most common type of malignancy encountered in women throughout the world (Ray and Mitra, 2003), is invariably accompanied by the cachexia (Knapp et al., 1991; Fox et al., 2009). The present investigation was undertaken to clarify the role of CART, an important anorexic neuropeptide, in the breast cancer-induced anorexia and weight loss. The female Sprague-Dawley rat model of MNU-induced mammary tumorigenesis was employed. The model has been widely used for such studies (Welsch, 1985; Kumar et al., 1990; Thompson and Adlakha, 1991; Lee et al., 2004) particularly because the histological structure of mammary gland tumor in this animal closely resembles to that in human mammary tumor (Tseng, 1980; Russo et al., 1990; Thompson et al., 1995). Fifty days old rats were used for MNU administration since at about this age (45-60 days), they become sexually mature, mammary epithelium proliferates rapidly and therefore increasing the probability of tumors development (Russo et al., 1990; Lee et al., 2004). Similar strategy was employed by the earlier researchers (Thompson and Adlakha, 1991; Roomi et al., 2005).

In the present study, MNU treated rats showed a progressive reduction in food intake and body weight. This is in agreement with the earlier studies (Grubbs et al., 1983; Ratko et al., 1991; Thompson and Adlakha, 1991). Analysis of blood plasma of cancerous animals showed a significant reduction in proteins, albumin, cholesterol and triglycerides contents, while a marginal decrease was noticed in the glucose concentrations. Earlier studies also reported similar changes during cachectic condition, and the effects were attributed to the factors like malnutrition (Fouladiun et al., 2005; Bovio et al., 2008; Krzystek-Korpacka et al., 2008), inhibition of the albumin gene expression (Brenner et al., 1990) or reduced synthesis of fatty acids/triglycerides (Kumar et al., 2003).

CART-immunoreactivity in cells and/or fibers of the DMH, VMH, LH, PVN and ARC of mammary tumor-bearing rats showed a considerable reduction as compared to that in normal animals. Hashimoto et al. (2007) observed a reduced CART mRNA in the ARC

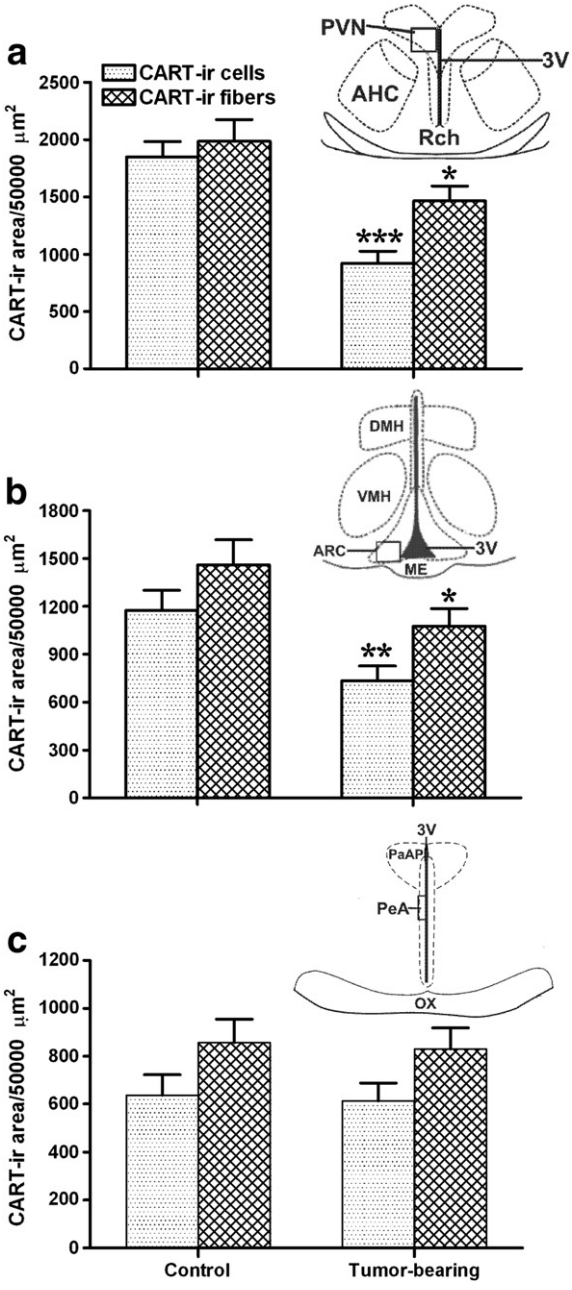



**Fig. 6.** Representation of the semiquantitative morphometric analysis of CART-immunoreactivity in cells and fibers of the PVN (Fig. 6a), ARC (Fig. 6b) and PeA (Fig. 6c) of control and mammary gland tumor-bearing rats. The outline of the transverse section through brain indicates the regions of the PVN, ARC and PeA at the co-ordinates −1.80 mm, −3.30 mm and −1.40 mm with reference to bregma respectively (Paxinos and Watson, 1998) from which the measurements were collated (square, not to scale). 3V, third ventricle; AHC, central part of the anterior hypothalamus; ARC, arcuate nucleus of hypothalamus; DMH, dorsomedial nucleus of hypothalamus; ir, immunoreactive; ME, median eminence; PaAP, anterior parvicellular part of the hypothalamic paraventricular nucleus; PeA, periventricular nucleus of the hypothalamus; PVN, paraventricular nucleus of hypothalamus; Rch, retrochiasmatic nucleus; VMH, ventromedial nucleus of hypothalamus; ox, optic chiasm. The bar values are shown as the mean ± SEM of five measurements from predetermined fields of the PVN, ARC and PeA on both the sides of each brain ($n = 6$/group). The data were analyzed by unpaired *t*-test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs* control rats.

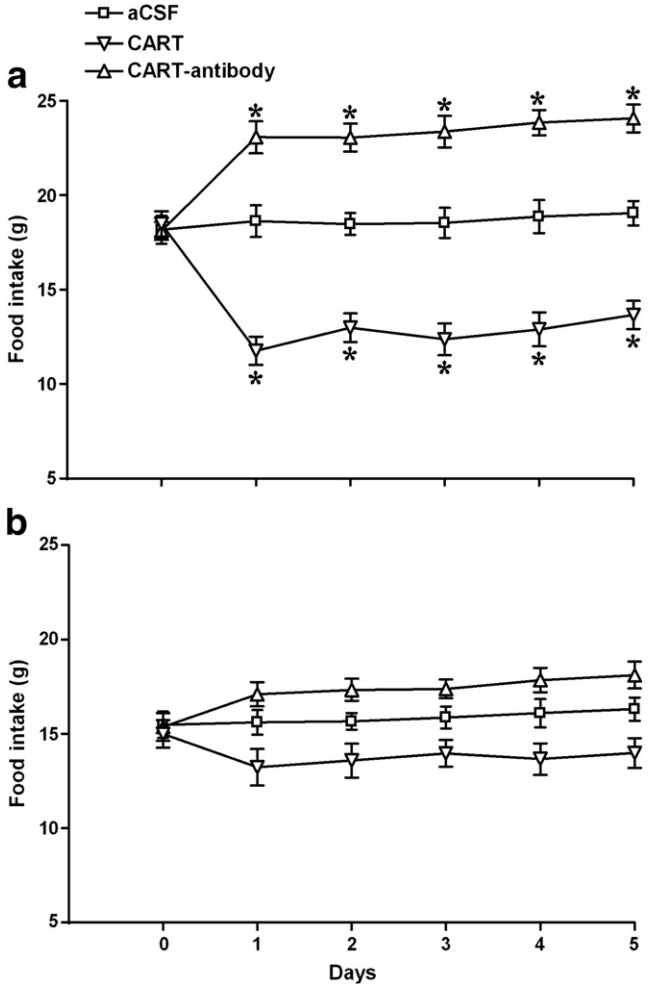



**Fig. 7.** Effect of CART or CART-antibody on food intake in normal (a) and mammary gland tumor-bearing (b) rats. Different sets of normal and tumor-bearing rats were administered daily, 10 min prior to the onset of the dark phase, with aCSF (5 μl/rat, i.c.v.), CART (1 μg/rat, i.c.v.) or CART-antibody (1:500 dilution, 5 μl/rat, i.c.v.) for a period of 5 days. Food intake was monitored (g) after an interval of 24 h, just prior to the next injection time-point. Each line and bar represents mean ± SEM ($n = 7$-8/group). The data were analyzed by two-way ANOVA followed by a post-hoc Bonferroni's multiple comparison test. $^{*}p<0.001$ *vs* aCSF.

of rats with the humoral hypercalcemia of malignancy. They also reported an increased expression of the orexigenic neuropeptides like agouti related peptide (AgRP) and NPY in the ARC. The malignancy-induced cachexia is known to produce a state of negative energy balance (Lelbach et al., 2007). Several animal studies reported a reduced CART expression and increased AgRP and NPY expression during a condition of acute or chronic negative energy balance (Adam et al., 2002; Bertile et al., 2003; Van Vugt et al., 2006). Johansen et al. (2000) reported a decreased expression of CART in the ARC of anorectic anx/anx mice. These authors proposed that there might be a compensatory down-regulation in response to the energy-deprived state, or the effects may be attributed to a molecular defect in the ARC. Collectively, the above data suggest that reduced CART contents in the hypothalamus of cancer-bearing animals might be an outcome of a normal homeostatic response to negative energy balance.

In the present study, CART (1 μg) and its antibody (1:500 dilution) were given via i.c.v. route. Similar treatments, in terms of dose range and route of administration, were used in the previous studies (Kristensen et al., 1998; Scruggs et al., 2003; Dandekar et al., 2008b). Since rats showed a peak feeding activity during the dark phase (Kimura et al., 1970), the injections of CART or CART-antibody were given at the onset of the dark phase. The same strategy was also employed in the earlier studies (Kristensen et al., 1998; Lambert et al., 1998). Daily injection of CART-antibody for 5 days caused a significant increase in food intake and body weight in normal rats, whereas CART produced opposite effects. This is in agreement with the previous reports (Kristensen et al., 1998; Lambert et al., 1998; Larsen et al., 2000; Rohner-Jeanrenaud et al., 2002). However, both the treatments,

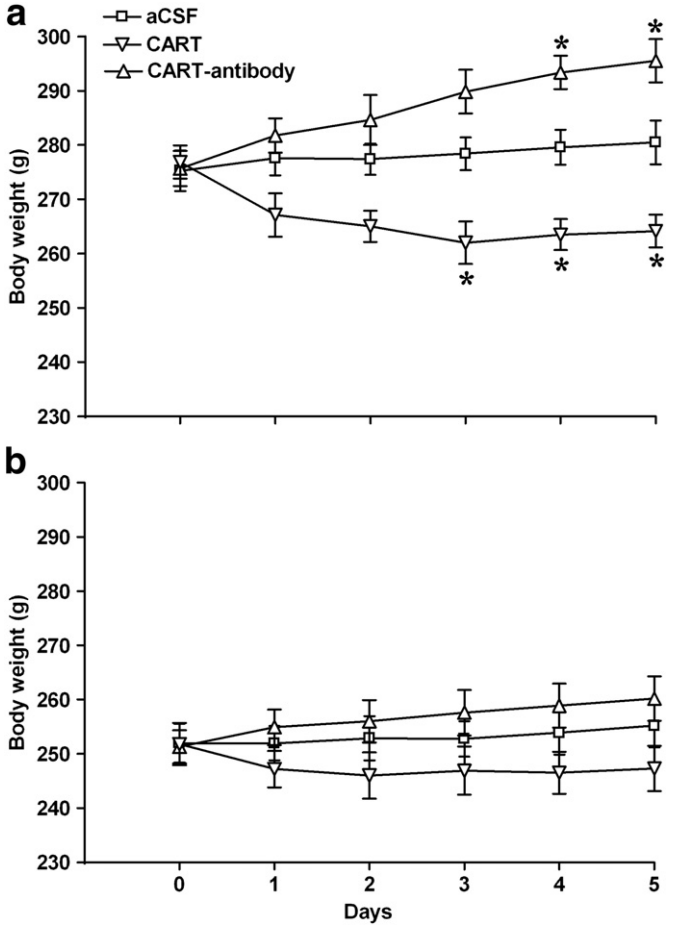



**Fig. 8.** Effect of CART or CART-antibody on body weight in normal (a) and mammary gland tumor-bearing (b) rats. Separate groups of normal and tumor-bearing rats were administered daily, 10 min prior to the onset of the dark phase, with aCSF (5 µl/rat, i.c.v.), CART (1 µg/rat, i.c.v.) or CART-antibody (1:500 dilution, 5 µl/rat, i.c.v.) for a period of 5 days. Body weight was monitored (g) after an interval of 24 h, just prior to the next injection time-point. Each line and bar represents mean ± SEM ($n = 7$-8/group). The data were analyzed by two-way ANOVA followed by a post-hoc Bonferroni's multiple comparison test. $^{*}p < 0.05$ vs aCSF.

given to tumor-bearing rats failed to influence food intake or body weight. It seems that, among other effects, tumor formation might bring about the down-regulation of endogenous CART system which in turn may render ineffective, the peptide or its antibody. Our immunohistochemistry data support this line of argument; the CART-immunoreactivity was much reduced in different hypothalamic nuclei of tumor-bearing rats.

Although the anorectic property of CART is well-established, some reports question the specificity of this effect. I.c.v. CART-induced anorexia was attributed to the motor abnormalities (Abbott et al., 2001) and altered palatability (Aja et al., 2001). However, reports from our lab (Dandekar et al., 2008b, 2009) and others (Kristensen et al., 1998; Kask et al., 2000) observed no locomotor abnormalities following i.c.v. CART administration. I.c.v. CART treated rats were clearly capable of eating the food normally (Kristensen et al., 1998; Kask et al., 2000). Some studies reported the orexigenic nature of CART. Injections of CART into discrete hypothalamic nuclei (Abbott et al., 2001) or increased CART expression in the ARC (Kong et al., 2003) and PVN (Smith et al., 2008), augmented feeding. I.p. injection of endocannabinoid antagonist, rimonabant produced anorexia in food-restricted mice, but not in their CART-deficient littermates (Osei-Hyiaman et al., 2005). Several theories have been proposed to elucidate these dissimilar responses of CART. Intra-hypothalamic injections might activate orexigenic CART circuits or might stimulate autoreceptors, whereas i.c.v. injection might activate anorectic CART circuits in the hindbrain (Abbott et al., 2001; Smith et al., 2008). It is also possible that hypothalamus itself might have two types of CART circuits, one orexigenic and another anorectic (Abbott et al., 2001). These speculations raise the possibility that the malignant condition may lead to a selective attenuation of orexigenic CART circuitry in the hypothalamus. However, additional studies are needed to clarify the exact mechanisms.

In contradistinction to the above reports, intra-PVN injection of CART not only produced a significant anorexia (Stanley et al., 2001), but also prevented the orexigenic response of NPY (Wang et al., 2000). Anorectic agents like nicotine and endocannabinoid agonist HU-210, increased CART levels in the PVN, PeA and DMH (Giuliani et al., 2000; Osei-Hyiaman et al., 2005; Kramer et al., 2007). It should also be noted that an up-regulation of CART expression in the ARC caused a reduction in food intake and body weight (Tian et al., 2005), whereas the deficiency of CART led to weight gain (Wierup et al., 2005; Bartell et al., 2008). However, in the present study, even though hypothalamic CART activity was declined in mammary tumor-bearing rats, their food intakes and body weights were significantly less than that in normal animals. According to Hashimoto et al. (2007), although cancerous rats might feel eagerness to consume the food, in effect, they may not be able to eat. It is also possible that cancer-bearing rats may satiate rapidly. This might also explain the observed reduction in food intake and body weight.

We may recall that the central injection of melanocortin-4 receptor antagonist significantly stimulated the food intake and body weight gain in tumor-bearing rats, whereas administration of ghrelin or NPY failed to reverse the cancer anorexia (Wisse et al., 2001). This finding suggests that melanocortin antagonists may be fruitful in the treatment of cancer cachexia. However, results of the present study suggest that CART receptor antagonists, even if they were available, would have no similar clinical potential.

In conclusion, the results of the present study suggest that malignant state may down-regulate the endogenous CART as well as its receptors system, which perhaps is an attempt to restore the energy balance or reflect a counter-regulatory mechanism to reverse the cachexia. However, more studies are warranted to elucidate the exact mechanisms underlying a decreased CART contents in the hypothalamus during cachectic conditions.

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