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PAPER

Chronic central infusion of cocaine- and amphetamine-regulated transcript (CART 55-102): effects on body weight homeostasis in lean and high-fat-fed obese rats

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BACKGROUND: Cocaine- and amphetamine-regulated transcript (CART) is expressed within hypothalamic nuclei implicated in the regulation of feeding behaviour. It is up-regulated by leptin, and CART-derived peptides acutely inhibit food intake.
OBJECTIVE: The present study was designed to assess the long-term effects of central CART administration on food intake, body weight, plasma levels of glucose, insulin, leptin, free fatty acids and triglycerides, and on fuel utilisation in normal and high-fat-fed obese rats.
DESIGN: Normal and high-fat-fed obese rats were cannulated intracerebroventricularly (i.c.v.) and infused for 6 days with CART (55–102) or its vehicle. At day 4, animals were placed in an indirect calorimeter for a 24 h period during which the respiratory quotient and the energy expenditure were determined hourly.
RESULTS: In both normal and obese animals, the chronic i.c.v. infusion of CART (55–102) had marked, sustained inhibitory effects on food intake and body weight gain that were accompanied by decreases in plasma insulin and leptin levels. Using indirect calorimetry, it was observed that CART infusion promoted an increase in lipid oxidation in normal and in obese animals, although this increase reached statistical significance only in the obese group. The hypothalamic CART mRNA expression was found to be higher in obese rats (displaying hyperleptinaemia) than in normal animals.
CONCLUSION: The data together show that chronic i.c.v. CART infusion is effective in inhibiting food intake, favouring lipid oxidation and limiting fat storage, both in normal and high-fat-diet-induced obese rats. The CART pathway thus seems to be an important determinant of body weight homeostasis in normal animals as well as in a model of nutritionally induced obesity.
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Keywords: food intake; indirect calorimetry; respiratory quotient (RQ); fuel utilisation; CART mRNA

Introduction

Cocaine- and amphetamine-regulated transcript (CART) mRNA was initially isolated from rat brain striatum and named according to the observation of its transcriptional regulation by the acute administration of cocaine or amphetamine.[1] In the rat brain, two alternatively spliced mRNA variants predict two different proteins of 116 and 129 amino acids.[1] The mature CART peptide contains several cleavage sites that allow for its post-translational processing into several biologically active fragments. Processing of CART has been shown to result in neuropeptides of different lengths that may have different biological properties, as this processing is tissue-specific.[2] Post-translational processing in the middle of the molecule at a Lys–Arg sequence generates an N-terminal CART(1–52) and a C-terminal CART(55–102) fragment.[3] The latter of these fragments has actually been isolated from ovine and rat hypothalamic extracts.[4,5]

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A role of CART in the control of body weight homeostasis is suggested by the observation that it is highly expressed in hypothalamic nuclei known to be implicated in the central control of feeding behaviour, the paraventricular, arcuate, dorsomedial nuclei, and the lateral hypothalamus.[1,6] In these hypothalamic areas, the presence of CART has been shown to be associated with that of orexigenic factors (eg NPY, melanin-concentrating hormone, MCH[7]), and with that of anorexigenic peptides (eg proopiomelanocortin (POMC), the precursor of α-MSH[8]). These observations suggest the likely importance of CART in the regulation of food intake and energy balance, all the more because CART and the orexigenic or anorexigenic peptides just mentioned are all regulated by leptin.[8–10] Although the CART receptor(s) have not been cloned yet, it is noteworthy that the hypothalamic distribution of the CART peptide matches that of the CART mRNA.[9,11]

Relatively few studies have dealt with the role of CART in the regulation of feeding. In normal rats, the acute intracerebroventricular (i.c.v) administration of a peptidic fragment, CART (42–89), has been shown to produce a dose-dependent decrease in food intake that was accompanied by the induction of *c*-fos expression in various hypothalamic and brainstem nuclei.[12,13] A transient decrease in food intake has also been reported to occur in fasted mice following the acute i.c.v. administration of CART (55–102).[3,9] Both CART (82–103) and CART (55–102) have been shown to transiently decrease the feeding response elicited by NPY.[14,15] Administration of antibodies to predicted CART peptides produced a significant increase in night-time feeding, indicating the presence of an endogenous inhibitory tone of CART on the food intake process.[9,14]

In the leptin-deficient obese hyperphagic *ob*/*ob* mouse, CART expression in the arcuate nucleus has been described to be low, and to substantially increase when leptin was chronically administered to these animals.[9] Hypothalamic CART expression was also found to be low in the leptin resistant genetically obese Zucker *fa*/*fa* rat.[9] A decreased CART expression and/or action could therefore be implicated in the hyperphagia of some animal models of obesity. This is in keeping with the recent observation that prolonged i.c.v. CART (42–89) administration inhibited food intake and decreased body weight in both lean and genetically obese *fa*/*fa* rats.[16]

The present study was undertaken to investigate the effects of chronic intracerebroventricular infusion of CART (55–102) on body weight homeostasis of normal rats, and of rats made obese by feeding them a high-fat diet. This model of nutritionally induced obesity was chosen as it occurs in the absence of any known mutation, thus having some potential resemblance to many human obesity syndromes. During these experiments, food intake, body weight, energy expenditure, respiratory quotient, some hormono-metabolic parameters, and hypothalamic CART mRNA were measured during or at the end of vehicle and CART infusion.

Methods

Intracerebroventricular infusions

Adult male Long–Evans rats were fed either a standard laboratory chow containing 6.5% fat and 23.5% protein (Formula chow 5008, 3.5 kcal/g, Harlan Teklad, Madison, WI, USA), or a high-fat diet containing 18.9% fat and 19.9% protein (TD 95217, 4.25 kcal/g, Harlan Teklad, Madison, WI, USA). At 15–16 weeks of age, the body weights of normal and obese rats were 431 ± 4 and 467 ± 5 g ($P < 0.001$), respectively, with the obese group exhibiting a significant increase in total body fat content as measured by dual-energy X-ray absorptiometry. At this age, they were implanted, under isoflurane anaesthesia, with a cannula placed in the right lateral cerebral ventricle and connected to an osmotic minipump (Alzet brain infusion kit, Alza Corporation, Palo Alto, CA, USA). Minipumps (Alzet Model 2001) were filled with saline containing 0.01% ascorbic acid for controls, or with a solution of 0.4 mg/ml CART (55–102) in the same vehicle. Minipumps were primed at 37°C in sterile saline for at least 20 h, then implanted subcutaneously in the interscapular region and connected to the intracerebroventricular (i.c.v.) cannulas with their catheter reaching the cerebral ventricles. Following this, rats were returned to their individual home cages. CART (55–102) was infused at a dose of 9 μg per day for up to 6 days, during which food intake and body weight were measured daily.

Indirect calorimetry

On experimental day 4, 24 h energy expenditure (EE) and respiratory quotient (RQ) were measured by indirect calorimetry using, for lean and obese rats, an open circuit calorimetry system (Oxymax, Columbus Instruments International Corporation, Columbus, OH, USA). The instrument was calibrated before the experiments using standard gas mixtures containing known concentrations of CO_2, N_2 and O_2. Gas sampled from each 16 chamber was first dried by a condenser. The volume of oxygen consumed (VO_2) and carbon dioxide produced (VCO_2) in 1 h was measured using a paramagnetic oxygen sensor and a spectrophotometric CO_2 sensor. Such measurement was obtained hourly for 24 h allowing the rats to acclimatize to the chambers during the first hour. RQ was calculated as the ratio of VCO_2 to VO_2 and was used to calculate the proportion of protein, fat and carbohydrates utilised during the 24 h period, as reported previously.[17,18] As a potential stimulatory effect of central CART infusion on lipid oxidation is of particular interest in obesity, the effect of CART on fuel utilisation, and that mediated by decreased food intake, were assessed in three groups of high-fat-fed obese rats by indirect calorimetry: *ad libitum*-fed obese rats i.c.v. infused with vehicle; *ad libitum*-fed obese animals i.c.v. infused with CART; obese rats i.c.v. infused with vehicle but pair-fed to the amount of food consumed by the obese CART-infused group.

On the morning of the last experimental day, rats were sacrificed by decapitation, and trunk blood collected in

EDTA and heparin-coated tubes for the measurements, using a Monarch 2000 Multianalyzer, of plasma levels of glucose (Instrumentation Laboratory, Lexington, MA, USA), free fatty acids (Waco, Neuss, Germany), and triglycerides (Sigma, St Louis, MO, USA). Plasma insulin and leptin levels were measured by RIA (Diagnostic Products Corp., Los Angeles, CA, USA and Linco, St Charles; MO, USA, respectively). Brains were also quickly removed and frozen. Mediobasal hypothalami were subsequently dissected from a frontal brain slice cut between the middle of the optic chiasm and the mammillary bodies.

mRNA isolation and Northern blot analysis

Total RNA was isolated from rat hypothalamus using Trizol procedure (Life Technologies Inc., Rockville, MD, USA). Fifteen micrograms of total RNA were denatured in formaldehyde/MOPS buffer at 65°C and separated on 1.25% SeaKem gold agarose gels (Ambion Inc., Austin, TX, USA). RNA was blotted onto Nytran Super Charge nylon membranes (Schleicher and Schuell Inc., Keene, NH, USA), and the membranes were UV crosslinked, hybridised, and washed as directed by the manufacturer. A CART cDNA amplicon was generated by PCR using a sense primer GCCCTACTGCTGCTGCTACCTTTG and an antisense primer CCTCTTCTTCCCAGAAAGGTCACAAG from the published rat CART mRNA coding sequence (Genbank accession no. U 10071). CART cDNA fragment was radiolabelled with [α-^{32}P] dCTP (3000 Ci/mmol) using a random priming system (Life Technologies Inc.). After hybridisation with the CART probe, the blot was stripped by washing in 0.5% SDS at 100°C for 15 min then rehybridised with a rat β-actin control probe (Clontech no. S0130) to normalise the quantity of RNA in each sample. CART and actin signals were digitised by exposing blots to storage phosphor screens (Molecular Dynamics/Amersham Pharmacia, Uppsala, Sweden) and scanning with a Biorad FX Molecular Imager (Biorad Laboratories, Hercules, CA, USA). Relative intensities were quantified using Biorad Quantity One Software. CART mRNA levels were determined by dividing the CART signal by the respective actin signal.

CART peptide

CART (55 – 102) was obtained from Phoenix Pharmaceuticals Inc., Mountain View, CA, USA. The mass spectrometry for the peptide was carried out in our laboratory and the mass found and the calculated mass were very similar to those given by the provider, as well as to those previously reported.[3]

Prior to its use in chronic experiments, the peptide was tested for its acute ability (using 5 μg given i.c.v.) to decrease food intake in satiated rats and in animals i.c.v. injected with 5 μg of NPY. These acute experiments were completed by the observation of an acute inhibitory effect (75% inhibition at 3 h) of the same i.c.v. dose of CART on i.c.v. MCH (5 μg)-elicited food intake (data not shown).

Finally, the *in vitro* stability of CART (55 – 102) at 37°C in the vehicle used (ie saline containing 0.01% ascorbic acid) was checked daily by HPLC and found to be 72% on day 7 as compared with the initial value measured at the time at which the CART solution was prepared for filling the minipumps.

Data analysis

All values are presented as means ± s.e.m. Statistical analysis was performed by one way analysis of variance followed, for dynamic curves, by Tukey tests for multiple comparisons.

Results

Chronic i.c.v CART (55 – 102) infusion in normal rats

The effect of the chronic i.c.v. infusion of CART (55 – 102) on food intake and body weight of normal rats is shown by Figure 1. Relative to controls, CART infusion resulted in a rapid decrease in food intake that progressively normalised. This was accompanied by a reduction in body weight that reached its maximum on day 2 (body weight loss of more than 30 g), and remained constant thereafter. These chronic i.c.v. effects of CART were accompanied, relative to vehicle-infused controls, by significant decreases in the plasma levels of glucose, insulin and leptin with no change in free fatty acids, nor in triglyceride levels (Table 1).

Respiratory quotients and energy expenditure were then measured over a 24 h period. As depicted by Figure 2, it was observed that, relative to vehicle-infused controls in which RQs were in the range of 0.82 – 0.92 (mean 0.877 ± 0.006), the central infusion of CART (55 – 102) in normal animals resulted in a prolonged decrease in RQ values (range 0.79 – 0.89, mean 0.840 ± 0.02). There was no difference in energy expenditure between the two groups at any time point during the 24 h period (data not shown). Upon calculation of the proportion of the fuels utilised, it was observed that CART infusion resulted in a trend toward an increase in lipid oxidation (vehicle, 36.2 ± 3.0; CART 47.0 ± 4.7 kcal/kg/day, $P = 0.076$), and a corresponding decrease in that of carbohydrates (vehicle, 69.2 ± 2.3; CART, 49.9 ± 9.5 kcal/kg/day, $P = 0.061$). These changes did not reach statistical significance.

Chronic i.c.v. CART (55 – 102) infusion in high-fat-fed obese rats

As chronic CART infusion was effective in reducing food intake and body weight in normal rats, it was tested in rats made obese by a high fat diet. As illustrated by Figure 3, relative to vehicle-infused controls, obese rats chronically i.c.v. infused with CART decreased their food intake and body weight, reaching a cumulative loss of about 30 g on the last experimental day. These effects of chronic i.c.v.

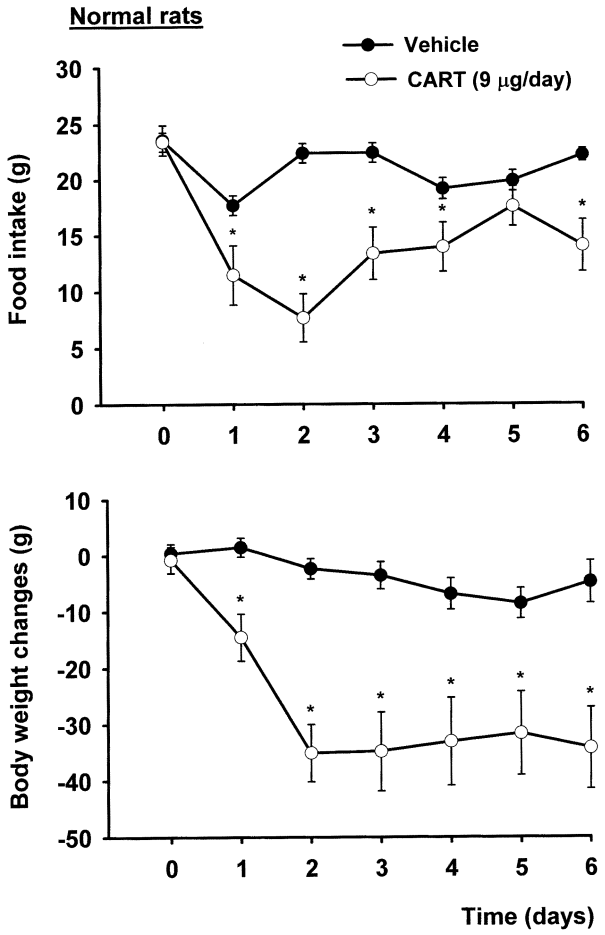

Figure 1 Effects of chronic (6 days) intracerebroventricular infusion of CART or its vehicle (saline with 0.01% ascorbic acid) on food intake and cumulative body weight changes in normal rats. Means ± s.e.m. of 5–6 animals per group. *P at least < 0.05.

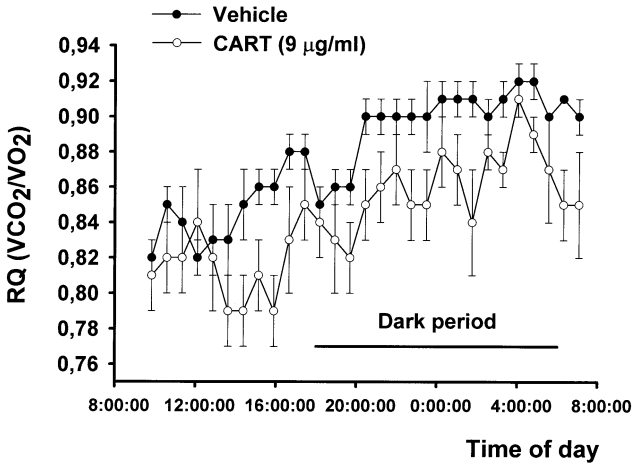

Figure 2 Effects of chronic (6 days) intracerebroventricular infusion of CART or its vehicle (saline with 0.01% ascorbic acid) on respiratory quotient (RQ) measured over 24 h in normal rats, on day 4 of the overall 6 day experimental period. Means ± s.e.m. of 5–6 animals per group.

CART infusion in obese rats were accompanied by decreases in the plasma levels of leptin and insulin, with no change in plasma glucose, free fatty acids, nor in triglyceride levels (Table 1). Of note is the observation that the i.c.v. CART infusion in high-fat-fed obese rats normalised their elevated leptinaemia to the levels measured in vehicle-infused normal animals (Table 1).

As mentioned in Methods, the measurements of the respective fuels utilised by obese animals were then performed via indirect calorimetry in three groups of animals: *ad libitum*-fed obese rats i.c.v. infused with vehicle; *ad libitum*-fed obese animals i.c.v. infused with CART; and obese animals i.c.v. infused with vehicle, but pair-fed to the amount of food consumed by the CART-infused group. During the experimental period, the body weight of the i.c.v vehicle-infused *ad libitum*-fed obese group increased by 3.3 ± 0.5 g, while that of the i.c.v. vehicle-infused pair-fed and of the i.c.v. CART-infused obese rats similarly decreased (loss of 11.5 ± 3.3 and 11.8 ± 1.9 g, respectively). As shown by Figure 4, protein utilisation was similar in the three groups of obese rats. Lipid and carbohydrate utilisation were not significantly different between *ad libitum*-fed and pair-fed vehicle-infused obese animals. However, obese rats i.c.v. infused with CART had a fat oxidation that was significantly

Table 1 Plasma glucose, insulin, leptin, free fatty acids and triglycerides in normal rats and in high-fat-fed rats chronically (6 days) intracerebroventricularly (i.c.v.) infused with vehicle or with CART (55–102) (9 μg/day)

	Normal rats		*High-fat-fed obese rats*	
	i.c.v. vehicle-infused	*i.c.v. CART-infused*	*i.c.v. vehicle-infused*	*i.c.v. CART-infused*
Glucose (mmol/l)	8.4 ± 0.1	7.6 ± 0.2*	9.7 ± 0.3	11.6 ± 1.2
Insulin (ng/ml)	3.7 ± 0.9	1.8 ± 0.2*	3.3 ± 0.2	2.6 ± 0.1*
Leptin (ng/ml)	9.3 ± 0.8	4.2 ± 0.4*	19.1 ± 2.6	9.7 ± 1.3*
Free fatty acids (mmol/l)	0.27 ± 0.03	0.32 ± 0.04	0.42 ± 0.08	0.46 ± 0.05
Triglycerides (mmol/l)	3.1 ± 0.5	2.9 ± 0.6	4.6 ± 0.7	3.4 ± 0.6

Values are means ± s.e.m. of 5–6 animals per group. Vehicle used was isotonic saline. *P at least < 0.05 compared to values of respective i.c.v. vehicle-infused rats.

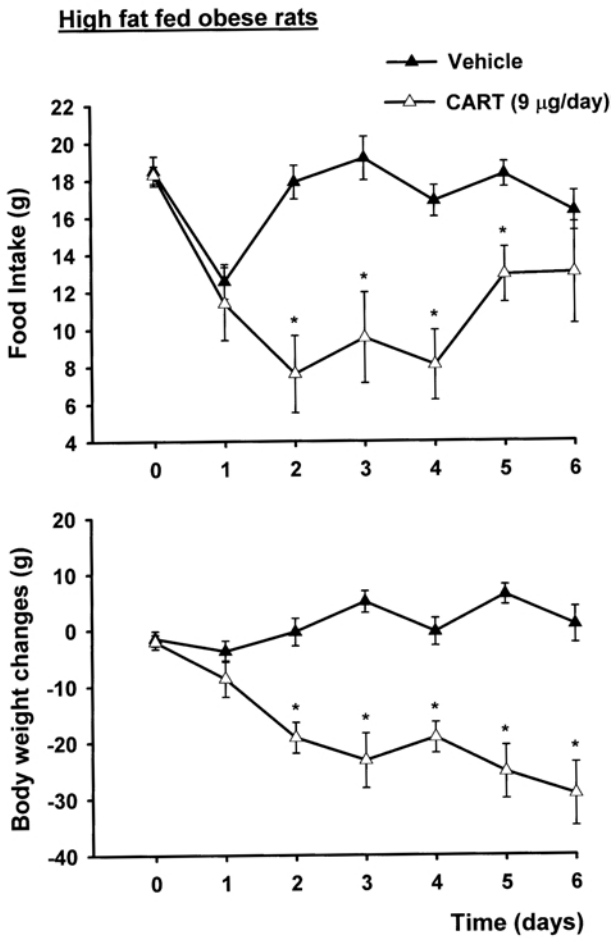

Figure 3 Effects of chronic (6 days) intracerebroventricular infusion of CART or its vehicle (saline with 0.01% ascorbic acid) on food intake and cumulative body weight changes in high-fat-fed obese rats. Means ± s.e.m. of 6 animals per group. *P at least < 0.05.

enhanced relative to *ad libitum*-fed obese control animals. There was no inter-group difference in energy expenditure at any time point during the 24 h experimental period (data not shown).

Hypothalamic expression of CART mRNA in normal and high-fat-fed obese rats

The expression of CART was determined in the mediobasal hypothalamus of normal and high fat fed obese rats. As can be seen on Figure 5, CART mRNA levels obtained by densitometry were higher in obese than in normal animals.

Discussion

These data represent the first long-term study on the effects of intracerebroventricular (i.c.v.) CART (55 – 102) infusion in rats made obese by feeding them a high-fat diet, and their

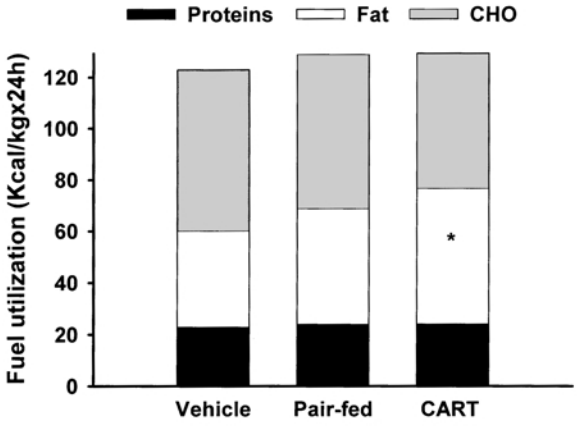

Figure 4 Twenty-four hour fuel utilisation in i.c.v. vehicle-infused *ad libitum*-fed obese rats (vehicle), i.c.v. vehicle-infused pair-fed obese rats (pair-fed), and i.c.v. CART-infused obese animals (CART) calculated from RQs measured by indirect calorimetry at day 4 of CART and vehicle infusion. Means ± s.e.m. of 5 – 6 animals per group. *$P < 0.05$ CART *vs* vehicle.

respective controls. The chronic i.c.v. CART infusion resulted in marked decreases in the respective food intake and body weight gain of the two groups of animals. In both normal and obese rats, the CART-induced decrease in food intake progressively normalised during the experiment. In normal rats, this was accompanied by a decrease in body weight that occurred rapidly, to remain constant subsequently. In the obese group, CART induced a more sustained loss of body weight as it persisted during the whole experimental period. Despite of these kinetic differences in body weight loss, both normal and high-fat-fed rats lost the same amount of body weight (-34.3 ± 7.2 and -29.3 ± 5.6 g, respectively; NS) The present data together are in agreement with another report[16] showing that another CART peptide, CART (42 – 89), given chronically into the cerebral ventricles of lean and genetically obese rats of the Zucker strain, also inhibited food intake and caused weight loss.

In the present study, the decreases in food intake and body weight produced by CART (55 – 102) infusion in normal and obese animals, were accompanied by decreases in plasma insulin and leptin levels. Decreased insulinaemia as produced by CART infusion may result from the decrease in food intake and/or indicate the occurrence of improved insulin sensitivity/responsiveness. The reduction in plasma leptin levels measured in lean and obese rats following i.c.v. CART infusion is the likely reflect of body fat loss. Of note is the observation that the high plasma leptin levels measured in high-fat-fed obese rats reflecting their elevated fat content compared to normal rats were normalised by the i.c.v. CART infusion. In both normal and obese rats, CART infusion appeared to initiate a shift in fuel utilisation away from carbohydrates, and toward fat oxidation. Such a CART-induced shift in fuel utilisation, with increased lipid at the

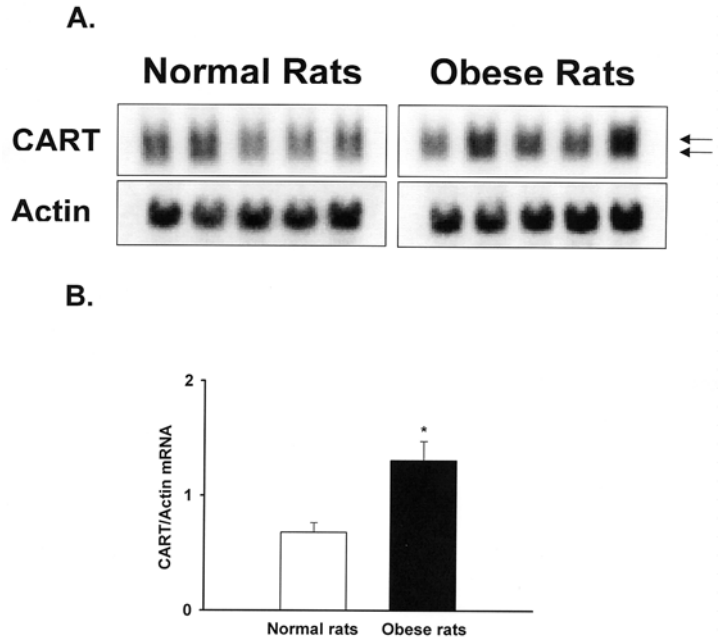

Figure 5 (A) Expression of hypothalamic CART mRNA in normal and high-fat-fed obese rats. (details of CART mRNA measurement, see Methods). Arrows indicate the 700 and 900 bp CART transcripts. (B) Quantification of the total (ie 700 and 900 bp) CART over the actin signal for the autoradiogramm shown on panel (A). Means ± s.e.m. of 5–6 animals per group. *P at least < 0.05.

expense of carbohydrate utilisation, was manifest in the high-fat-fed obese group when compared to *ad libitum*-fed vehicle-infused controls.

As the existence of interrelationships between hypothalamic CART neurones and sympathetic neurones of the spinal cord has been reported,[8] it is conceivable that the chronic i.c.v. CART infusion ultimately increases the activity of the sympathetic nervous system. This would be in keeping with other observations showing that acute i.c.v. CART injection activates central CRH neurones,[13,19] CRH being known to stimulate sympathetic-mediated processes.[20–22] The observation that CART acutely induced the expression of uncoupling protein-1 (UCP1) in brown adipose tissue, of UCP2 in white adipose tissue and of UCP3 in skeletal muscle[15] suggests that the peptide might contribute to favour lipid utilisation via an activation of the sympathetic nervous system. In the present study, measurements of actual energy dissipation in lean and high-fat-fed obese animals failed to show that CART induces an increase in energy expenditure.

It has been reported that the low hypothalamic CART expression of the leptin deficient obese *ob/ob* mouse was increased by the administration of leptin.[9] However in the present study, hypothalamic CART expression was greater in high-fat-fed obese than in normal rats. This is in agreement with a previous report which suggested the occurrence, due to the hyperleptinaemia of the obese animals, of some leptin transport across the blood–brain barrier, with partial activation of the CART pathway by leptin, limiting the degree of this nutritionally induced obesity syndrome.[23] It should however be mentioned that, in the present study, the CART mRNA measurements were performed on whole mediobasal hypothalami, not excluding the possibility of discrete changes within specific hypothalamic nuclei. In conclusion, the present study demonstrates that chronic i.c.v. CART (55–102) infusion has marked inhibitory effects on food intake and body weight gain in normal and high-fat-fed obese rats. Such an exogenous CART infusion appears to favour lipid oxidation at the expense of carbohydrate utilisation, an effect that is most marked in high-fat-fed obese animals. As CART (55–102) is efficient on most parameters measured, not only in normal, but also in obese animals exhibiting hyperleptinaemia and concomitant leptin resistance, it appears possible that CART may exert its anorectic and body weight-lowering action downstream of the leptin signalling pathway.

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