

# Quantification and synthesis of cocaine- and amphetamine-regulated transcript peptide (79–102)-like immunoreactivity and mRNA in rat tissues

K G Murphy, C R Abbott, M Mahmoudi, R Hunter<sup>1</sup>,  
J V Gardiner, M Rossi, S A Stanley, M A Ghatei, M J Kuhar<sup>1</sup>  
and S R Bloom

Endocrine Unit, Division of Investigative Sciences, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK

<sup>1</sup>Yerkes Regional Primate Research Center, Division of Neuroscience, Emory University, Atlanta, Georgia 30322, USA

(Requests for offprints should be addressed to S R Bloom; Email: sbloom@ic.ac.uk)

## Abstract

The distribution of cocaine- and amphetamine-regulated transcript peptide (79–102)-like immunoreactivity (CART-LI) was quantified in brain and peripheral tissues of male and female Wistar rats, and male obese (fa/fa) and heterozygous (Fa/+) Zucker rats using a specific RIA. CART-LI tissue levels have not been quantified previously. The assay, using cocaine- and amphetamine-regulated transcript (CART) (79–102) as a standard and radioactive tracer and an antibody to CART (79–102) fragment, detected CART-LI in all brain regions examined, the anterior and posterior pituitary, the spinal cord and throughout the gastrointestinal tract of both male and female Wistar rats. The highest concentrations were found in the hypothalamus, duodenum, anterior pituitary and posterior pituitary ( $50.6 \pm 4.4$ ,  $26.1 \pm 4.2$ ,  $50.0 \pm 1.3$  and  $373.0 \pm 55.2$  pmol/g wet tissue respectively, means  $\pm$  S.E.M.,  $n=6-10$  male animals). There was no significant variation between the sexes. The concentrations of

CART-LI in hypothalami and anterior and posterior pituitaries from fa/fa rats were significantly ( $P<0.002$ ) lower than those of Fa/+ controls ( $35.9 \pm 2.1$  vs  $53.9 \pm 4.9$ ,  $<0.6$  vs  $1.8 \pm 0.4$  and  $114 \pm 9.1$  vs  $255.5 \pm 20.9$  pmol/g wet tissue respectively, means  $\pm$  S.E.M.,  $n=7$ ).

Gel permeation chromatography of regions of rat brain and gastrointestinal tract showed possible differential processing between regions. CART-LI was released from hypothalamic tissue slices in a calcium-dependent fashion by potassium-induced depolarisation. Northern blot analysis detected CART mRNA in the hypothalamus, anterior pituitary, brain stem, cerebellum and spinal cord.

The pattern of distribution of CART mRNA and CART-LI in various neural and other tissues is in accord with a role for CART as a neurotransmitter.

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## Introduction

Cocaine- and amphetamine-regulated transcript (CART) cDNA was originally isolated from rat brain using PCR differential display as a transcript whose expression is regulated by acute administration of cocaine or amphetamine (Douglass *et al.* 1995). It was not until 3 years after its initial discovery that CART was reported as a hypothalamic satiety factor (Kristensen *et al.* 1998, Lambert *et al.* 1998). However, the more widespread distribution of CART suggests that this is not its only physiological role. Immunohistochemical and *in situ* hybridisation studies have revealed the presence of the transcript and the encoded peptide in various regions of the central nervous system (CNS) and peripheral nervous system (Douglass *et al.* 1995, Couceyro *et al.* 1997, 1998, Koylu *et al.* 1997,

1998, Smith *et al.* 1997). CART mRNA and peptides have been found in the adrenal medulla and the anterior pituitary of the rat (Douglass & Daoud 1996, Couceyro *et al.* 1997, Koylu *et al.* 1997) and also in the paraventricular nucleus (PVN), supraoptic nucleus (SON), arcuate nucleus (ARC) and perifornical area of the hypothalamus (Koylu *et al.* 1997). However, the encoded peptide has not previously been quantified.

In addition, CART is co-localised with a number of neurotransmitters, including neuropeptide Y (NPY) in the PVN (Lambert *et al.* 1998). CART immunoreactivity is present in  $\gamma$  aminobutyric acid-containing neurones in the nucleus accumbens (Smith *et al.* 1997), in noradrenergic neurones in the locus coeruleus (Koylu *et al.* 1999) and cholinergic neurones in the ileum (Couceyro *et al.* 1998). The majority of CART-containing neurones in the

retrochiasmatic area and ARC of the hypothalamus also contain pro-opiomelanocortin mRNA (Elias *et al.* 1998). The CART system may represent a new neurotransmitter/neuromodulator system (Kuhar & Dall Vechia 1999).

CART mRNA has been proposed as the third most abundant region-specific mRNA in the rat hypothalamus (Gautvik *et al.* 1996). In the rat, alternate splicing and polyadenylation of CART gives rise to four transcripts, the alternately spliced versions of which result in two different prepro-peptides of 116 or 129 amino acids. The cleavage of a 27 amino acid signal sequence at the N-terminus of the CART prepro-peptide results in pro-peptides of 89 and 102 amino acids in length (Douglass *et al.* 1995). These pro-peptides are believed to be processed to several of the same mature forms (Thim *et al.* 1999). There are several nomenclatures used to describe CART peptides. Following nomenclature previously used in the literature (Kristensen *et al.* 1998), all CART peptides referred to in this paper are numbered as if they are products of the longer 102 residue peptide unless otherwise stated. It should be noted that the peptides CART 42–89 and CART 49–89 described using nomenclature based on the 89 residue pro-peptide (Thim *et al.* 1999) have the same primary structure as CART 55–102 and CART 62–102 (Kristensen *et al.* 1998) respectively.

In human CART, no alternate splicing occurs and the mRNA is 92% homologous to the shorter rat form, with 95% homology at the amino acid level (Douglass & Daoud 1996). This evolutionary conservation suggests that CART has an important role in mammalian physiology.

The CART amino acid sequence contains several pairs of basic residues, which are potential cleavage sites for pro-hormone convertases 1 and 2. These include one at Lys-53–Arg-54 that would result in the production of the fragments CART (1–51) and CART (55–102). CART (55–102) was initially isolated from ovine brain extract (Speiss *et al.* 1981), and then from rat hypothalamus, nucleus accumbens and anterior pituitary (Kuhar & Yoho 1999, Thim *et al.* 1999). CART (55–102) is believed to be at least one of the processed forms of CART peptide that affects food intake. Intracerebroventricular injection of recombinant CART (55–102) inhibits feeding in non-fasted rats in a dose-dependent manner (Kristensen *et al.* 1998). Although CART (55–102) is biologically active, it is likely that there are other functional forms of CART created by processing at alternative cleavage sites. It has been shown recently that endogenous CART also exists in the forms described by the 89 residue nomenclature (1–89), (10–89) and (49–89) (Kuhar & Yoho 1999, Thim *et al.* 1999) in different rat tissues. The RIA used in this study is directed to the C-terminal end of all these forms. To further investigate differential processing of CART peptide we used Sephadex G-50 and FPLC to separate tissue extract CART (79–102)-like immunoreactivity (CART-LI) into distinct peaks. To determine if

CART-LI distribution varied between male and female Wistar rats, tissue levels in both sexes were measured.

One of the criteria for the definition of a peptide neurotransmitter is that its release from nerve fibres is calcium-dependent. We therefore investigated whether the potassium-stimulated release of CART-LI from static incubated rat hypothalami was dependent on the presence of calcium.

Obese Zucker rats (fa/fa) have a leptin receptor mutation resulting in an attenuated response to leptin administration compared with that of Zucker control rats (Fa/+) (Cusin *et al.* 1996). Significantly lower CART mRNA levels have been found in the ARC of the hypothalamus of fa/fa rats compared with Fa/+ rats (Kristensen *et al.* 1998). To see if this difference in mRNA message translated into a difference in peptide, we compared the CART-LI content of various tissues, including the hypothalamus, from Fa/+ and fa/fa rats.

## Materials and Methods

### Materials

Bolton and Hunter reagent, <sup>125</sup>I-Na and [<sup>32</sup>P]CTP were obtained from Amersham International plc, Amersham, Bucks, UK, aprotinin (Trasylol) was from R Bayer, Haywards Heath, W Sussex, UK, Sephadex G-50 Superfine was from Pharmacia, Uppsala, Sweden, synthetic CART (55–102) was obtained from The Peptide Institute, Osaka, Japan and T7 RNA polymerase was from Promega, Southampton, Hants, UK.

### Rat tissue

All animals were caged in groups of five under controlled temperature (21–23 °C) and light (12 h light:12 h darkness, lights on at 0700 h) with *ad libitum* access to food (RM1 diet; SDS UK Ltd, Witham, Essex, UK) and water. Animals were killed by decapitation in the early light phase between 0900 and 1200 h. Adult male and female Wistar rats weighing 250–300 g (Imperial College School of Medicine, London, UK) were decapitated and various brain regions, spinal cord, pituitary glands, adrenal gland, gastrointestinal (GI) tract, testis, ovary, liver, trachea, uterus, pancreas, lung, heart, spleen and kidney were rapidly dissected. Tissues were immediately extracted by boiling for 15 min in 0.5 M acetic acid (approximately 10 ml/g wet weight apart from the anterior and posterior pituitary glands which were each extracted in 0.2 ml). Extracts were stored at –20 °C until assayed.

Adult male Zucker rats (Charles River, Bicester, Oxon, UK), Fa/+ (250–300 g) and fa/fa (450–500 g) were decapitated. The hypothalamus, pituitary glands and GI tract were rapidly dissected. In order to determine the stability of CART-LI when subjected to freezing prior to

extraction, additional Wistar rats (details as above) were also decapitated and the hypothalamus, pituitary gland and liver dissected. Tissues were immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . They were extracted as above prior to assay.

There is no established protocol for the tissue extraction of CART peptide. An additional tissue extraction method was therefore tested. A further group of Wistar rats (details as above) were decapitated and the hypothalamus and pituitary gland dissected. Tissues were immediately placed in 1 ml acid/ethanol solution (0.15% HCl/25% ethanol/74.85%  $\text{H}_2\text{O}$  (v/v)) and stored for 24 h at  $4^{\circ}\text{C}$ . They were then homogenised using an Ultra-Turrax T25 homogeniser (IKA Labor Technik, Staufen, Germany) and stored at  $-20^{\circ}\text{C}$  until assayed.

#### Radioimmunoassay

An RIA directed against CART (79–102) was developed. The peptide was synthesised on an Advanced Chemtech 396 multiple peptide synthesiser (Advanced Chemtech Europe, Cambridge, Cambs, UK) by batch method fluorenylmethoxycarbonyl-polyamide solid phase synthesis, and purified by HPLC on a C8 (Phenomenex, Macclesfield, Cheshire, UK) column over a 20–40% 30-min gradient of acetonitrile (ACN) plus 0.1% (v/v) trifluoroacetic acid (TFA)/0.1% (v/v) TFA. The identity of the peptide was confirmed by a matrix-assisted laser desorption ionisation-time of flight mass spectrometer (Kratos, Manchester, UK).

The CART (79–102) antiserum was produced as described previously (Koyle *et al.* 1997). Briefly, CART peptide (79–102) was conjugated to keyhole limpet haemocyanin via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Three New Zealand white rabbits were initially inoculated with 200  $\mu\text{g}$  peptide conjugate mixed with an equal volume of complete Freund's adjuvant. Inoculations were administered at multiple sites subcutaneously and intramuscularly. Boosts consisted of 100  $\mu\text{g}$  peptide conjugate in an equal volume of incomplete Freund's adjuvant. The antiserum showed full cross-reactivity with synthetic CART (55–102). Synthetic CART (79–102) was iodinated by the Bolton & Hunter (1973) method. Briefly, 5 nmol CART (79–102) in 50  $\mu\text{l}$  0.1 M borate buffer (pH 8.2) was added to 1 mCi dry Bolton and Hunter reagent. The reaction was incubated at room temperature for 120 min and the iodinated peptide separated by RP-HPLC on a C18 (Waters, Milford, CT, USA) column over a 15–45% 90-min gradient of ACN/water/0.1% (v/v) TFA.

The assay was set up as previously described (Adrian 1982). The final antibody dilution used was 1:80 000. CART (79–102) label was used at 30 Bq/tube. The assay was performed in 0.06 M phosphate buffer (pH 7.2) containing 0.3% (v/v) bovine serum albumin (BSA) and 0.02% (v/v) Tween 20. After 3 days of incubation at  $4^{\circ}\text{C}$ ,

antibody-bound and free fractions were separated by charcoal adsorption of the free fraction.

Concentrations up to 1 nmol/tube of agouti-related peptide, arginine vasopressin, bombesin, brain natriuretic peptide, calcitonin gene-related peptide, corticotrophin-releasing factor, endothelin-1, follicle-stimulating hormone, galanin, glucagon, growth hormone-releasing hormone, NPY, oxytocin, prolactin, prolactin-releasing peptide, somatostatin and substance P were added to the assay to assess potential cross-reactivity of the antibody. Concentrations from 20 to 2000 fmol/tube CART (55–102) were added to 200  $\mu\text{l}$  rat liver homogenate, extracted (see above) and assayed to assess the recovery efficiency of the assay. A standard curve of 2–200 fmol/tube CART (79–102) was set up in conjunction with dilution curves of hypothalamus, posterior pituitary and stomach extracts to assess their parallelism.

To verify that the tissue extraction had been successful, the substance P immunoreactivity content of the Wistar, Fa/+ and fa/fa anterior pituitary acetic acid extracts were measured using methods described previously (McGregor & Bloom 1983).

#### Chromatography

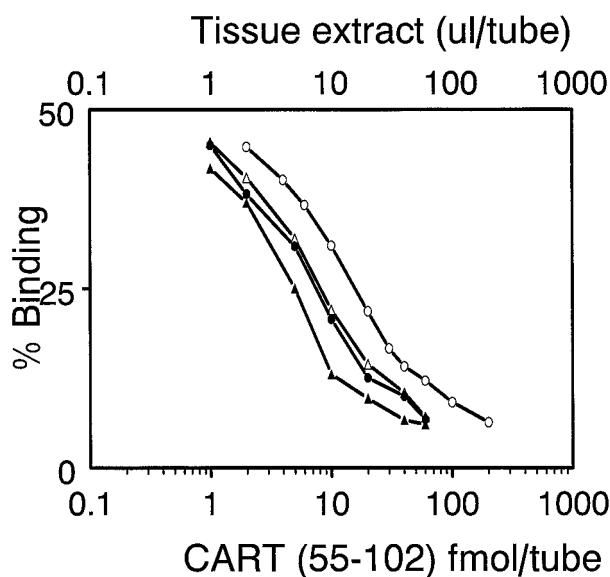
Fractionation of the CART-LI in extracts of Wistar rat hypothalamus, ileum, duodenum, stomach, anterior and posterior pituitary was carried out with Sephadex G-50 Superfine on a  $1.5 \times 200$  cm column and FPLC using a high-resolution reverse-phase (Pep RPC HR 5/5) C-18 column (Pharmacia, Uppsala, Sweden).

The Sephadex G-50 column was eluted at a flow rate of 3.2 ml/h at  $4^{\circ}\text{C}$  in 0.06 M phosphate buffer containing 0.2 M NaCl and 0.3% (v/v) BSA, and 0.8 ml fractions were collected. To determine the relative elution coefficient ( $K_{av}$ ) of CART-LI (see below) dextran blue (molecular weight (MW) 2 000 000, 30 mg/ml), horse heart cytochrome C (MW 12 384, 30 mg/ml) and  $^{125}\text{I}$ -Na were added to each sample extract ( $n=3$  for each tissue type), which was loaded as a volume of 0.7 ml. RIA was used to observe the elution profile of CART-LI.

Tissue extracts ( $n=3$  for each tissue type) were centrifuged at 15 000 g for 3 min and the supernatants filtered through 0.2  $\mu\text{m}$  hydrophilic membranes (Sartorius, Göttingen, Germany). Samples of 0.5–1 ml were then loaded onto the FPLC column and eluted with a 15–50% gradient of ACN plus 0.1% (v/v) TFA/0.1% (v/v) TFA over 50 min at a flow rate of 1 ml/min per fraction. Fractions from all runs were freeze-dried, reconstituted in assay buffer and the CART-LI content determined by RIA.

#### Release experiments

The static hypothalamic incubation system used was a modification of the method previously described (Beak



**Figure 1** Parallelism of standard and tissue extract novel CART-LI RIA. Assay conditions are as described in Materials and Methods. CART (55–102) (○); hypothalamus (●); posterior pituitary (△); stomach (▲) ( $n=5$ ).

*et al.* 1998). Male Wistar rats were decapitated and the whole brain immediately removed. The brain was mounted with ventral surface uppermost and placed in a vibrating microtome (BioRad, York, N Yorks, UK). A 1.5 mm slice was taken from the base of the brain and immediately transferred into individual tubes containing 1 ml artificial cerebrospinal fluid (aCSF; 20 mM  $\text{NaHCO}_3$ , 126 mM  $\text{NaCl}$ , 0.09 mM  $\text{Na}_2\text{HPO}_4$ , 6 mM  $\text{KCl}$ , 1.4 mM  $\text{Ca}_2\text{Cl}$ , 0.09 mM  $\text{MgSO}_4$ , 8 mM glucose, 18 mg/ml ascorbic acid and 100  $\mu\text{g}/\text{ml}$  aprotinin) equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . The tubes were placed on a shaking platform in a water bath maintained at 37 °C. After an initial 2-h equilibration period, with aCSF replaced every 60 min, the hypothalami were incubated for 45 min in 500  $\mu\text{l}$  calcium-free aCSF (basal period) followed by 56 mM  $\text{KCl}$  in 500  $\mu\text{l}$  low calcium aCSF. Finally, the tissue was exposed for 45 min to 56 mM  $\text{KCl}$  in 500  $\mu\text{l}$  aCSF containing 1.4 mM  $\text{Ca}_2\text{Cl}$ ; isotonicity was maintained by substituting  $\text{Na}^+$  for  $\text{K}^+$ . At the end of each period, the aCSF was removed and frozen at -20 °C until measurement of CART-LI by RIA.

#### Northern blot analysis

Total RNA was isolated using the single step guanidinium thiocyanate-phenol-chloroform method (Chomcynski & Sacchi 1987). The integrity of the RNA was verified on a denaturing 3-(*n*-morpholino) propane-sulphonic acid (MOPS)/formaldehyde gel (1% agarose) (Southern 1975, Lehrach *et al.* 1977). Fifty micrograms total RNA from each tissue was then size-separated on another denaturing

MOPS propane-sulphonic acid/formaldehyde gel (1% agarose) (as above) and transferred to a Hybond-N membrane (Amersham International plc). The RNA was fixed by baking at 80 °C for 2 h before probing with a riboprobe corresponding to nucleotides 100–409 of rat CART (Douglass *et al.* 1995). The riboprobe was synthesised using [ $^{32}\text{P}$ ]-CTP using T7 RNA polymerase. Hybridisation was carried out overnight at 55 °C in 5 × Denhardt's, 50% (w/v) deionised formamide, 100 g/ml denatured sonicated herring sperm DNA and 100 g/ml yeast tRNA with 1.5 MBq labelled riboprobe. Non-specific hybridisation was removed by increasingly stringent washes, the final one being in 0.1 × SSC/0.1% (w/v) SDS at 70 °C for 30 min.

Autoradiography of the filter was carried out at room temperature using Kodak X-OMAT and an intensifying screen for 7 h.

#### Statistical analysis

The concentrations of CART-LI in tissue extracts are expressed as means  $\pm$  s.e.m.

All RIA results were compared using a two-tailed homoscedastic Student's *t*-test.

The  $K_{av}$  was calculated for each immunoreactive peak on G-50 column chromatography according to a modification (Andrews 1970) of the equation proposed by Laurent & Killander (1964):  $K_{av} = (V_e - V_o) / (V_t - V_o)$  where  $V_o$  = void volume, the volume of mobile phase in the column not associated with the gel matrix, determined by the elution of dextran blue,  $V_t$  = total volume of the gel matrix and mobile phase in the column, determined by the elution of  $^{125}\text{I}$ -Na, detected by gamma counter and  $V_e$  = elution volume of the analyte, between  $V_o$  and  $V_t$ , determined by RIA.

## Results

#### Radioimmunoassay

The assay had a sensitivity of  $2.0 \pm 0.4$  fmol/tube (means  $\pm$  s.e.m.),  $n=5$ , with 95% confidence. The least detectable tissue concentration was  $<0.6$  pmol/g and the midrange was  $24.9 \pm 0.8$  fmol/tube (means  $\pm$  s.e.m.),  $n=14$ . Inter- and intra-assay variation were established to be  $8.6\% \pm 0.7$  and  $5.6\% \pm 1.7$  respectively,  $n=5$ . The assay showed less than 0.1% cross-reaction with up to 1 nmol/tube of all peptides tested (see methods above). Recovery of CART (79–102) from liver homogenate was between 76 and 84%, with no significant difference between homogenates of liver stored at -70 °C prior to acetic acid extraction ( $n=5$ ) and the concentration of CART-LI in those tissues extracted immediately after death (data not shown). The specific activity of freshly prepared CART (79–102) peptide label, as estimated by

**Table 1** Regional distribution of CART-LI in male and female rat tissues. Values are means  $\pm$  S.E.M.;  $n=4-10$ 

Region	CART-LI (79–102) (pmol/g wet weight)	
	Male	Female
Hypothalamus	50.6 $\pm$ 4.4	54.5 $\pm$ 8.0
Hippocampus	7.5 $\pm$ 2.5	6.2 $\pm$ 1.2
Thalamus	17.1 $\pm$ 2.5	11.9 $\pm$ 1.7
Cerebellum	0.5 $\pm$ 0.1	0.8 $\pm$ 0.7
Brain cortex	3.5 $\pm$ 0.3	3.4 $\pm$ 0.6
Brain stem	5.0 $\pm$ 0.3	5.9 $\pm$ 0.5
Anterior pituitary <sup>a</sup>	50 $\pm$ 1.25	55.4 $\pm$ 7.1
Posterior pituitary <sup>a</sup>	373.0 $\pm$ 55.2	304.2 46.7
Cervical spinal cord	2.3 $\pm$ 0.3	2.3 $\pm$ 0.6
Thoracic spinal cord	1.5 $\pm$ 0.5	1.8 $\pm$ 0.6
Lumbar spinal cord	2.1 $\pm$ 0.6	1.7 $\pm$ 0.4
Sacral spinal cord	1.8 $\pm$ 0.6	4.0 $\pm$ 1.4
Adrenal glands <sup>a</sup>	10.6 $\pm$ 1	8.5 $\pm$ 2.7
Stomach	16.8 $\pm$ 3.3	26.3 $\pm$ 4.6
Duodenum	26.1 $\pm$ 4.2	32.7 $\pm$ 4.4
Jejunum	15.5 $\pm$ 1.5	17.1 $\pm$ 2.4
Ileum	9.4 $\pm$ 0.7	13.2 $\pm$ 2.1
Caecum	1.9 $\pm$ 0.4	2.2 $\pm$ 0.5
Ascending colon	8.2 $\pm$ 0.7	12.6 $\pm$ 0.9
Descending colon	5.2 $\pm$ 0.7	11.4 $\pm$ 2.2
Rectum	6.8 $\pm$ 0.9	7.7 $\pm$ 1.5
Testis	ND	—
Ovary	—	ND
Oesophagus	ND	ND
Trachea	ND	ND
Uterus	—	ND
Pancreas	ND	ND
Lung	ND	ND
Heart (ventricle)	ND	ND
Heart (atria)	ND	ND
Spleen	ND	ND
Kidney (medulla)	ND	ND
Kidney (cortex)	ND	ND

ND, not detectable.

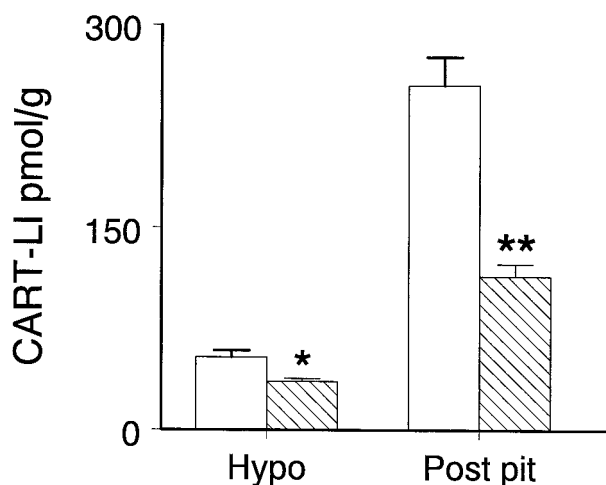
<sup>a</sup>Based on average weights of 8 mg, 1.5 mg and 50 mg for anterior pituitary, posterior pituitary and adrenal gland respectively.

$P>0.05$ , male compared with female (Student's *t*-test).

self-displacement in the assay, was 52 Bq/fmol. The dilution curves for hypothalamus, posterior pituitary and stomach extracts were almost parallel to that of CART (79–102) standard,  $n=5$  (Fig. 1). There was no significant difference between the CART-LI concentration of the Wistar hypothalami and pituitaries frozen and stored at  $-70^{\circ}\text{C}$  prior to acetic acid extraction ( $n=5$ ) and the concentration of CART-LI in those tissues extracted immediately after death (e.g. hypothalamus  $54 \pm 2.5$  vs  $50.6 \pm 4.4$  pmol/g wet tissue respectively,  $n=5$ ,  $P>0.05$ , other data not shown). Acid/ethanol solution extracts of hypothalami and pituitaries contained less than 2% of the concentrations of CART-LI obtained by acid extraction (e.g. hypothalamus  $0.6 \pm 0.3$  pmol/g wet tissue,  $n=5$ , other data not shown).

CART-LI was detected in the CNS, pituitary gland, adrenal gland and GI tract. It was not detectable in the testis, ovary, trachea, oesophagus, uterus, lung, heart, liver, kidney, spleen or pancreas. The highest concentration of CART-LI was detected in the posterior pituitary. In the GI tract, the highest concentrations were found in the stomach, duodenum and rectum. There was no significant difference between the concentrations of CART-LI found in male and female animals (Table 1).

The concentrations of CART-LI in hypothalami and anterior and posterior pituitaries from fa/fa rats were significantly lower than that of the Fa/+ controls (Fig. 2). The fa/fa anterior pituitaries contained significantly lower CART-LI than the Fa/+ anterior pituitaries ( $<0.6$  vs  $1.8 \pm 0.3$  pmol/g,  $n=7$ ,  $P=0.002$ ). There was no



**Figure 2** Quantification of CART-LI (79–102) extracted from the hypothalamic (Hypo) and posterior pituitaries (Post pit) of Fa/+ (open bars) and fa/fa (hatched bars) Zucker rats. Values are means  $\pm$  S.E.M.;  $n=7$ . Fa/+ versus fa/fa: \* $P=0.03$ , hypothalamus; \*\* $P=0.0001$ , posterior pituitary (Student's *t*-test).

significant difference in concentrations of CART-LI in the GI tract between these two groups (data not shown).

There was no significant difference found between the concentration of substance P in Wistar, Fa/+ and fa/fa rat anterior pituitary extracts ( $36.5 \pm 4.3$ ,  $34.5 \pm 0.8$ ,  $33.8 \pm 3.7$  pmol/g respectively,  $n=7$ ).

#### Chromatography

Sephadex G-50 chromatography showed different CART-LI elution profiles for separate tissue extracts (Fig. 3a–f). The synthetic CART peptide (55–102) used as a standard has a  $K_{av}$  of approximately 0.4. The hypothalamic extract CART-LI (Fig. 3a) eluted as two major immunoreactive (IR) peaks. The second, larger IR peak eluted with a  $K_{av}$  of 0.4, comparable with that of the CART (55–102) standard. The smaller, broader IR peak eluted with a  $K_{av}$  of approximately 0.15. The posterior pituitary extract elution profile (Fig. 3b) showed elution of one major IR peak with a  $K_{av}$  of approximately 0.4. In contrast, the anterior pituitary extract elution profile (Fig. 3c) indicated the presence of at least three separate CART-LI fragments. The two major IR peaks eluted with a  $K_{av}$  of 0.15 and 0.4. The stomach extract elution profile (Fig. 3d) eluted as two major IR peaks with  $K_{av}$  values of 0.15 and 0.4. The third minor IR peak had a  $K_{av}$  of 0.9. The duodenum extract elution profile (Fig. 3e) eluted in a profile similar to the stomach, except that the final minor IR peak is absent. The ileum extract elution profile (Fig. 3f) was also similar to the stomach extract elution profile. However, the first IR peak at  $K_{av}$  0.15 is smaller in relation to the second (0.4), which eluted as a

broad IR peak with apparent smaller IR peaks forming shoulders on the later eluting slope.

FPLC of tissue extracts gave separate distinct CART-LI peaks (see Fig. 4a–f for representative profiles). The hypothalamic extract gave two major IR peaks at 27 and 36% ACN (Fig. 4a). The anterior (Fig. 4b) and posterior pituitaries (4c) extract elution profiles gave a single major IR peak at 27% ACN, though the posterior pituitary extract elution profile had shoulders on either side of this IR peak. The stomach (Fig. 4d), duodenum (Fig. 4e) and ileum (Fig. 4f) extract elution profiles showed major IR peaks at 27 and 35% ACN, with the later IR peak being relatively larger than the first on the duodenum extract elution profile. In addition, the stomach and duodenum extract elution profiles showed a third IR peak at 33% ACN. The recovery of CART-LI in the tissue extract from each column run was above 78% (data not shown).

#### Release experiments

Exposure to 56 mM  $K^+$  in the superfusing medium containing 1.4 mM  $Ca^{2+}$  for 15 min significantly ( $P<0.05$  by paired *t*-test) increased the release of CART-LI compared with basal CART-LI release. Exposure to 56 mM  $K^+$  in  $Ca^{2+}$ -free superfusing medium did not significantly increase the release of CART-LI (Fig. 5).

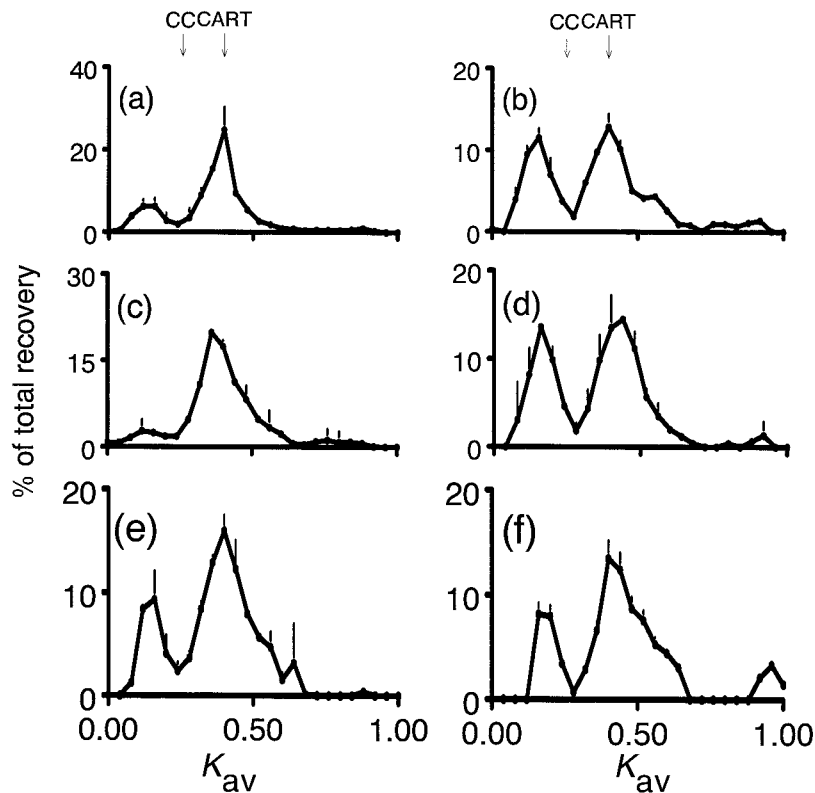
#### Northern blot analysis

Northern blot analysis detected two bands of approximately 900 and 700 base pairs in the hypothalamus, anterior pituitary, brain stem, cerebral cortex and spinal cord. No bands were detected in any of the other tissues examined, including posterior pituitary, stomach, duodenum, colon, rectum, liver and adrenals (Fig. 6 and other data not shown).

#### Discussion

In the present study, we have confirmed the presence of, and quantified, CART-LI in different regions of the brain, the pituitary gland, the adrenal gland and the spinal cord, and demonstrated and quantified its presence throughout the GI tract of male and female rats. Quantification of CART peptide in the rat shows that it is present in greatest concentrations in the hypothalamus, which is in accord with the postulated role of CART peptide as a hypothalamic satiety factor, and the pituitary. There was no significant difference in concentration or distribution between the sexes.

There is, as yet, no established independent assay for CART peptide, so our RIA cannot be compared with previous results. However, CART-LI has been detected in the brain, pituitary, adrenal medulla (Couceyro *et al.* 1997), thoracic spinal cord (Elias *et al.* 1998) and ileum



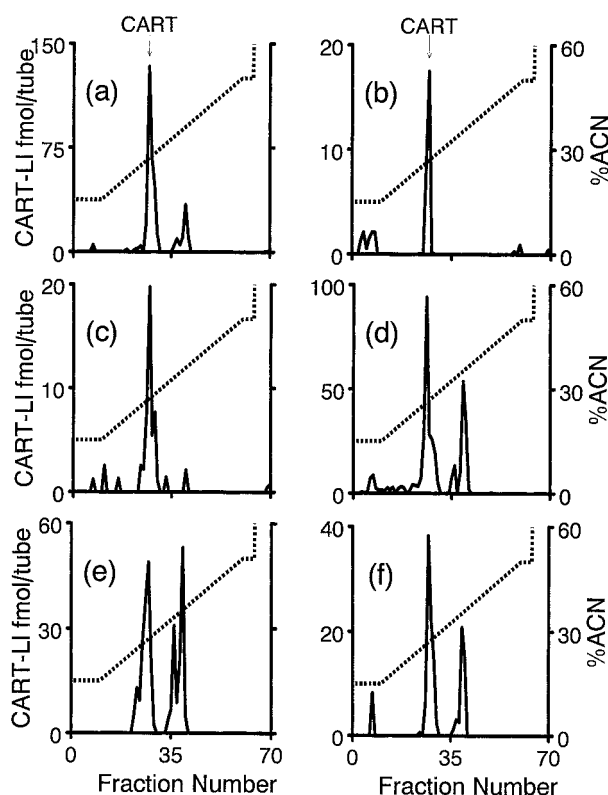
**Figure 3** Sephadex G-50 column chromatography of rat (a) hypothalamus, (b) anterior pituitary, (c) posterior pituitary, (d) stomach, (e) duodenum and (f) ileum extracts (means  $\pm$  S.E.M.;  $n=3$ ). CART=the elution position of CART (55–102); CC=the elution position of horse heart cytochrome C.

(Couceyro *et al.* 1998) by immunocytochemistry. A particularly high density of CART peptide containing fibres has been observed by immunohistochemistry in the posterior pituitary (Koylu *et al.* 1997), which is in accord with the very high concentrations of CART-LI that we detect. The function of CART peptide in the posterior pituitary is at present unknown and requires further investigation. Western blotting has been used to estimate CART peptide levels in rat hypothalamus and gave a concentration of 1  $\mu\text{g/g}$ , a level similar to that which we found here (Kuhar & Yoho 1999).

The G-50 and Northern blot results are less straightforward. It seems possible that the IR peaks observed at approximately  $K_{av}$  0.15 correspond to the partially processed CART peptide (1–102) and/or (10–102). The IR peaks with  $K_{av}$  values of 0.4 seem likely to correspond to the CART peptide fragment (55–102) and/or (62–102), as the CART (55–102) standard that we used eluted with the same approximate  $K_{av}$ .

However, Northern blot reveals the presence of CART mRNA in only the hypothalamus, anterior pituitary, brain stem, cerebellum and spinal cord. CART is postulated to be synthesised as pro-CART (1–102), so the presence of

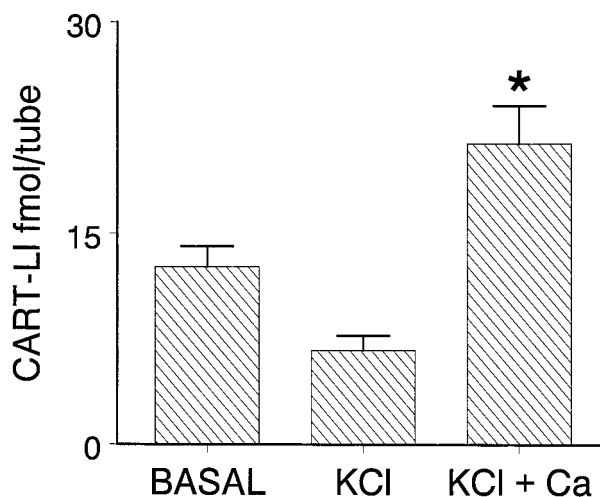
a larger CART-LI peptide fragment that may correspond to CART (1–102) peptide is expected in these regions. But the G-50 profiles show that a CART-LI peptide fragment with approximately the same  $K_{av}$  is also found in the stomach, duodenum and ileum. The posterior pituitary shows only one major IR peak at a  $K_{av}$  of 0.4, which is in accord with the lack of mRNA detected by Northern blot. The posterior pituitary consists primarily of nerve terminals extending from the PVN and the SON of the hypothalamus. These nuclei are known to contain CART mRNA (Vrang *et al.* 1999), and are presumably the source of CART-LI peptide detected in the posterior pituitary. It has been suggested that previous attempts to detect CART mRNA in the rat small intestine (Douglass *et al.* 1995) failed because of its presence at only low levels (Couceyro *et al.* 1998). Immunohistochemical studies have shown CART peptide-immunoreactive neuronal cell bodies in rat ileum, which suggests that CART peptide may be synthesised in the ileum (Couceyro *et al.* 1998). However, in this report we have demonstrated that CART mRNA was detectable in brain stem, cerebellum and spinal cord, which show much lower concentrations of CART-LI than the majority of the GI tract, but that CART mRNA was



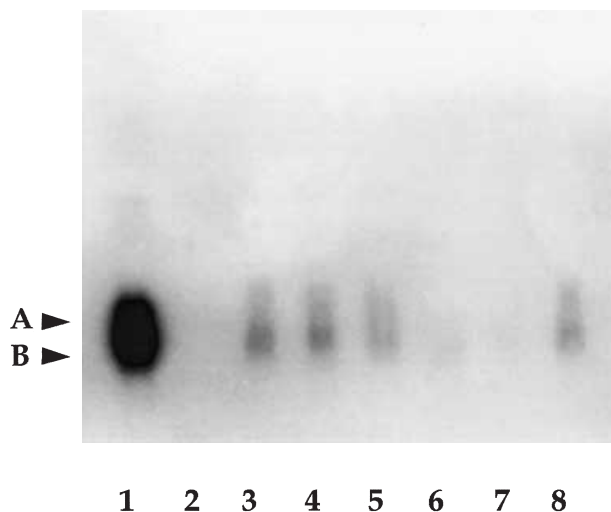
**Figure 4** Representative FPLC profiles of (a) hypothalamus, (b) anterior pituitary, (c) posterior pituitary, (d) stomach, (e) duodenum and (f) ileum extracts. Solid lines = CART-LI concentration; broken lines = %ACN. CART = the elution position of CART (55–102).

undetectable in the GI tract. This suggests that the CART-LI detected in the GI tract may be synthesised in other tissues, for example, the spinal cord. Further investigation is required to clarify whether the CART-LI is present in sensory fibres, and therefore perhaps dorsal root ganglia. A possible approach might be to study the presence of CART-LI in the GI tract of neonatally capsaicin-treated rats (Mulderry *et al.* 1988). It is also possible that the mRNA detection techniques used were not sufficiently sensitive. Another possibility is that alternative transcript(s) exist in the GI tract, but further investigation of this was beyond the scope of this study. We did not check for consistency in loading exactly the same amount of total mRNA in each lane, because the primary aim was detection of mRNA rather than quantification. The two bands seen in Northern blot analysis are thought to correspond to the alternate forms of rat CART mRNA produced by differential polyadenylation (Douglass *et al.* 1995).

FPLC of tissue extracts revealed that CART-LI elution profiles differed between regions. It is possible to discern a trend for higher proportions of the CART-LI fragment



**Figure 5** Release of CART-LI from medial basal hypothalamic explants. CSF CART-LI levels were measured after 45-min incubation alone in low  $\text{Ca}^{2+}$ -aCSF, in the presence of KCl (56 mmol/l) without  $\text{Ca}^{2+}$  and then KCl (56 mmol/l) with 1.4 mmol/l  $\text{CaCl}_2$ . The results are the means  $\pm$  S.E.M. of ten experiments. \* $P < 0.05$  compared with basal.



**Figure 6** Northern blot analysis of 50 µg total RNA extracted from the tissues listed below. The filter was hybridised with a probe corresponding to nucleotides 100–409 of rat CART. A, 900 bp band; B, 700 bp band. Lane 1, hypothalamus; 2, liver; 3, pituitary; 4, brain stem; 5, brain cortex; 6, stomach; 7, duodenum; 8, spinal cord.

eluting at 35% ACN in the GI tract, which may be equivalent to the small IR peak eluting at 36% in the hypothalamic extract profile. In addition, the CART-LI fragment eluting at 33% ACN in stomach and duodenum extract may be equivalent to another, smaller hypothalamic IR peak. It is possible that the relevant CART peptide fragments have very similar hydrophobicity and that separation based on hydrophobicity is of limited use.

Previous work with RP-HPLC has demonstrated that the four CART peptide fragments (49–102), (54–102), (61–102) and (62–102) elute with very similar retention times (Thim *et al.* 1998). The major IR peaks from anterior and posterior pituitary extracts elute at the same %ACN, when we would expect them to represent the peptides CART (55–102) and CART (62–102) respectively (Thim *et al.* 1999). We would therefore not expect fragments differing by only a few amino acids to be resolvable by FPLC.

Significantly lower hypothalamic, anterior and posterior pituitary CART-LI peptide levels were found in fa/fa rats than in Fa/+ rats, as might be expected from the lowered mRNA levels previously observed (Kristensen *et al.* 1998). Unexpectedly, CART immunoreactivity was undetectable (<0.6 pmol/g) in the anterior pituitary of fa/fa rats and almost absent from those of Fa/+ rats. The low anterior pituitary CART-LI concentration in male Fa/+ rats when compared with male Wistar rats is probably a result of genetic variation between the two inbred strains. This implies that the anterior pituitary CART-LI content may be dependent on factors other than the effects of leptin that influence Zucker, but not Wistar, rats. The anterior pituitary extracts contained levels of the anterior pituitary peptide substance P which did not differ significantly from control Wistar rats, suggesting that failed extraction or unviable tissue is not responsible for the different concentration of CART-LI in the extracts.

Other groups have observed that CART immunoreactivity is found in the dense core vesicles of dendrites (Smith *et al.* 1997), and is co-localised with other neurotransmitters (Elias *et al.* 1998). The calcium-dependent release of CART-LI from static incubated hypothalami by potassium stimulation is also in accord with a role for CART peptide as a neurotransmitter (Smith *et al.* 1997).

The results of the present study demonstrate that rat hypothalamus, cortex, cerebellum, brain stem, pituitary gland, adrenal gland, spinal cord, stomach, duodenum, jejunum, ileum, caecum, ascending and descending colon and rectum contain CART-LI. This distinctive distribution pattern involves tissues separate from the hypothalamus and the hypothalamic–pituitary–adrenal (HPA) axis, and suggests that CART peptide may be involved in various physiological processes besides appetite regulation, and, as previously postulated, modulation of the HPA axis (Koylu *et al.* 1997, 1998, Kuhar & Dall Vechia 1999).

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