The hypothalamic satiety peptide CART is expressed in anorectic and non-anorectic pancreatic islet tumors and in the normal islet of Langerhans

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Received 4 February 1999

Abstract The hypothalamic satiety peptide CART (cocaine and amphetamine regulated transcript) is expressed at high levels in anorectic rat glucagonomas but not in hypoglycemic insulinomas. However, a non-anorectic metastasis derived from the glucagonoma retained high CART expression levels and produced circulating CART levels comparable to that of the anorectic tumors. Moreover, distinct glucagonoma lines derived by stable HES-1 transfection of the insulinoma caused severe anorexia but retained low circulating levels of CART comparable to that of insulinoma bearing or control rats. Islet tumor associated anorexia and circulating CART levels are thus not correlated, and in line with this peripheral administration of CART (5–50 mg/kg) produced no effect on feeding behavior. In the rat two alternatively spliced forms of CART mRNA exist and quantitative PCR revealed expression of both forms in the hypothalamus, in the different islet tumors, and in the islets of Langerhans. Immunocytochemistry as well as in situ hybridization localized CART expression to the somatostatin producing islet D cell. A potential endocrine/paracrine role of islet CART remains to be clarified.

Key words: CART (cocaine and amphetamine regulated transcript); Islet of Langerhans; Islet tumor; Anorexia; Somatostatin

1. Introduction

Our recent work on the characterization of CART (cocaine and amphetamine regulated transcript) as a potent anorectic hypothalamic peptide [1,2] originated from attempts to characterize the active principle in transplantable rat glucagonomas causing severe anorexia [3,4]. As we had derived anorectic as well as non-anorectic glucagonomas of common origin [5,6], such representative in vitro cultures were screened by subtraction cloning to identify unique mRNAs associated with the anorectic phenotype. CART was thus identified and subsequently cloned, expressed in yeast [2] and recombinant CART was shown to have powerful effects on feeding behavior when administered centrally [1]. CART was originally identified as a brain peptide with high expression in the hypothalamus [7] but immunoreactive CART and CART mRNA have subsequently been localized to a number of central peripheral neurons as well as to pituitary and adrenal glands [8–11].

In the present study we show that islet tumor expression of CART is not confined to the anorectic phenotype and thus possibly does not at all contribute to the observed anorexia. Additionally, we show that CART is a product of the normal islet of Langerhans, where the rat pre-mRNA is differentially spliced to give rise to two transcripts encoding distinct CART polypeptides differing by a 13 amino acid insert. Both mRNA forms are expressed in a balanced ratio in islet as first demonstrated in the hypothalamus [7]. We also present evidence that mRNA and immunoreactive CART are confined to the somatostatin producing islet D cell in the rat.

2. Materials and methods

2.1. CART mRNA expression analysis by multiplex RT-PCR

RNA was prepared as previously described [12] using the RNAzol method (Cinna Biotecx). Tissues included rat hypothalamus ($n=6$), isolated newborn rat islets ($n=5$ (batches)), a panel of islet tumors [12], and liver, kidney, heart, and white adipose tissue as negative controls. First-strand cDNA was prepared from 1 μg total RNA using random primers (Gibco) as described [12]. The multiplex PCR was run in 50 μl volume, using 1 μl cDNA and a standard PCR mix, with buffer (1×) including $MgCl_2$ (1.5 mM) and a dNTP mix with 40 μM of dCTP, dGTP, dTTP, and 20 μM dATP, 2.5 units of Taq polymerase (all Promega, all final concentrations) and the PCR products were labeled by the addition of 2.5 μCi of 1000–3000 Ci/mmol [α-^{33}P]dATP (Amersham). 10 pmol of each oligo was used and the PCR reaction run with the simultaneous addition of two primer sets, one specific for rat CART and the other for the internal standard G6PDH. Rat CART oligos: 5′-oligo 5′-GGATGATGCGTCCCATG-3′, 3′-oligo 5′-GGAACCGAAGGAGGCTGTCACC-3′; rat G6PDH oligos: 5′-oligo 5′-GACCTGCAGAGCTCCAATCAAC-3′, 3′-oligo 5′-CACGACCCTCAGTACCAAAGGG-3′. 50 μl mineral oil was added to each tube. PCR conditions were: denaturation at 95°C for 1.5 min, followed by 22 cycles of 94°C/30 s, 55°C/1 min, and 72°C/1 min. The PCR reactions were analyzed on a 6% polyacrylamide/7 M urea gel and then exposed overnight to a Phosphor-Imager screen for quantification. The method and the use of MPX-PCR for the quantification of mRNA have been thoroughly described and discussed in [12], but in short the simultaneous use of two primer sets with the one specific for an internal standard ensures than any variation in set-up of cDNA and PCR is reflected also in the internal standard, so a result is always related to the amount of standard. We furthermore limited the number of PCR cycles to ensure that we were in the linear range of amplification for both products.

2.2. Immunocytochemistry and in situ hybridization

2.2.1. Immunocytochemistry. The CART rabbit serum 2055A was raised against a glutathione *S*-transferase-CART fusion protein as

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PII: S0014-5793(99)00291-4

described [1] using the full length coding region of the short transcript CART(1–89). CART rabbit serum 2025A was raised against the 13 amino acid insert unique to the long isoform of CART (PRRQLRAPGAVLQ) coupled to ovalbumin as carrier. Microwave pretreated (2×500 W for 5 min), dewaxed paraffin sections (4 μm) of rat pancreas were double stained by overnight incubations of combinations of polyclonal rabbit anti-rat CART (2055A or 2025A) and monoclonal anti-islet hormone antibodies (GLU-001 anti-glucagon, SOM-018 anti-somatostatin, or HUI 18 anti-insulin, all Novo Nordisk A/S). CART and islet hormone immunoreactivity were visualized by incubation with combinations of fluorochrome conjugated species specific secondary antibodies (FITC anti-rabbit and Texas red anti-mouse, both from Jackson Laboratories). Single antibody staining (indirect immunoperoxidase) was performed by using the Histo-stain kit from Zymed Laboratories, San Francisco, CA. Specificity controls included (1) antigen preabsorption (25 μg/ml of immunizing or irrelevant antigen preincubated with diluted antiserum for 1 h prior to application on section); and (2) single (rabbit or mouse) primary antisera, developed with the mixture of fluorochrome-conjugated secondary antibodies to verify the species specificity of the latter.

2.2.2. Combined in situ hybridization and immunocytochemistry. In situ hybridization analysis was performed on cryostat sections of rat pancreas [13] using antisense RNA probes against the rat CART cDNA (bp 226–411; GenBank accession number U10071) as described in detail [1]. Following post-hybridization washes and RNase treatment, sections were stained using antibody to either insulin (ICN 65-1041), glucagon (DAKO A565), or somatostatin (DAKO A-0566). Bound antibodies were detected using secondary antibodies coupled with biotin followed by Streptavidin Texas red. Following immunohistochemistry sections were dehydrated in alcohol, dipped in liquid emulsion, and exposed in the dark for 28 days. Following development of the photographic emulsion sections were examined for colocalization of emulsion grains and immunofluorescent signal and images collected. Combined images of light-field emulsion grains and Texas red fluorescence was prepared using Adobe Photoshop.

2.3. Animal experiments

The appetite suppression activity of CART(42–89) was evaluated using the following method. Female NMRI mice (9 weeks old) were fasted for 24 h after exposure to the test diet, a nutritionally complete liquid diet (Complan). Thirty minutes before testing, a solution containing CART peptide or vehicle (phosphate buffered saline) was injected intraperitoneally (5, 10, 20, or 50 mg/kg, i.e. 125–1250 μg/mouse) in a volume of 0.25 ml (e.g. 0.01 ml/g body weight), or doses of 0.1, 0.2, 0.5, or 1 μg were injected into the lateral cerebral ventricle in a volume of 10 μl. Each mouse was placed in a 15×15×15 cm test box with a grid floor and a glass drinking tube containing the liquid diet. Food consumption was measured for 10 min by electronically recording the total amount of contact with the liquid diet during the test session. The degree of appetite suppression produced by a dose of CART was evaluated by comparing the duration of consumption by control mice with that of treated mice [2].

2.4. Radioimmunoassay of CART in plasma

Immunoreactive CART in plasma was measured by radioimmunoassay, against standards of recombinant CART(42–89) [2] using the polyclonal 2055A CART antiserum. CART(42–89) was radioiodinated by the lactoperoxidase method [14,15] and used as tracer. Assay buffer was 0.05 mol/l sodium phosphate containing in addition 0.1% human serum albumin (Behringwerke, Marburg, Germany) and 0.6 mol/l thimerosal (Sigma). 80 μl plasma samples or standard (+80 μl of charcoal treated plasma) were preincubated with 100 μl antibody solution (diluted 1000 times) in a total volume of 400 μl. After 24 h preincubation at 4°C, 100 μl of tracer (approximately 1 fmol labeled CART) was added. After a further 24 h, free and bound peptide were separated using plasma coated charcoal [16]. Under these conditions, the assay had a detection limit of 5 pmol/l, 50% displacement of label at 80 pmol/l, and an intra-assay coefficient of variation of 3–7% in the working range (5–320 pmol/l). Samples with high concentrations were assayed after dilution with charcoal treated plasma. Recovery of CART added to plasma deviated less than 15% from expected values, and dilutions of samples with high concentrations paralleled the standard curve.

CART levels were quantified in a collection of sera from NEDH rats carrying transplantable islet tumors, previously characterized to be associated with or without anorexia, as follows: MSL-A-M3 is a non-anorectic metastatic variant of MSL-A, an anorectic glucagonoma [5]; MSL-G-AN and MSL-G2-IN are well characterized anorectic glucagonomas and hypoglycemic insulinomas, respectively [3,4,17,18]; IN-HES control and IN-HES-AN represent insulinoma derived clones with (AN) or without (control) expression of recombinant HES-1 (hairy-enhancer of split-1). We have shown that constitutive expression of HES-1 converts hypoglycemic insulinomas into anorectic glucagonomas (J. Jensen and O.D. Madsen, unpublished data).

3. Results

3.1. CART mRNA level of expression in different rat islet tumors does not correlate with anorexia

We previously identified CART as being selectively expressed in anorectic versus non-anorectic glucagonoma cultures in vitro [2]. However, when testing corresponding in vivo propagated tumors we found expression in both types and therefore there was no obvious correlation with anorexia. Thus, the non-anorectic tumor had high CART mRNA expression levels comparable to the anorectic glucagonoma (MSL-A-M3 versus MSL-G-AN, Fig. 1) while the insulinoma (MSL-G2-IN, Fig. 1) had low levels. CART mRNA was undetectable in kidney, heart, white adipose tissue, and liver extracts (only shown for liver, Fig. 1).

3.2. Circulating level of islet tumor derived CART-like immunoreactivity does not correlate with anorexia

Using iodinated recombinant CART as tracer a radioimmunoassay was established allowing determination of circulating levels of CART-like immunoreactivity. Normal rat plasma levels were in the range of 14–184 pM (Table 1). Plasma from rats carrying transplantable islet glucagonomas MSL-G-AN and MSL-A-M3 all had moderately to highly elevated

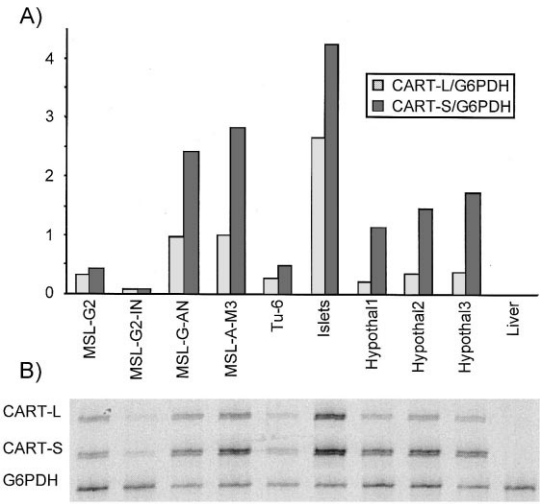

Fig. 1. RT-PCR measurement of CART mRNA. The two CART mRNA isoforms were quantified in total RNA extracts from various islet derived tumors, isolated normal islets, liver, and hypothalami by multiplex RT-PCR analysis (see Section 2). The primer set used for amplifying CART detects both splice variants (33 nucleotides in difference) and produces the two upper bands (CART-L and CART-S in B). G6PDH amplicon (glucose 6-phosphate dehydrogenase) serves as internal control and migrates as the lower band (in B). Quantification (% of G6PDH expression level) of the two splice variants in each sample from B is plotted as a bar diagram in A. Note that a similar balanced expression of the two variants are present in hypothalamus as well as in islets and islet derived tumors.

Table 1
Circulating CART and anorexia

Islet tumor (phenotype)	Rat ID	CART (pM)	Anorexia
MSL-A-M3	1	470	No
(non-anorectic glucagonoma)	2	475	No
	3	405	No
MSL-G-AN	4	421	Yes
(anorectic glucagonoma)	5	460	Yes
	6	292	Yes
	7	503	Yes
	8	138	Pre-onset
	9	190	Yes
MSL-G2-IN	10	52	No
(insulinoma)	11	62	No
	12	90	No
	13	37	No
	14	190	No
	15	17	No
IN-HES control	16	13	No
(insulinoma)	17	9	No
	18	31	No
	19	58	No
IN-HES-AN	20	139	Yes
(anorectic glucagonoma)	21	114	Yes
	22	53	Yes
	23	55	Yes
Control NEDH rats	24	131	No
	25	142	No
	26	57	No
	27	45	No
	28	14	No
	29	184	No

A collection of serum samples from rats transplanted with various types of islet derived tumors with or without severe anorexia (see Section 2) were analyzed for immunoreactive CART. Note that the anorectic (MSL-G-AN) as well as non-anorectic (MSL-A-M3) glucagonomas are associated with highly elevated CART levels compared to controls. Interestingly, the anorectic glucagonoma (IN-HES-AN) derived from an insulinoma (see Section 2) retains unchanged circulating CART levels when compared to control and insulinoma bearing rats.

levels of CART-like immunoreactivity. MSL-G-AN consistently produces anorexia in transplanted rats while MSL-A-M3 has little or no influence on food intake. Both of these tumors with similar high CART mRNA levels also produced the highest levels of circulating CART observed (Table 1). The MSL-G2-IN (insulinoma) and IN-HES transplanted rats had circulating levels of CART comparable to control rats. IN-HES in fact represents a glucagonoma phenotype induced by stable transfection of IN (insulinoma) in vitro cultures with HES-1 expression vector. While the presence of HES-1 strongly affects the regulation of the islet hormone genes and actually converts hypoglycemic insulinomas into anorectic glucagonomas (J. Jensen and O.D. Madsen, unpublished data), it apparently does not affect CART gene regulation.

In summary, there was no correlation between tumor induced anorexia (glucagonoma associated) and circulating levels of CART immunoreactivity (Table 1).

3.3. Peripheral administration of CART does not affect feeding

When CART(42–89) was injected intraperitoneally in mice at doses of 125–1250 μg food intake was not affected at any dose (ANOVA: $P > 0.1$). This is in distinct contrast to the effects of intracerebral injection of CART in the same experiment, which significantly inhibited food intake (ANOVA: $P < 0.05$) at doses as low as 0.2 μg (64% inhibition, $P < 0.001$). In this experiment the maximum appetite suppressive effect was a 76% reduction of liquid diet intake after 0.5 μg ($P < 0.01$).

3.4. CART is expressed by the somatostatin producing islet D cells

CART mRNA was clearly detectable in all RNA preparations from normal islets of Langerhans (only shown for one of five batch preparations, Fig. 1) with a relative abundance comparable to that found in RNA extracts from six individually dissected rat hypothalami (three shown, Fig. 1). CART mRNA is alternatively spliced in a similar balanced ratio in rat hypothalamus as well as in islets and derived endocrine tumors (Fig. 1). Immunocytochemistry using two different antisera raised against recombinant CART(42–89) (Ab 2055A) and the synthetic 13 amino acid peptide (Ab 2025A) representing the rat specific alternative splice variant produced identical staining on sections of rat pancreas (only shown for 2025A, Fig. 2). Double labeling with islet hormone antisera showed colocalization of CART and somatostatin immunoreactivity (Fig. 2C–E). This D cell specific staining was selectively abolished by preabsorption to the immunizing antigen but not to e.g. somatostatin (Fig. 2A,B). Finally, in situ hybridization combined with immunocytochemical staining for somatostatin confirmed the D cell specific expression of the CART mRNA (Fig. 2F–H).

4. Discussion

The pluripotent nature of MSL cells – transformed endocrine pancreatic cultures derived from a liver metastasis [6] of an X-ray induced insulinoma in the rat [19] – has been well documented (reviewed in [20]). We have thus established transplantable insulinomas [17], glucagonomas [3] as well as a somatostatin producing culture in vitro – all of common origin. Of particular interest has been the feature of severe anorexia associated only with the transplantable glucagonoma [3,4]. Our recent identification of yet another glucagonoma devoid of anorexia suggested that a non-proglucagon derived peptide could be involved. Transformed islet tissue has previously been the source of identification of crucial neuropeptides, such as GHRH [21,22], due to apparent ectopic expression. CART was similarly identified as a potential candidate for causing the observed glucagonoma associated anorexia due to its selective expression in the 'anorectic' in vitro culture [2].

CART proved to be a very powerful satiety peptide when administered intracerebroventricularly [1], a function compatible with its normal hypothalamic expression [7,8,23]. We have now shown that CART is also expressed in non-anorectic islet tumors and that we can find high levels of circulating CART immunoreactivity in blood from islet tumor transplanted rats with normal weight gain. These data suggest that the CART produced by the tumor does not contribute to the observed acute onset of anorexia. This is further supported by the fact that peripherally administered CART does not affect feeding. Finally, the HES-1 induced phenotypic conversion of insulinomas to glucagonomas is associated with the same severe anorexia despite unchanged low circulating CART levels – strongly arguing that the anorectic factor is distinct from CART. Islet tumor expression of CART is not

ectopic since CART is identified as a normal islet product – which we find selectively colocalized with somatostatin to the islet D cell. This highly cell specific expression in the islet contrasts with the broader expression in the different MSL tumor phenotypes, which by other features have been shown to reflect the corresponding non-transformed islet phenotype [12,18]. The function of islet CART remains unknown but could be paracrine as it may be co-secreted with somatostatin. CART was recently localized also to the pituitary and adrenal glands [8] but whether these peripheral sources of CART are also regulated by leptin as shown for hypothalamic CART [1] is unknown.

The original molecular cloning of rat CART [7] identified a series of potential dibasic sites for proteolytic processing. By alternative splicing to yield small (CART-S) and large (CART-L) isoforms of CART differing by a 13 aa insert [7] the larger isoform has yet an additional dibasic site to add to complexity in processing. In 1981 Spiess et al. identified and sequenced a CART fragment from ovine hypothalamic extracts [24] with an N-terminus starting at position 42 (of the short isoform). Recombinant and bioactive CART(42–89) appeared to fold into a unique structure containing three disulfide bridges [2]. This secondary structure is mandatory for biological activity measured as anorectic potential following i.c.v. administration [1] and has moreover very recently been verified to exist as a major extractable product (in addition to the N-terminally truncated CART(49–89) from isolated rat hypothalami [25]). Both of these products suppress feeding

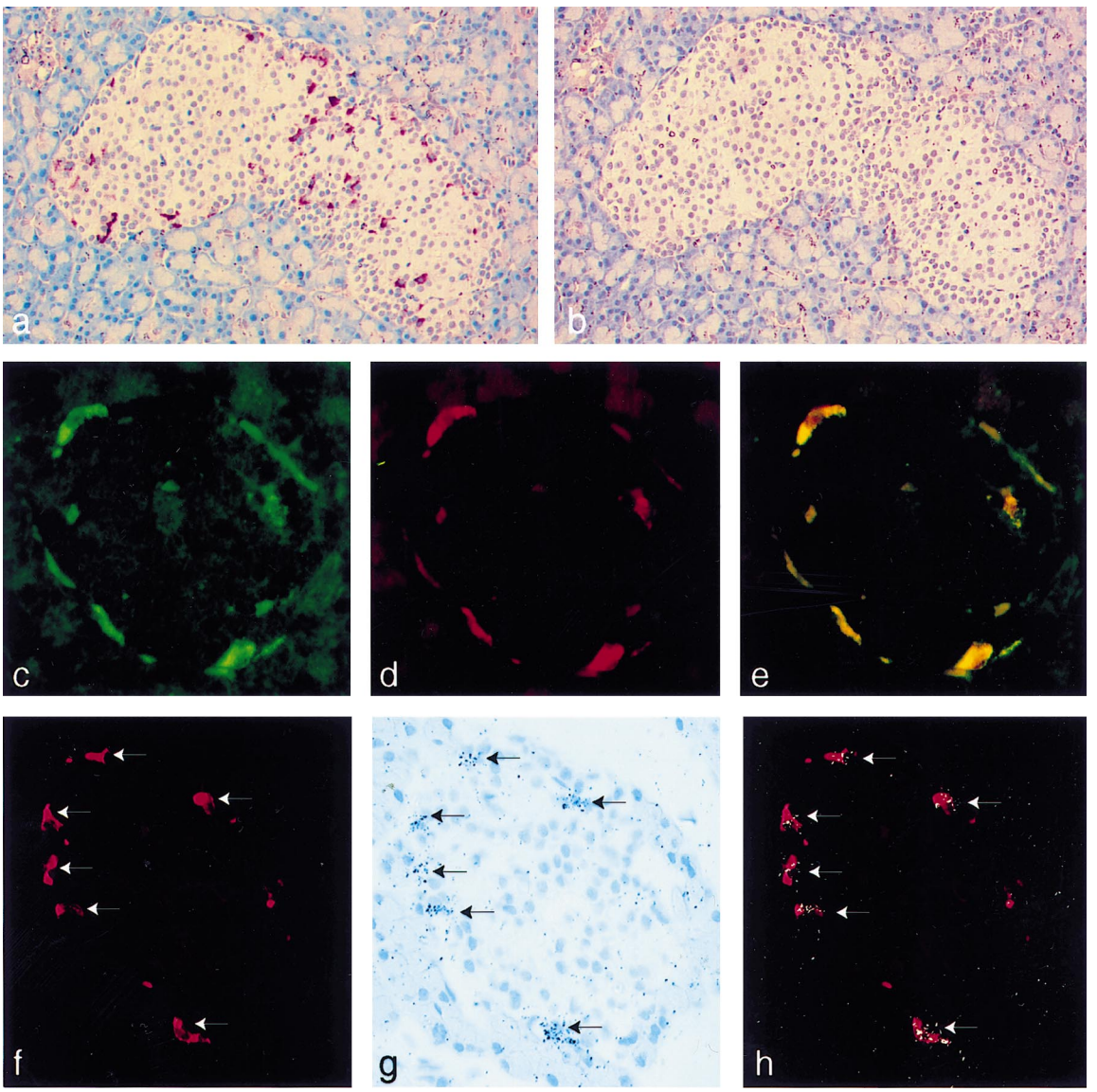

Fig. 2. Islet D cell expression of CART-like immunoreactivity and mRNA. Paraffin sections of rat pancreas (A–E) were stained for CART and islet hormones as follows. A: CART immunoperoxidase staining of a peripheral islet cell population using the peptide specific 2025A antiserum. B: Preabsorption of antiserum 2025A with the immunizing peptide antigen completely abolishes staining. Preabsorption to synthetic somatostatin had no effect (not shown). C–E: Rat pancreas double stained for CART (in green, C) and somatostatin (in red, D). Double exposure (in yellow, E) reveals complete overlap in the distribution of somatostatin and CART-like immunoreactivity. F–H: Cryostat sections of rat pancreas double labeled for immunoreactive somatostatin (in red, F) and CART mRNA by radioactive in situ hybridization (black grains (arrows), G). The overlay plot reveals complete overlap of immunoreactive somatostatin (in red, H) and CART mRNA (white grains, H) as indicated by the arrows.

when administered i.c.v. [2] – and curiously, a synthetic peptide representing CART(42–63) was also reported to have some activity [26] despite the fact that Cys-55 and Cys-61 normally would be engaged in disulfide bond formation with Cys-73 and Cys-81, respectively [2].

Interestingly, both CART-S and CART-L will contain the bioactive C-terminal domain – and it is not clear why differential N-terminal extensions exist since both can be processed to bioactive CART(42–89). Moreover, alternative splicing of CART mRNA does not take place in man where only the short peptide is found [27]. By the use of antisera raised specifically against the 13 aa insert and thus specific for pro-CART-L derived peptides we have clearly demonstrated that CART-L is translated into immunoreactive protein in the rat. The processing of islet CART is at present unknown but preliminary data from tumor extracts suggest an efficient processing similar to hypothalamic tissue. We thus believe that a substantial fraction of quantifiable CART by our RIA is likely equivalent to CART(42–89) or CART(49–89) – and that these species apparently do not cross the blood-brain barrier to any considerable extent.

In conclusion, the hypothalamic satiety peptide, CART, is expressed in the islet of Langerhans, where it is localized by immunocytochemistry and in situ hybridization to the somatostatin producing D cell. CART is produced by a variety of islet derived transplantable tumors but does apparently not contribute to acute onset of anorexia caused by certain glucagonoma lines. CART transcripts are differentially spliced to yield two mRNAs of 39 nucleotides in difference leading to a unique 13 amino acid insert in one isoform. Only the short form occurs in man but we have clearly demonstrated that the larger isoform is translated to CART-like immunoreactivity in rat islets. The function of islet derived CART is unknown. Elevated circulating CART may be diagnostic for neuro-endocrine tumors.

Acknowledgements: Erna Engholm Pedersen, Tove Funder Nielsen, Heidi Ingeman Jensen, Helle Petersen, Lene Albæk and Jette Mandelbaum are thanked for skilful technical assistance. This work was in part supported by the Danish Cancer Society and the Danish National Research Foundation (P.B.J., O.D.M.). Work related to the HES-1 mediated islet tumor phenotype conversion was supported by NIH Grant DK-55284. Hagedorn Research Institute is an independent basic research component of Novo Nordisk A/S.

References

[1] Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., Larsen, P.J. and Hastrup, S. (1998) Nature 393, 72–76.
[2] Thim, L., Nielsen, P.F., Judge, M.E., Andersen, A.S., Diers, I., Egel-Mitani, M. and Hastrup, S. (1998) FEBS Lett. 428, 263–268.
[3] Madsen, O.D., Karlsen, C., Nielsen, E., Lund, K., Kofod, H., Welinder, B., Rehfeld, J.F., Larsson, L., Steiner, D.F., Holst, J.J. and Michelsen, B.K. (1993) Endocrinology 133, 2022–2030.
[4] Jensen, P.B., Blume, N., Mikkelsen, J.D., Larsen, P.J., Jensen, H.I., Holst, J.J. and Madsen, O.D. (1998) J. Clin. Invest. 101, 503–510.
[5] Madsen, O.D., Karlsen, C., Blume, N., Jensen, H.I., Larsson, L.-I. and Holst, J.J. (1995) Scand. J. Clin. Lab. Invest. 55, (Suppl. 220) 27–36.
[6] Madsen, O.D., Larsson, L.-I., Rehfeld, J.F., Schwartz, T., Lernmark, Å., Labrecque, A. and Steiner, D.F. (1986) J. Cell Biol. 103, 2025–2034.
[7] Douglass, J., McKinzie, A.A. and Couceyro, P.R. (1995) J. Neurosci. 15, 2471–2481.
[8] Koylu, E.O., Couceyro, P.R., Lambert, P.D., Ling, N.C., Desouza, E.B. and Kuhar, M.J. (1997) J. Neuroendocrinol. 9, 823–833.
[9] Couceyro, P.R., Koylu, E.O. and Kuhar, M.J. (1997) J. Chem. Neuroanat. 12, 229–241.
[10] Couceyro, P.R., Paquet, M., Koylu, E.O., Kuhar, M.J. and Smith, Y. (1998) Synapse 30, 1–8.
[11] Koylu, E.O., Couceyro, P.R., Lambert, P.D. and Kuhar, M.J. (1998) J. Comp. Neurol. 391, 115–132.
[12] Jensen, J., Serup, P., Karlsen, C., Funder, T.F. and Madsen, O.D. (1996) J. Biol. Chem. 271, 18749–18758.
[13] Kristensen, P., Eriksen, J. and Danø, K. (1991) J. Histochem. Cytochem. 39, 341–349.
[14] Thorell, J.I. and Johansson, B.G. (1971) Biochim. Biophys. Acta 251, 363–369.
[15] Jørgensen, K.H. and Larsen, U.D. (1980) Diabetologia 19, 546–554.
[16] Holst, J.J. and Bersani, M. (1991) in: Methods in Neurosciences (Conn, P.M., Ed.), pp. 3–22, Academic Press, New York.
[17] Madsen, O.D., Andersen, L.C., Michelsen, B., Owerbach, D., Larsson, L.-I., Lernmark, Å. and Steiner, D.F. (1988) Proc. Natl. Acad. Sci. USA 85, 6652–6656.
[18] Blume, N., Skouv, J., Larsson, L.-I., Holst, J.J. and Madsen, O.D. (1995) J. Clin. Invest. 96, 2227–2235.
[19] Chick, W.L., Warren, S., Chute, R.N., Like, A.A., Lauris, V. and Kitchen, K.C. (1977) Proc. Natl. Acad. Sci. USA 74, 628–632.
[20] Madsen, O.D., Jensen, J., Blume, N., Petersen, H.V., Lund, K., Karlsen, C., Andersen, F.G., Jensen, P.B., Larsson, L.-I. and Serup, P. (1996) Eur. J. Biochem. 242, 435–445.
[21] Guillemin, R., Brazeau, P., Bohlen, P., Esch, F., Ling, N. and Wehrenberg, W.B. (1982) Science 218, 585–587.
[22] Rivier, J., Spiess, J., Thorner, M. and Vale, W. (1982) Nature 300, 276–278.
[23] Gautvik, K.M., Lecea, L., Gautvik, V.T., Danielson, P.E., Tranque, P., Dopazo, A., Bloom, F.E. and Sutcliffe, J.G. (1996) Proc. Natl. Acad. Sci. USA 93, 8733–8738.
[24] Spiess, J., Villarreal, J. and Vale, W. (1981) Biochemistry 20, 1982–1988.
[25] Thim, L., Kristensen, P., Nielsen, P.F., Wulff, B.S. and Clausen, J.T. (1999) Proc. Natl. Acad. Sci. USA (in press).
[26] Lambert, P.D., Couceyro, P.R., McGirr, K.M., Vechia, S.E.D., Smith, Y. and Kuhar, M.J. (1998) Synapse 29, 293–298.
[27] Douglass, J. and Daoud, S. (1996) Gene 169, 241–245.