Cocaine- and amphetamine-regulated transcript in the rat vagus nerve: A putative mediator of cholecystokinin-induced satiety

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Cocaine- and amphetamine-regulated transcript (CART) is widely expressed in the central nervous system. Recent studies have pointed to a role for CART-derived peptides in inhibiting feeding behavior. Although these actions have generally been attributed to hypothalamic CART, it remains to be determined whether additional CART pathways exist that link signals from the gastrointestinal tract to the central control of food intake. In the present study, we have investigated the presence of CART in the rat vagus nerve and nodose ganglion. In the viscerosensory nodose ganglion, half of the neuron profiles expressed CART and its predicted peptide, as determined by *in situ* hybridization and immunohistochemistry. CART expression was markedly attenuated after vagotomy, but no modulation was observed after food restriction or high-fat regimes. A large proportion of CART-labeled neuron profiles also expressed cholecystokinin A receptor mRNA. CART-peptide-like immunoreactivity was transported in the vagus nerve and found in a dense fiber plexus in the nucleus tractus solitarii. Studies on CART in the spinal somatosensory system revealed strong immunostaining of the dorsal horn but only a small number of stained cell bodies in dorsal root ganglia. The present results suggest that CART-derived peptides are present in vagal afferent neurons sensitive to cholecystokinin, suggesting that the role of these peptides in feeding may be explained partly by mediating postprandial satiety effects of cholecystokinin.

area postrema | feeding | nodose ganglion | nucleus of the solitary tract | spinal cord

Cocaine- and amphetamine-regulated transcript (CART; refs. 1 and 2) and its putative peptide(s) (CARTp; refs. 3 and 4) are widely distributed along the neuraxis. One prominent function of CARTp in the central nervous system seems to be inhibition of feeding. Thus, intracerebroventricular injection of predicted CART protein fragments blocks spontaneous feeding, as well as feeding induced by treatment with neuropeptide Y (5, 6). Furthermore, administration of antibodies to predicted CARTps stimulates feeding (5, 6), indicating that endogenous CARTp contributes to dampening of the feeding drive. Several possible anatomical substrates for these effects have been described. In the hypothalamus, CART is expressed in the paraventricular, arcuate, dorsomedial, and lateral hypothalamic nuclei (1–3, 6), all of which are areas implicated in the control of feeding.

In addition to the hypothalamus, the vagus nerve plays a major role in determining the parameters of energy metabolism. The vagus nerve transmits mechanoceptive and chemoceptive information from organs of the upper gastrointestinal tract to the nucleus of the solitary tract (NTS; refs. 7–9; see also refs. 10 and 11). This information has been postulated to regulate food intake on a short-term basis, in particular by inducing meal termination (12). In addition, signals influencing the central control of, e.g., blood pressure and respiration are also transmitted through this pathway (see ref. 13). To investigate whether vagal signaling may be partly CART encoded, we have, in the present study, analyzed the presence of CART and its putative peptides in the nodose ganglion, where cell bodies of vagal afferents reside, as well as in the vagus nerve proper. Because the gastrointestinal hormone cholecystokinin (CCK) has also been shown to induce satiety (14), likely through actions on vagal afferents (15), we have also studied the relation between CART and CCK_A receptor mRNA in nodose ganglion neurons. Finally, we have employed CARTp histochemistry on dorsal root ganglia (DRGs) to compare the expression of this peptide in viscerosensory and somatosensory ganglia.

Materials and Methods

Animals. Male Sprague–Dawley rats (250–300 g; B & K Universal, Sollentuna, Sweden) were divided into treatment groups as follows. In one group, animals ($n = 5$; vagotomy) were unilaterally vagotomized by transection of the left proximal vagus nerve (approximately 15 mm distal to the ganglion) 1 week before dissection and processing for *in situ* hybridization. In a second group, three animals underwent the same procedure and were used for immunohistochemistry. A third group of animals ($n = 5$; food restriction) was served only 10 g of standard rodent chow in pellet form (B & K Universal) per day for 14 days. Control animals for this group ($n = 5$) were allowed to feed on the same chow ad libitum. A fourth group of animals ($n = 6$; high-fat diet) were allowed to feed ad libitum on a diet consisting of 33% fat (B & K Universal); control animals for this group ($n = 6$) were allowed to feed ad libitum on standard rodent chow (B & K Universal) in powder form, which was combined with water to achieve the same consistency as that of the high-fat diet. A fifth group of three naïve animals was used for double-label *in situ* hybridization. In the sixth group of rats ($n = 3$), the left proximal vagus nerve was exposed and pinched with a watchmaker's forceps for 30 s, and these rats were sutured and returned to home cages for 24 h before perfusion for immunohistochemistry. A seventh group of animals ($n = 2$) were stereotactically injected with colchicine (120 μg in 20 μl of 0.9% NaCl; Sigma) in the lateral ventricle 24 h before perfusion. Finally, in the eighth group, four naïve rats were perfused, and their nodose ganglia and lumbar DRGs were excised and processed for immunohistochemistry. The experiments were approved by the local ethical committee, Stockholm's norra djurförsöksetiska nämnd. For *in situ* hybridization, the ganglia were extirpated under deep anesthesia and frozen. For immunohistochemistry, rats were anesthetized with 0.5 ml of sodium pentobarbital (Mebumal, i.p.) and perfused via the ascending aorta with formalin-picric acid. The tissue was excised, immersion fixed, and rinsed (16). All tissues were cut to 14-μm

Abbreviations: CART, cocaine- and amphetamine-regulated transcript; CARTp, CART-predicted peptide; CCK, cholecystokinin; DRG, dorsal root ganglion; NTS, nucleus of the solitary tract; -LI, -like immunoreactivity; -ir, -immunoreactive.

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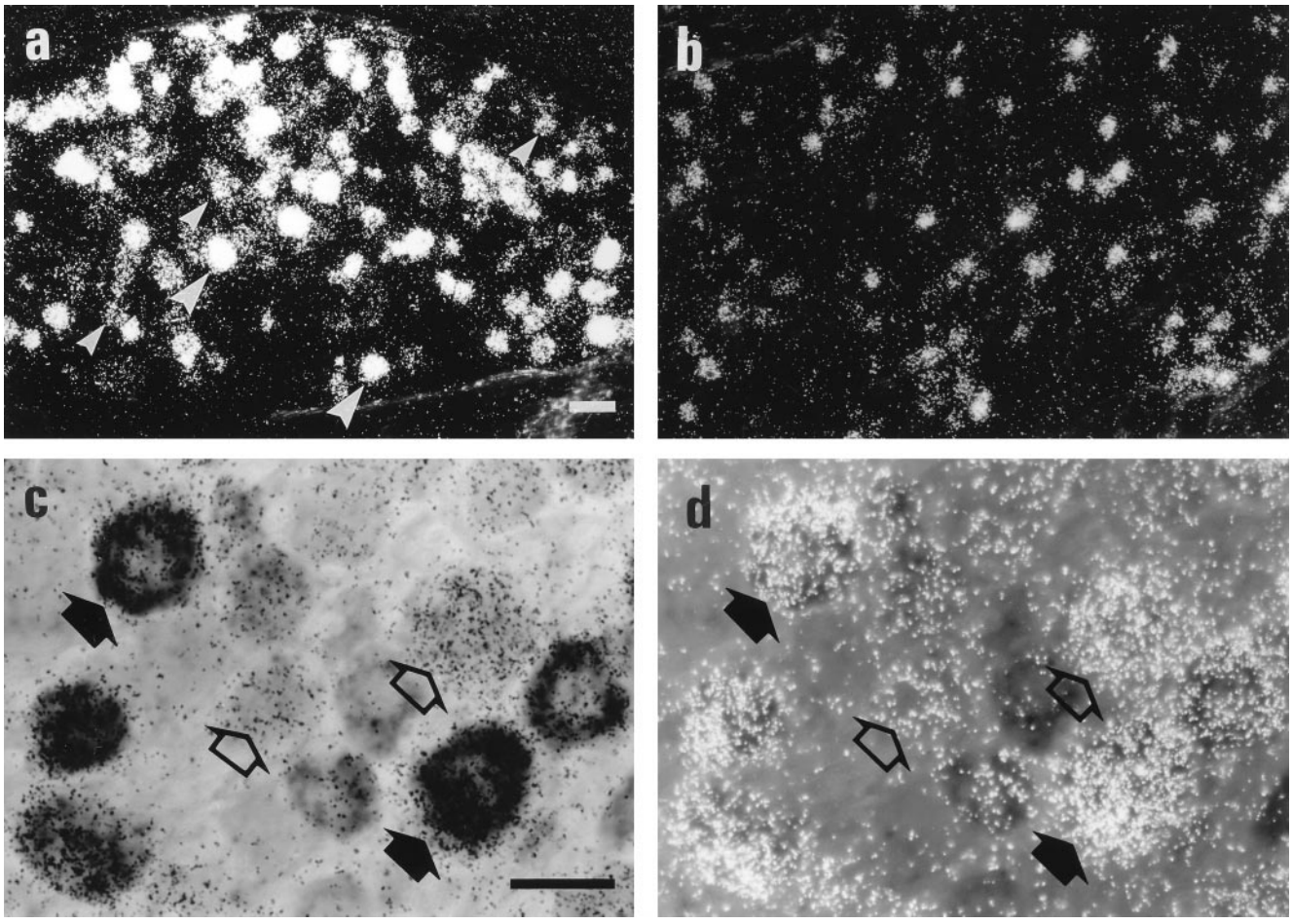

Fig. 1. Darkfield micrographs of sections of nodose ganglia hybridized for CART from normal (*a*) and ipsilaterally vagotomized (*b*) rats. Large arrowheads indicate strongly labeled neuron profile, and small arrowheads indicate weakly labeled profiles in *a*. (For *a* and *b*, bar in *a* = 50 μm.) Brightfield micrographs (*c* and *d*) show a section of rat nodose ganglion hybridized simultaneously for CART (silver grains) and CCK_A receptor mRNA (dark precipitate) viewed without (*c*) or with (*d*) epiillumination. Note CART signal in all CCK_A receptor mRNA-labeled neuron profiles (filled arrows), as well as several CART single-labeled profiles (open arrows). (For *c* and *d*, bar in *c* = 25 μm.)

thicknesses in a cryostat and thaw mounted onto precoated glass slides.

***In Situ* Hybridization.** Probes complementary to nucleotides 223–270 of the rat CART mRNA (1) and to nucleotides 192–239, 279–326, 922–969, and 969-1016 of the rat CCK_A receptor mRNA (17) were synthesized (Scandinavian Gene Synthesis, Köping, Sweden). Terminal deoxynucleotidyl transferase (Amersham Pharmacia) was used for 3′ end labeling of the CART probe with [^{35}S]dATP (New England Nuclear; ref. 18) to a specific activity of 5×10^9 cpm/μg, followed by purification with Qiaquick Nucleotide Removal Kit (Qiagen, Chatsworth, CA) and labeling of the oligonucleotides complementary to CCK_A receptor mRNA with digoxigenin-11-dUTP (16, 19). The ganglion sections were processed as described (16, 18): incubation for 16 h at 42°C with 1.5 ng of CART probe and (for double-labeled sections) with 12.5 nmol/liter CCK_A receptor probes diluted in a hybridization solution (18) followed by extensive rinsing in SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7). Single-labeled slides were then dehydrated before coating with photographic emulsion. Double-labeled sections were preincubated with BSA and incubated overnight at 4°C with alkaline-phosphatase-conjugated antidigoxigenin F(ab) fragment (1:5,000; Roche Molecular Biochemicals). Alkaline phosphatase activity was developed by incubating the sections with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (GIBCO/BRL). The sections were air dried, dipped in Kodak NTB2 (single-labeled sections) or Ilford K5 nuclear emulsion (double-labeled sections), exposed for 2 weeks, developed, and fixed. After counterstaining with toluidine blue and mounting in glycerol, the sections were analyzed in a Nikon Microphot-FX microscope equipped with a darkfield condenser. Photomicrographs were taken with Kodak T-max 100 film.

Quantification and Statistics. The percentage of labeled neuron profiles in the nodose ganglia was counted in toluidine blue-counterstained sections under epiillumination combined with brightfield illumination. Neuron profiles could be clearly distinguished, with autoradiographic signal visible as silver grains overlying the cytoplasm. Profiles containing at least three times more grains than the background were considered to be labeled. For each marker (mRNA), five sections per ganglion were counted, the sections being separated by a minimum distance of 80 μm. Counting was performed through a $\times$20 objective by using a rectangular box placed in the eye-piece that was systematically moved over the specimen. Neuron profiles crossing the lower and right-hand edge of the box were excluded from counting. The relative density levels were measured over a total of 75 neuron profiles from five sections per ganglion. Each image was digitized with 256 grey levels for each picture element by

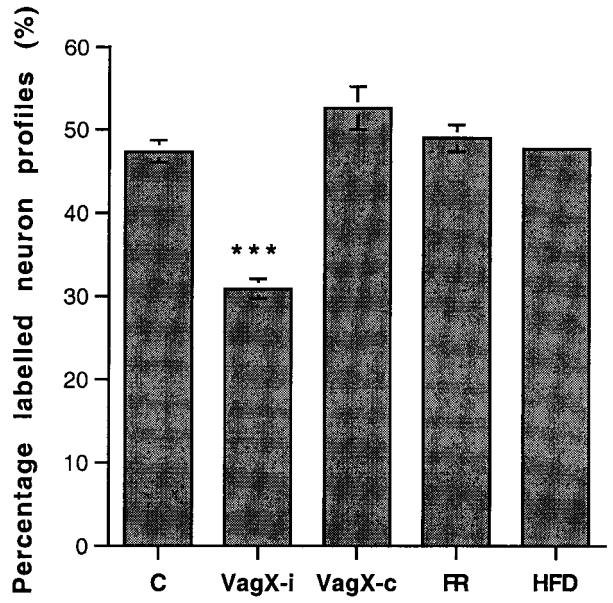

Fig. 2. Bar graph showing percentage of labeled neuron profiles in control (C), ipsilaterally (VagX-i), and contralaterally (VagX-c) vagotomized rats and in rats exposed to food restriction (FR) or high-fat diet (HFD). The data are presented as means ± SEM. ***, $P < 0.001$.

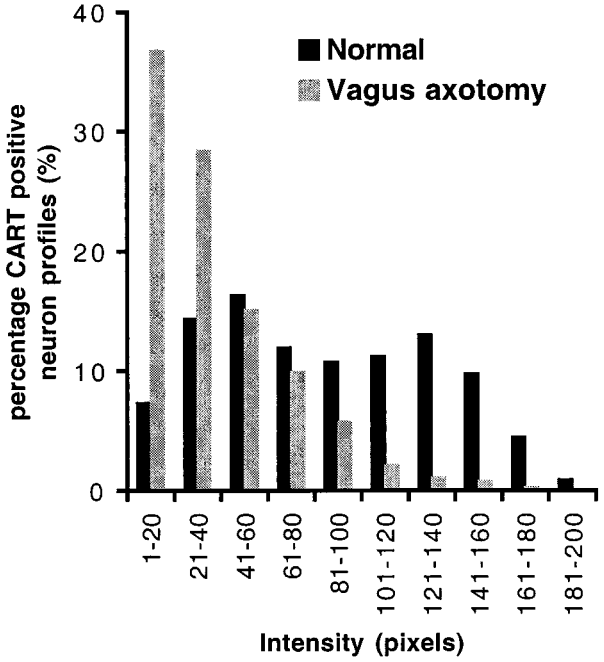

Fig. 3. Histogram showing relative density levels for CART over nodose neuron profiles in controls and 7 days after ipsilateral vagotomy; 75 profiles were measured in five sections from each ganglion in each group.

using a Macintosh IIx computer-imaging system equipped with a DAGE-MTI CCD-72 series camera connected with a Nikon Microphot-FX microscope through ×20 magnification. Statistics were performed by using an unpaired *t* test, ANOVA, and Dunnett's post hoc test. Data are expressed as means ± SEM.

Immunohistochemistry. Sections were processed for indirect immunofluorescence staining by using the Renaissance kit (New England Nuclear) for the tyramide signal amplification method (20) as described (16). Sections were incubated with CART antiserum (C4 antiserum; ref. 3), diluted 1:5,000 at 4°C overnight, rinsed, preincubated with Blocking Reagent (New England Nuclear), incubated for 30 min with horseradish-peroxidase-conjugated swine anti-rabbit Ig (1:100; Dako A/S), rinsed, and incubated for 8 min with a 1:50 dilution of biotinyl tyramide in 1× amplification diluent (New England Nuclear). After rinsing, the sections were incubated for 30 min with fluorescein-conjugated streptavidin (Amersham Pharmacia) diluted at 1:200, rinsed, mounted, and examined in a Nikon Microphot-FX microscope. Photographs were taken on black-and-white Kodak Tri-X film. Confocal microscopy samples were recorded by using a Bio-Rad Radiance Plus confocal microscope with 488-nm excitation. Optical slices of 0.5-μm thicknesses (n = 15) were sampled and combined to produce composite confocal images, which were printed on an Epson Stylus PhotoEX printer.

Controls. To control for specificity, some sections were incubated with a hybridization mixture containing an excess (100×) of nonlabeled probe. Specificity of the CARTp immunostaining was established by preabsorption of the antiserum with a synthetic peptide corresponding to the predicted amino acids 106–129 of rat CART (3) before immunohistochemistry.

Results

***In Situ* Hybridization.** In normal nodose ganglia, labeling for CART was seen in a large population (47.4 ± 1.3%) of neuron profiles (Figs. 1*a* and 2). Measurements of signal intensity indicated both strongly and more moderately labeled cells in the nodose ganglion, resulting in a slightly bimodal intensity curve (Fig. 3). In vagotomized nodose ganglia, a significantly lower number (30.9 ± 1.2%) of neuron profiles expressing CART was observed (Figs. 1*b* and 2). A prominent decrease in signal intensity over individual profiles could also be observed (Fig. 1, compare *b* with *a*; Fig. 3). No significant differences in number of CART-labeled neuron profiles were found in ganglia contralateral to the lesioned vagus nerve (Fig. 2; 52.6 ± 2.6%), in nodose ganglia from animals subjected to a 2-week food restriction (49.0 ± 1.6%), or in nodose ganglia from animals subjected to feeding with a high-fat diet (47.7 ± 0.5%; Fig. 2). In nodose ganglia double-labeled for CART and CCK_A receptor mRNA, CCK_A receptor mRNA signal was observed in a large number of neuron profiles (Fig. 1 *c* and *d*). All CCK_A receptor mRNA-expressing profiles also contained CART (Fig. 1 *c* and *d*). The majority of CART-expressing neuron profiles also expressed CCK_A receptor mRNA.

Immunohistochemistry. CARTp-like immunoreactivity (-LI) of varying intensity was observed in about half of the neuron profiles in nodose ganglia from normal animals (Fig. 4*a*) in a Golgi-like, granular configuration (Fig. 4*b*). In nodose ganglia vagotomized 7 days before perfusion, CARTp-LI was more intense and distributed diffusely throughout the cytoplasm, extending to the initial segment of the axon (Fig. 4*c*). However, no apparent difference in the total proportion of CARTp-ir neuron profiles could be detected in vagotomized as compared with contralateral and normal ganglia. CARTp-ir fibers were abundant within the ganglia, intermingled with the perikarya (Fig. 4*a*), as well as in the peripheral nerve, distal of the ganglion (Fig. 4*d*). Nerve fibers surrounding both CARTp-LI-positive and CARTp-LI-negative neuron profiles could be observed. Pinched peripheral vagus nerves displayed accumulation of CART-LI both on the proximal side of the lesion and, less prominently, on the distal side (Fig. 4*e*). In contrast to the large proportion of CARTp-ir neuron profiles in the nodose ganglion, only about four to five cells containing CARTp-LI could be observed per section in DRGs (Fig. 5).

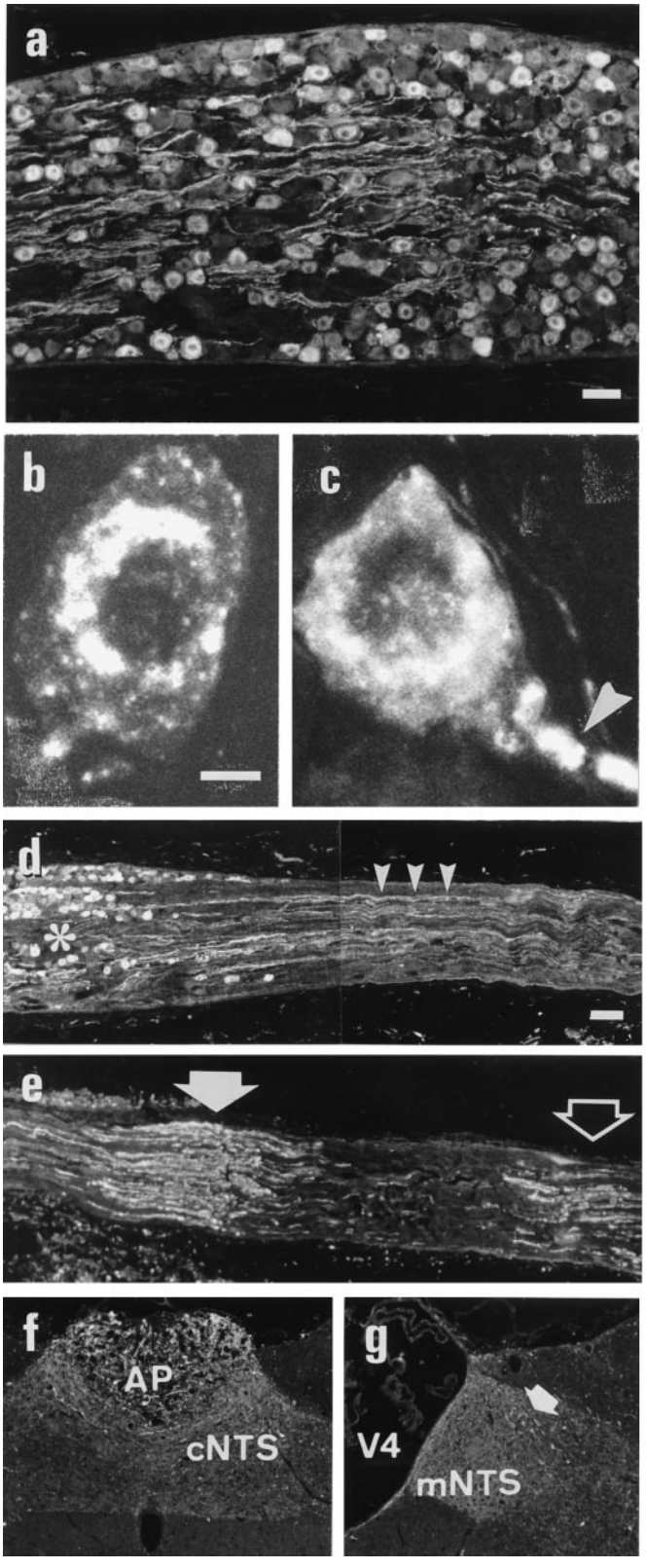

Fig. 4. Immunofluorescence (*a* and *d–g*) and confocal (*b* and *c*) micrographs of sections of rat control (*a* and *b*) and ipsilaterally vagotomized (*c*) nodose ganglia, normal (*d*) and pinched (*e*) rat peripheral vagus nerve, area postrema (*f*), and a more rostral section of the brain stem (*g*) stained with antiserum against CARTp. Stained fibers are seen both in the nodose ganglion (*a*) and vagus nerve (small arrowheads in *d*). Note accumulation of CARTp-LI in the cell body and the initial segment of vagotomized cell body (large arrowhead in *c*), as well as on the proximal (large filled arrow in *e*) and distal (large open arrow in *e*) side in the vagus nerve. Small filled arrows in *g* indicate CARTp-immunoreactive (-ir) cell bodies in the NTS. The asterisk in *d* indicates nodose ganglion. AP, area postrema; cNTS, commissural part of the NTS; medial part of the NTS; V4, fourth ventricle. (*a*, Bar = 50 μm for *a*; *b*, bar = 5 μm for *b* and *c*; *d*, bar = 100 μm for *d* and *e*, and 200 μm for *f* and *g*.)

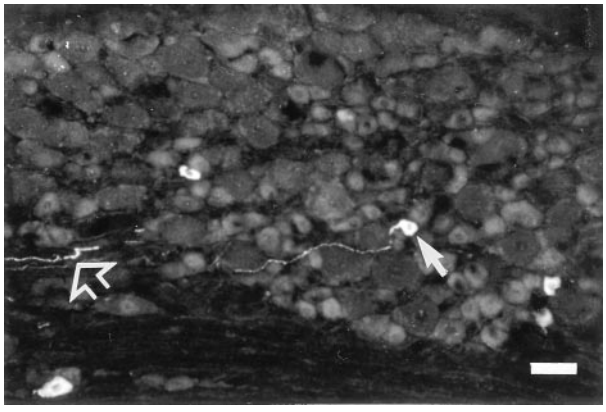

Fig. 5. Immunofluorescence micrograph of rat DRG stained with an antiserum against CARTp. Filled arrow indicates CARTp-ir cell body. Open arrow indicates stained axon. (Bar = 50 μm.)

In the caudal medulla oblongata of colchicine-treated rats (bregma −15.5 mm), CARTp-ir varicose fibers were observed in moderate densities in the commissural part of the NTS and in lower densities in its medial posterior part (Fig. 4 *f* and *g*). Stained fibers were also present in high density in the superficial layers of the spinal nucleus of the trigeminal nerve with scattered fibers in the trigeminal tract. Scattered, strongly CARTp-ir cell bodies with prominent dendrites were seen lateral to the central canal in the lateral reticular nucleus. More rostrally (bregma −14.0 mm), the area postrema contained high densities of intensely CARTp-ir varicose fibers and scattered cell bodies (Fig. 4*f*). The moderate fiber densities in the commissural and medial NTS sharply delineated the ventral border but decreased diffusely toward the lateral NTS. Further rostrally, as the NTS assumes a more lateral position (bregma −13.0 mm), fiber densities were decreased in all portions of the nucleus and were virtually absent rostral of bregma −12.0 mm. Stained cell bodies and processes were observed in dorsal parts of the NTS, particularly in the gelatinous part (Fig. 4*g*).

In the spinal cord, dense CARTp-ir terminal networks were found in superficial laminae of the dorsal horn and in the lateral spinal nucleus. CARTp-ir fibers were also seen in deeper layers as well as in the ventral horn. Occasional cell bodies containing CARTp-LI, with richly arborized, spinous dendrites were seen in the lateral spinal nucleus and adjacent to the central canal (not shown).

Controls. Addition of an excess (100×) of unlabeled CART or CCK_A receptor cDNA probe to the hybridization mixture abolished hybridization signal. The same probe has also been used on brain sections, where it gives the expected pattern of labeling (not shown). Immunohistochemistry performed after preincubation of the CARTp antiserum with corresponding synthetic peptide failed to produce normal staining patterns.

Discussion

It is well established that the vagus nerve plays an important role in regulating feeding behavior by transmitting chemosensory and mechanosensory information from the viscera (see refs. 10 and 11). Although it has been suggested that glutamate is a dominant

transmitter in this pathway (21), the identity of the signaling molecules used by vagal afferents still remains to be defined exactly. In the present paper, we show that CART and its putative peptides are present in high amounts in about half of the vagal afferent neurons. We also report the presence of CARTp-LI in the vagus nerve itself and that pinching of the nerve caused accumulation on both sides of the compression, indicating both anterograde and retrograde transport. Thus, nodose ganglion cells may give rise to at least part of the dense network of CARTp-ir fibers in the NTS, described in the present and a previous study (3). Although this explanation seems likely, considering the dense projection supplied to the NTS by vagal afferent fibers (8, 9), the present results do not exclude the possibility that other sources, e.g., the local CARTp-ir NTS cell bodies reported here, also contribute to those fibers.

These data are intriguing in light of the potent attenuation of feeding observed on intracerebroventricular injection of predicted CARTp and the stimulation of food intake that follows intracerebroventricular injection of anti-CARTp antibodies (5, 6). Although the abundance of hypothalamic cell populations expressing CART (1–3) and its regulation by leptin (5) make the hypothalamus a likely mediator of these effects, there is no conclusive evidence pin-pointing the brain site of action of CART-mediated modulation of feeding. The present data suggest that CARTp may also relay gastrointestinal meal-induced viscerosensation that acts to terminate food intake. The rapid anorexigenic effects of a single intracerebroventricular injection of CARTp (5, 6) are indeed compatible with a role as a short-term satiety signal.

We also show that a high percentage of CART mRNA-expressing neuron profiles in the nodose ganglion coexpress CCK_A receptor mRNA. The nodose ganglion and the vagus nerve display high levels of CCK_A-type binding (22–25). Recently, the presence of both CCK_A and CCK_B receptor mRNAs has been shown in the nodose ganglion (ref. 26 and C.B., K.H., T.-J. Shi, and T.H., unpublished work). These receptors have been suggested as mediators of the potent satiety-inducing effect of CCK (14, 27). Increased CCK plasma levels, probably from enteroendocrine cells (28), are seen in response to a meal. Thus, CCK may activate vagal input into the NTS (29–32). Based on the present data, it is tempting to speculate that CARTp may be a mediator released from central vagal afferents on CCK_A receptor stimulation. Interestingly, the pattern of CCK_A-type binding in the NTS is similar to the distribution of CARTp-LI seen in the present study, being restricted to the area postrema and the medial NTS in the caudal pole of the nucleus, both in rat (33–35) and human (36) brain. In fact, this site is also the site of termination of afferents from the gastrointestinal tract (9).

We failed to observe any significant difference in CART expression in nodose ganglia from rats subjected either to food restriction or to high-fat diet feeding for 2 weeks. It should be noted, however, that despite the cessation of feeding that can be achieved by a single peripheral injection of CCK, administering this peptide to animals chronically for a longer period of time does not alter total food intake or body weight (37). Although each meal is interrupted after CCK injection, the animal will instead increase its total number of meals (38). This result would suggest that the vagal CCK-responsive pathway is resistant to developing tolerance to CCK, possibly because of an absence of transcriptional changes. Similarly, we have not observed any difference in nodose CCK_A receptor mRNA levels in food-deprived or high-fat diet animals (C.B., K.H., T.-J. Shi, and T.H., unpublished work).

However, we detected a marked decrease in nodose ganglion CART mRNA expression after vagotomy. It is well established that primary sensory neurons dramatically alter their neurochemical makeup when challenged with nerve lesions (see refs. 39–41). In general, it seems that peptides and transmitter-related enzymes present under normal conditions (and thus possibly involved in the normal signaling of these cells) are down-regulated in response to nerve injury. Conversely, molecules previously not present, or present only at low levels, appear, and they may play a part in the survival-regeneration process (see refs. 42–44). Thus, CARTp is presumably a component of the normal transmitter repertoire. However, we did not observe any vagotomy-induced decrease in CARTp-LI, similar to the *in situ* hybridization data. The noted increased cytoplasmic immunoreactivity may reflect accumulation of peptide caused by proximity of the axotomy to the cell bodies.

In comparison to the impressive CART-ir cell population in nodose ganglia, only scattered (albeit strongly) stained cell bodies could be observed in DRGs. We have also failed to observe any larger number of CART mRNA-expressing neuron profiles in DRGs by using *in situ* hybridization (C.B. and T.H., unpublished observations). This result correlates to previous studies that showed that, despite many similarities, significant differences in the expression patterns of several messenger molecules in viscerosensory vs. somatosensory ganglia exist (see ref. 41 for review). Such discrepancies may reflect the different embryological origins and different functions of these classes of neurons (45, 46) and suggest that signaling in these two systems can be pharmacologically dissociated. Based on the sparse distribution of CARTp-ir DRG cell bodies described here, it seems likely that the dense plexuses of CARTp-ir fibers in the dorsal horn and trigeminal tract observed by ourselves and others (4) receive contributions also from CART-expressing populations other than primary afferent neurons. One such contributor may be the strongly CARTp-ir cell bodies in the lateral spinal nucleus (ref. 4 and present data). The possibility of descending CARTp pathways also remains to be studied.

The present data indicate high levels of CART expression in the viscerosensory vagal pathway under the potential influence of enteroendocrine CCK acting via CCK_A receptors. These findings suggest that CARTp-induced suppression of feeding may be caused by meal-related termination of food intake, as well as the possible participation of CARTp in other vagal transmission, e.g., cardiovascular control (13).

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