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# Cocaine- and amphetamine-regulated transcript: distribution and function in rat gastrointestinal tract

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**Abstract** Cocaine- and amphetamine-regulated transcript (CART) peptide, originally isolated from brain, is also expressed in the peripheral nervous system. The distribution, origin and projections of CART-expressing enteric neurones by immunocytochemistry and *in situ* hybridization in rat gastrointestinal (GI) tract were studied. Possible motor functions of CART were studied *in vitro* using longitudinal muscle strips from stomach, ileum and colon. Cocaine- and amphetamine-regulated transcript peptide was found in numerous myenteric neurones throughout the GI tract while CART-expressing submucous neurones were scarce. Cocaine- and amphetamine-regulated transcript was also expressed in the antral gastrin cells. Myenteric CART-expressing neurones in both small and large intestine issued short descending projections. In atrophic ileum, CART mRNA-expressing neurones increased in number while neurones containing CART peptide decreased. In hypertrophied ileum, no change in CART peptide or CART mRNA containing myenteric neurones was detected. Cocaine- and amphetamine-regulated transcript  $55\text{--}102$  ( $10^{-9}\text{--}10^{-7}$  mol L<sup>-1</sup>) did not induce any contractile or relaxatory responses in the muscle strips, neither did it affect responses induced by vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide or neuronal stimulation. In colonic, but not in ileal, strips addition of CART attenuated nitric oxide (NO) donor-induced relaxations. Although CART does not seem to play a pivotal role in classic neurotransmission to the longitudinal muscle, it may serve a modulatory role in NO transmission. It may, moreover, be involved in intestinal

adaptation, and an additional hormonal role is also possible.

**Keywords** cocaine- and amphetamine-regulated transcript, CART, enteric neurones, gastrin cells, intestinal adaptation, nitric oxide, vasoactive intestinal peptide.

## INTRODUCTION

Cocaine- and amphetamine-regulated transcript (CART) was identified by polymerase chain reaction (PCR) differential display in rat striatum after acute administration of cocaine or amphetamine.<sup>1</sup> Subsequent studies have identified CART in a number of other locations in the brain such as the hypothalamus and thalamus,<sup>1</sup> and also in the spinal cord,<sup>2</sup> in sympathetic and sensory neurones,<sup>3</sup> in enteric neurones<sup>4</sup> and in pancreatic islet (somatostatin) cells.<sup>5</sup> In rats and mice, the CART gene codes for either a long (129 amino acids) or a short (116 amino acids) form by alternative mRNA splicing,<sup>1</sup> while in human beings only one major transcript, the short form, is expressed.<sup>6</sup> A high degree of homology exists between rat and human CART cDNA (92%) and between the predicted rat and human CART protein (95%).<sup>6</sup>

In addition to its role in drug abuse,<sup>7</sup> CART has also been shown to be a satiety factor regulated by leptin.<sup>8,9</sup> Neuronal plasticity involves the regulated transcription of unique sets of genes in the brain and is considered the basis of drug tolerance, dependence and sensitization. In the peripheral nervous system, including the enteric nervous system (ENS), plasticity in terms of changes in the expression of various neurotransmitters is common in response to nerve injury or neuronal adaptation.<sup>10–12</sup>

The aims of this study were to outline the expression of CART in the gastrointestinal (GI) tract with particular emphasis on the origin, distribution and projection pattern of CART-expressing enteric neurones in rat. The possibility that CART-expressing enteric neurones

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display plasticity in terms of an up- or down-regulation of this peptide was tested in the adult rat by inducing intestinal hypertrophy or atrophy. The role for CART in intestinal motor activity was also investigated.

## MATERIALS AND METHODS

### Animals, surgical procedures and tissue processing

Adult female Sprague–Dawley rats (170–200 g) were used. For surgery the rats were anaesthetized using ketamine (Ketalar<sup>®</sup>, Parke—Davis, Warner—Lambert, Nordic AB, Solna, Sweden; 100 mg kg<sup>-1</sup>) and xylazine (Rompun<sup>®</sup>, Bayer AB, Gothenburg, Sweden; 15 mg kg<sup>-1</sup>). Postoperatively the animals were housed individually with free access to standard rat chow and water. They were monitored daily with regard to weight, general well-being and circumference of the abdomen. The Animal Ethics Committee, Lund and Malmö approved the procedures.

*Extrinsic denervation of small and large intestine* Extrinsic denervation of a 5–10 cm segment of the jejunum ( $n = 4$ ) or midcolon ( $n = 4$ ) was performed by clamping mesenteric blood vessels and surrounding mesentery for 10 min with a pair of forceps (nerve crush).<sup>13,14</sup> The animals were left for 10 days after the operations.

*Severing of intrinsic neuronal pathways in small and large intestine* Enteric neuronal pathways were disrupted by circumferential clamping with a pair of forceps (3–4 mm for 10 min) of the jejunum ( $n = 4$ ) or midcolon ( $n = 4$ ).<sup>13,14</sup> The animals were left for 10 days after the operations.

*Atrophic ileum* In five rats, atrophy of the distal ileum was created by a bypass operation. The distal ileum was cut approximately 10 cm from the ileo-caecal valve and the distal end of the divided small intestine was transposed and sutured with fine silk-sutures by an end-to-side anastomosis to the proximal colon. The distal ileum, still connected to the caecum, was closed proximally. The animals were left for 10 weeks after the operations. The bypassed ileal segment displayed marked atrophy as previously described.<sup>15</sup>

*Hypertrophic ileum* Partial obstruction of the ileum was performed in five rats using a technique modified from Gabella<sup>16</sup> and Ekblad *et al.*<sup>17</sup> A loop of ileum approximately 5 cm from the ileo-colic junction was exposed and a band of cotton (width 3 mm) was fixed

around it and closed with silk. The band was 1–2 mm wider than the intestine and thus causes no strangulation. The rats were killed 3 weeks postoperatively. From the induced obstruction and 10–15 cm proximally the small intestine was greatly distended with an increased wall thickness.

*Tissue processing* The small and large intestinal segments of interest from operated rats and GI segments (oxyntic and pyloric portions of stomach, duodenum, jejunum, ileum, midcolon) from age-matched controls ( $n = 10$ ) were opened along the mesenteric border and pinned flat, without stretching, on pieces of balsa wood. The specimens were fixed overnight in a mixture of 2% formaldehyde and 0.2% picric acid in phosphate buffer (pH 7.2) followed by thorough rinsing in Tyrode's solution containing 10% sucrose. Whole-wall specimens, approximately 10 mm long, were then frozen on dry ice and cut in a cryostat to a thickness of 10  $\mu$ m. Sections were placed on chrome alum-coated slides and processed for immunocytochemistry, *in situ* hybridization or ethidium bromide (EtBr) staining. Whole mount preparations consisting of the longitudinal muscle layers with attached myenteric ganglia were prepared from stomach, small and large intestine. The preparations were attached to slides and stored at -20 °C for subsequent immunocytochemistry and *in situ* hybridization.

### Immunocytochemistry

To study the presence of CART in neurones and endocrine cells, we used a previously characterized<sup>18</sup> polyclonal antiserum raised in rabbit against the CART peptide fragment 106–129 (Cocalico Corp., Reamstown, PA, USA). For double-immunolabelling, the CART antiserum was used in combination with one of the following antibodies: monoclonal antibodies against vasoactive intestinal peptide (VIP; dilution 1 : 640, code MaVIP; East Acres Biologicals, Southbridge, MA, USA),<sup>17</sup> antiserum raised in guinea-pig against human gastrin 2–17 (dilution 1 : 1280, code 8818; Euro Diagnostica, Malmö, Sweden),<sup>19</sup> or antiserum against rat neuronal nitric oxide synthase (NOS) sequence 1409–1429 raised in sheep (dilution 1 : 3200, code AB1529; Chemicon International, Ltd, Harrow, UK). The sections were incubated overnight simultaneously with two of the primary antibodies raised in different species. The site of the antigen–antibody reaction was visualized by fluorescein isothiocyanate (FITC)- or Texas Red-conjugated antibodies to rabbit immunoglobulin G (IgG) raised in pigs (DAKO, Copenhagen, Denmark), affinity purified FITC-conjugated

antibodies to mouse IgG raised in goat (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA), FITC-conjugated antibodies to sheep IgG raised in donkey or FITC-conjugated antibodies to guinea-pig IgG raised in goat (Sigma, St Louis, MO, USA). For controls, antisera inactivated by the addition of an excess amount of antigen (10–100  $\mu\text{g}$  of synthetic peptide  $\text{mL}^{-1}$  diluted antiserum) were used. Inappropriate binding of the secondary antibodies upon double immunostaining was also tested and found to be insignificant.

### ***In situ* hybridization**

To study the presence of CART mRNA we used a mixture of two oligoprobes complementary to rat CART mRNA 133–162 and 192–221. The probes were 3'-endtailed with  $^{35}\text{S}$ -dATP by use of terminal transferase (both supplied by NEN, DuPont, Stockholm, Sweden), yielding a specific activity of approximately  $2 \times 10^9$  CPM  $\mu\text{g}^{-1}$ . The hybridization protocol has previously been described in detail.<sup>17</sup> Briefly, after clearing in chloroform, the sections were acetylated and hybridized with the probes (1 pmol  $\text{mL}^{-1}$ ) overnight at 37 °C. The slides were washed (1  $\times$  SSC, 4  $\times$  15 min, 55 °C), dehydrated and dipped in emulsion (Ilford K5). The exposure time was 3 weeks after which the slides were developed in Kodak D-19. For control purposes, hybridization was also performed after incubation in RNase A (45  $\mu\text{g mL}^{-1}$ , Sigma; 30 min at 37 °C) or in the presence of a 100-fold excess of unlabelled probe in the hybridization buffer. In the control experiments, autoradiographic labelling of enteric neurones or endocrine cells was not obtained.

### **Ethidium bromide staining**

Counterstaining using 0.1% of EtBr in Tris-HCl was used in order to visualize all nerve cell bodies.<sup>17,20</sup>

### **Cell counting**

Ethidium bromide-stained, CART-immunoreactive, or autoradiographically labelled (CART mRNA containing) nerve cell bodies in myenteric ganglia were counted per visual field (1.2 mm) in sequentially cut whole-wall longitudinal sections from control ( $n = 5$ ), atrophic ( $n = 5$ ) and hypertrophic ( $n = 5$ ) ileum. Each section was approximately 10 mm long and from each animal three sections cut at different depths were analysed. The results were expressed as the mean of the total number of nerve cell bodies per visual field and 60 visual fields were counted per animal. Statistical evaluation was by

ANOVA followed by a post-test using Bonferroni correction to adjust for multiple comparisons.  $P < 0.05$  was considered to be statistically significant.

### **Estimation of projection distances**

The average lengths of the oro-anal projections of CART-immunoreactive myenteric neurones in rat small and large intestine were established after axonal degeneration upon severing the intrinsic neuronal pathways, by measuring the distance from the lesion to the region showing a normal fibre frequency. Distances were measured by inserting a micrometer scale in the visual field. Both cryostat sections and whole mounts were used to establish the projection distances of the CART-immunoreactive neurones.

### ***In vitro* studies of motor activity**

Twenty-four rats were used in the study. The animals were anaesthetized in vaporized diethylether and killed by bleeding. Strips of longitudinal smooth muscle with adherent myenteric ganglia from stomach, ileum and distal colon were dissected, under microscope, and the strips (5–7 mm) were placed in ice-cold modified Krebs solution (in  $\text{mmol L}^{-1}$ ) NaCl 133, KCl 4.7,  $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$  2.5,  $\text{MgCl}_2$  1.0,  $\text{NaHCO}_3$  16.3,  $\text{NaH}_2\text{PO}_4$  1.4 and glucose 7.8. The strips were mounted in organ baths (37 °C) containing 5 mL of Krebs solution (pH 7.35–7.45) aerated with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Isometric tension was continuously recorded with a Grass FTO3C force-displacement transducer and registered on a Grass Model 79D polygraph (Grass Instrument Co., Quincy, MA, USA). The strips were given an initial passive load of 25–30 mN (ileum) or 35–40 mN (stomach and colon) and allowed to equilibrate for 1 h (ileum) or 1.5 h (stomach and colon) before experimentation started. During this period, the strips gradually relaxed and the basal tension stabilized at approximately 5 mN (stomach and ileum) and 10 mN (colon). Rhythmic spontaneous contractions of varying amplitude developed in all preparations. The viability of the strips was tested by the addition of acetylcholine ( $10^{-5}$  mol  $\text{L}^{-1}$ ) to the baths followed by washout and 15-min recovery and the addition of a buffer with 124 mmol  $\text{L}^{-1}$   $\text{K}^+$ , after which the strips were allowed to recover for another 15 min. Strips not evoking a contractile response to both of these test substances were excluded from the study.

*Effects of CART on contractile or relaxatory responses*  
Cocaine- and amphetamine-regulated transcript 55–102 ( $10^{-9}$  to  $3 \times 10^{-7}$  mol  $\text{L}^{-1}$ ) was added to

stomach, ileal and colonic strips in order to study possible contractile responses. In order to study relaxatory responses gastric and ileal preparations were precontracted with prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) ( $10^{-9}$ – $10^{-7}$  mol  $L^{-1}$ ; chosen individually for each muscle strip to ensure the same contraction level). Prostaglandin  $F_{2\alpha}$  elicited only a weak and unstable contraction in the colonic preparations. Therefore, precontraction was achieved by a submaximal concentration of acetylcholine ( $10^{-6}$  mol  $L^{-1}$ ). When the level of precontraction was stable, CART 55–102 ( $10^{-9}$ – $10^{-7}$  mol  $L^{-1}$ ) was added. Isoprenaline ( $10^{-6}$  mol  $L^{-1}$ ) was added to the precontracted strips at the end of experimentation and used as a reference of maximal relaxation. Each strip was exposed to 4–6 different concentrations of CART, always starting with the lowest.

*Effects of CART on relaxations evoked by NO donor, VIP or pituitary adenylate cyclase-activating peptide*  
In separate experiments on strips from both ileum and colon, we evaluated possible influence of CART 55–102 on relaxatory responses induced by the NO-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP), VIP and pituitary adenylate cyclase-activating peptide (PACAP). After testing for viability, strips from both ileum and colon were precontracted (as described above) and SNAP ( $10^{-8}$ – $10^{-4}$  mol  $L^{-1}$ ), VIP ( $10^{-6}$  mol  $L^{-1}$ ) or PACAP ( $10^{-7}$  mol  $L^{-1}$ )-evoked relaxatory responses were registered. This was followed by recovery for 60 min in combination with extensive washouts after which the strips were exposed to CART 55–102 ( $10^{-7}$  mol  $L^{-1}$ ) 5 min before precontraction followed by the addition of SNAP ( $10^{-8}$ – $10^{-4}$  mol  $L^{-1}$ ), VIP ( $10^{-6}$  mol  $L^{-1}$ ) or PACAP ( $10^{-7}$  mol  $L^{-1}$ ). Strips not receiving CART were run in parallel and served as 'run-down' controls. Evaluation of differences was by Student's unpaired *t*-test.

*Effects of CART on motor activity induced by neuronal stimulation*  
Platinum electrodes (0.5 mm in diameter and 10 mm apart) were mounted on both sides of muscle strips from ileum and colon. The electrodes were connected to a Grass S4C stimulator for field stimulation with square wave pulses (4 V over the electrodes, 400 mA, 1 ms duration, frequency 20 Hz); pulse trains lasting for 5 s. After equilibration and testing for viability muscle strips, with or without CART ( $10^{-7}$  mol  $L^{-1}$ ), at basal tension were subjected to electric field stimulation (EFS). These experiments were used to study CART effects on EFS-induced contractions. To study the possibility that CART modulates neuronally induced relaxatory responses, ileal strips were exposed to atropine ( $10^{-6}$  mol  $L^{-1}$ ); to

eliminate cholinergic contractions evoked by EFS) and CART, 5 min prior to precontraction with  $PGF_{2\alpha}$  (see above) and, when the precontraction was stable the strips were subjected to EFS (as described above). The substance of choice for rat colonic muscle strips is acetylcholine, which prevented the use of atropine. To overcome this, nerve stimulation leading to a relaxation of colonic strips was by high  $K^+$  instead of EFS.<sup>21</sup> Pretreatment with CART 55–102 ( $10^{-7}$  mol  $L^{-1}$ ) was by addition of the peptide 5–90 min prior to EFS or high  $K^+$ . When used, tetrodotoxin (TTX;  $10^{-6}$  mol  $L^{-1}$ ) and atropine ( $10^{-6}$  mol  $L^{-1}$ ) were added 5 min prior to EFS; L-N-nitro-arginine methyl ester (L-NAME;  $10^{-4}$  mol  $L^{-1}$ ) was added 30 min prior to EFS.

*Drugs used*  
Rat CART 55–102 was from American Peptide (Sunnyvale, CA, USA). This CART fragment has, in previous studies<sup>8,22</sup> been shown to be biologically active. Atropine sulphate, TTX, isoprenaline, acetylcholine, L-NAME, rat VIP, rat PACAP-27 were purchased from Sigma. Prostaglandin  $F_{2\alpha}$  (Prostin) was from Upjohn Co. (Kalamazoo, MI, USA). All substances were dissolved in 0.9% saline.

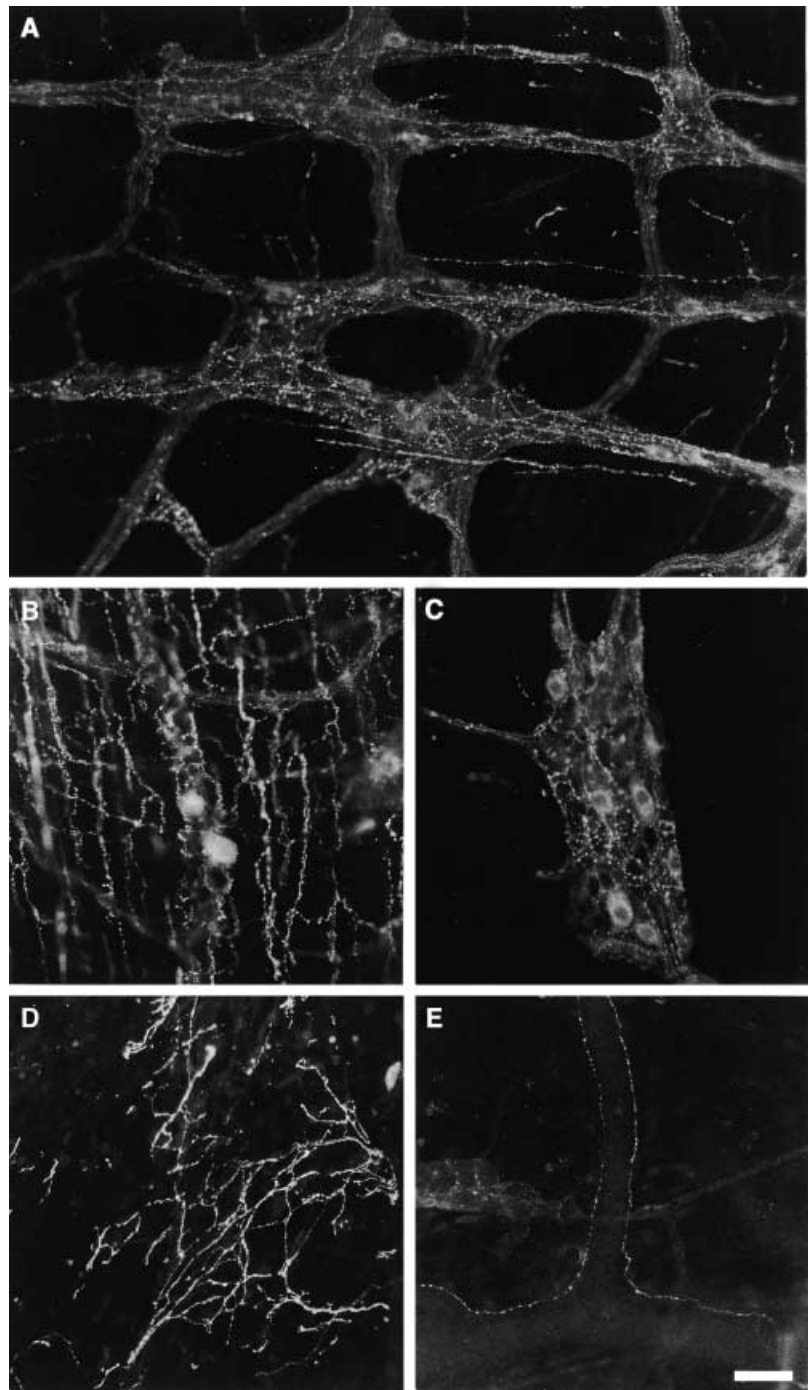
## Nomenclature

Different studies have used different nomenclatures for CART peptides. In this paper, 'CART 55–102' refers to the long form of rat proCART and corresponds to CART 42–89 which is numbered according to the short form of the transcript.<sup>23</sup> The N-terminal amino acid sequence of CART 55–102 begins with IPIYE ... and ends in ... KCL. Cocaine- and amphetamine-regulated transcript 55–102 is found in tissues<sup>24,25</sup> and fits into a typical scheme of propeptide processing and gene regulation.<sup>26</sup> The batch of peptide used in this study was verified to have locomotor-inducing activity as described by Kimmel *et al.*<sup>27</sup>

## RESULTS

### Distribution of CART-expressing neurones and endocrine cells

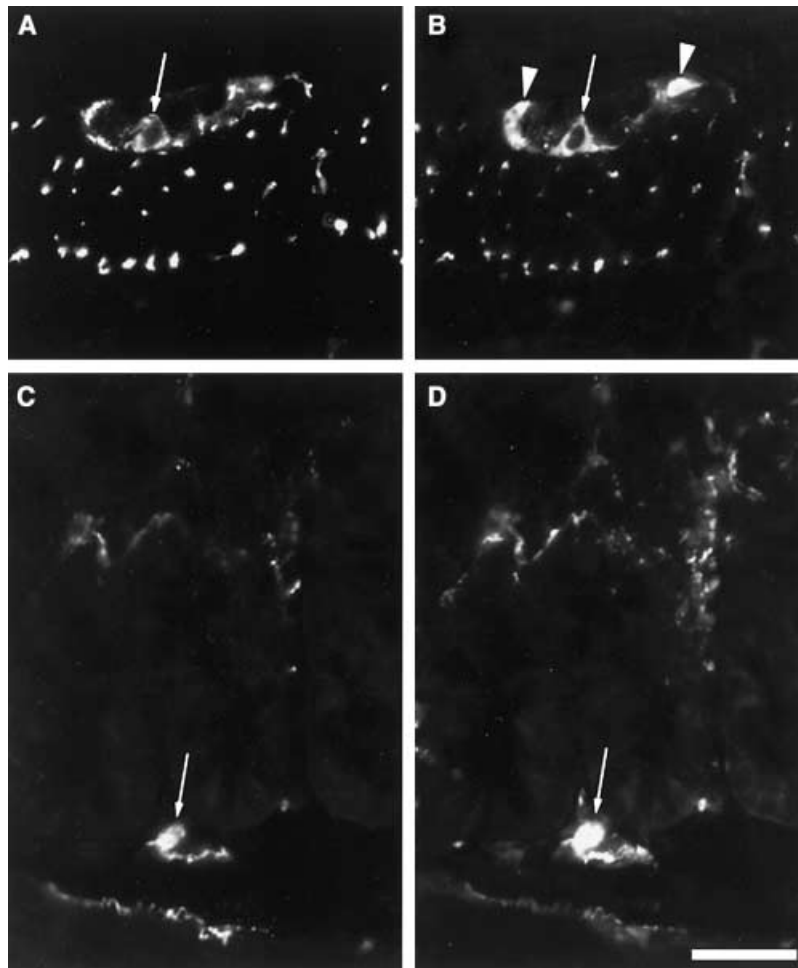
Numerous CART-immunoreactive and autoradiographically labelled CART mRNA containing nerve cell bodies were found in myenteric ganglia throughout the GI tract (Figs 1–3). In intestinal submucous ganglia, only a few scattered CART-expressing (peptide and mRNA) nerve cell bodies could be detected; none was found in gastric submucous ganglia. Cocaine- and amphetamine-regulated transcript-immunoreactive nerve fibres were numerous in the smooth muscle



**Figure 1** Whole mount preparations of longitudinal muscle and myenteric ganglia from rat small (A and B) and large (C) intestine and of the submucosa from large intestine (D and E) showing cocaine- and amphetamine-regulated transcript (CART)-immunoreactive nerve cell bodies and nerve fibres. Cocaine- and amphetamine-regulated transcript-immunoreactive nerve cell bodies are found in myenteric ganglia in both small and large intestine. Within the submucosa CART-immunoreactive nerve fibres are moderate in number and some are found to innervate submucous blood vessels (E). The scale bar in E represents 70  $\mu\text{m}$  (A) and 50  $\mu\text{m}$  (B-E).

layers, particularly the circular muscle, and within the myenteric ganglia throughout the GI tract. A moderate number of CART-immunoreactive nerve fibres were found in the submucosa mainly innervating the submucous ganglia and blood vessels. The small intestinal mucosa received a sparse number of CART-immunoreactive fibres while the gastric and colonic mucosa was more densely innervated.

Double immunostaining (CART/VIP and CART/NOS) revealed that some CART-immunoreactive myenteric nerve cell bodies in stomach and small and large intestine also contained VIP or NOS (Fig. 2A and B). Nitric oxide synthase-immunoreactive myenteric nerve cell bodies were numerous throughout the GI tract and most of them were devoid of CART (Fig. 2A and B). Vasoactive intestinal peptide-immunoreactive



**Figure 2** Cryostat sections of myenteric ganglion from small intestine (A and B) and submucous ganglion from large intestine (C and D) double-immunostained for cocaine- and amphetamine-regulated transcript (CART) (A and C) and nitric oxide synthase (NOS) (B) or vasoactive intestinal peptide (VIP) (D). In myenteric ganglia CART-immunoreactivity is found in a subpopulation of NOS-immunoreactive neurones (CART/NOS-immunoreactive neurone indicated by arrows, NOS-immunoreactive neurones without CART indicated by arrow heads). In submucous ganglia, all CART-immunoreactive neurones also contain VIP (arrows). The scale bar in D represents 40  $\mu\text{m}$  (A–D).

myenteric nerve cell bodies were few and approximately half of them also contained CART. All the submucous CART-immunoreactive nerve cell bodies (small and large intestine) were also found to contain VIP (Fig. 2C and D), while none were NOS-immunoreactive. The majority of CART-immunoreactive nerve fibres within the smooth muscle throughout the entire GI tract contained both VIP and NOS (Fig. 2). In the myenteric ganglia, approximately half of all CART-immunoreactive fibres contained VIP and some contained NOS. In the submucous ganglia and in the mucosa/submucosa, all CART-immunoreactive nerve fibres were also VIP-immunoreactive (Fig. 2C and D) while none contained NOS.

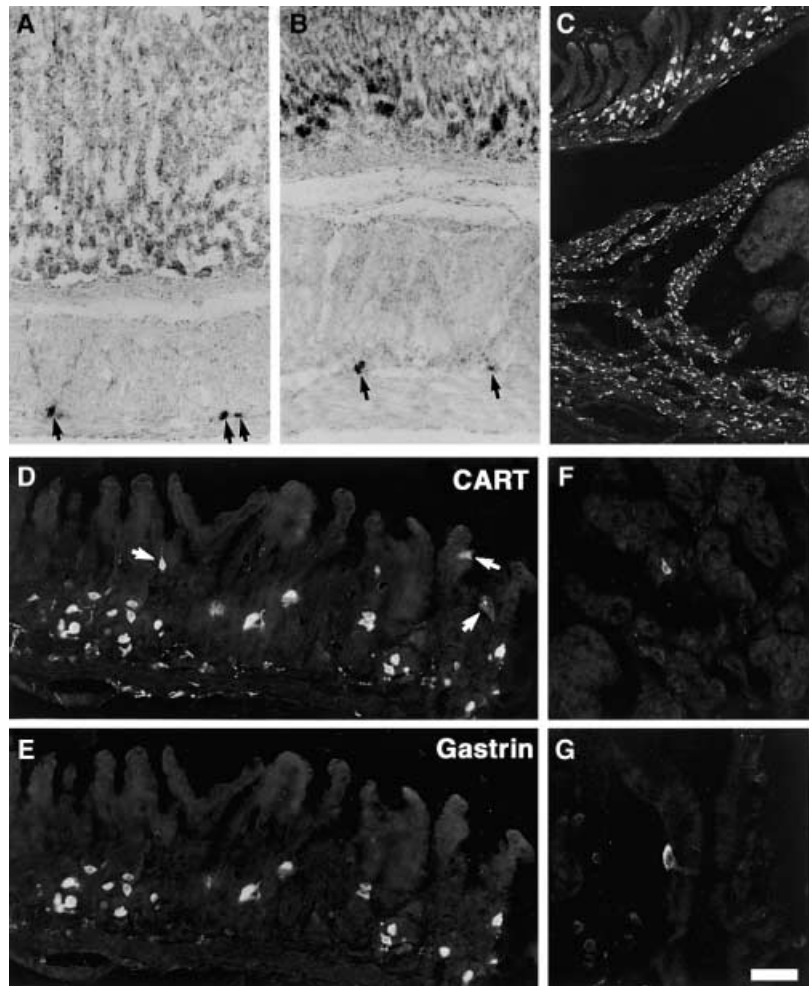
In addition to enteric neurones, CART was found in a dense population of endocrine cells in the antral mucosa of the stomach (Fig. 3D), in scattered endocrine cells in the duodenal mucosa (Fig. 3F and G) and in Brunner glands. These cells displayed both CART peptide, as revealed by immunocytochemistry, and CART mRNA, as demonstrated by *in situ* hybridization. The vast

majority of the CART-expressing cells were found to be identical with the gastrin cells; however, a subpopulation was devoid of gastrin (Fig. 3D and E). Cocaine- and amphetamine-regulated transcript-expressing endocrine cells could not be detected in the oxyntic mucosa, jejunum, ileum or large intestine.

### Origin and projections of myenteric CART-expressing neurones

Extrinsic denervation (nerve crush) caused no overt change in the number of CART-immunoreactive nerve fibres (Fig. 4 upper panel) and thus, an intrinsic origin of CART-immunoreactive nerve fibres in both small and large intestine can be suggested.

After local disruption of intramural nerve fibres by circumferential intestinal clamping, CART-immunoreactive fibres were found to be absent or markedly reduced in number in myenteric ganglia and smooth muscle up to 1.5 mm anally to the lesion (Fig. 4 lower panel). Further distally the CART-immunoreactive



**Figure 3** Cryostat sections of whole wall from rat stomach (A, oxyntic region; B, pyloric region) and pyloric sphincter (C), and mucosa from pyloric region (D and E) and duodenum (F and G). (A) and (B) show autoradiographic labelling of cocaine- and amphetamine-regulated transcript (CART) mRNA in nerve cell bodies in myenteric ganglia by *in situ* hybridization (arrows); (C) shows the dense innervation of CART-immunoreactive nerve fibres in the pyloric sphincter; (D) and (E) demonstrate by immunocytochemical double-staining co-localization of CART and gastrin in the same endocrine cells in the pyloric mucosa. Note a few CART-immunoreactive endocrine cells that are non-immunoreactive to gastrin (arrows). A few CART-immunoreactive endocrine cells are also found in the duodenal mucosa (F and G). The scale bar in G represents 70  $\mu\text{m}$  (A–C) and 50  $\mu\text{m}$  (D–G).

fibres had a normal frequency. In the mucosa and submucosa, CART-expressing nerve fibres were too few to allow determination of projections.

### Plasticity of CART neurones in hypertrophic and atrophic intestine

In order to study the role of CART in intestinal adaptation, two well-defined experimental models involving ileum were used. One leads to hypertrophic changes in the ileal wall<sup>16,17</sup> and the other leads to atrophy.<sup>15</sup>

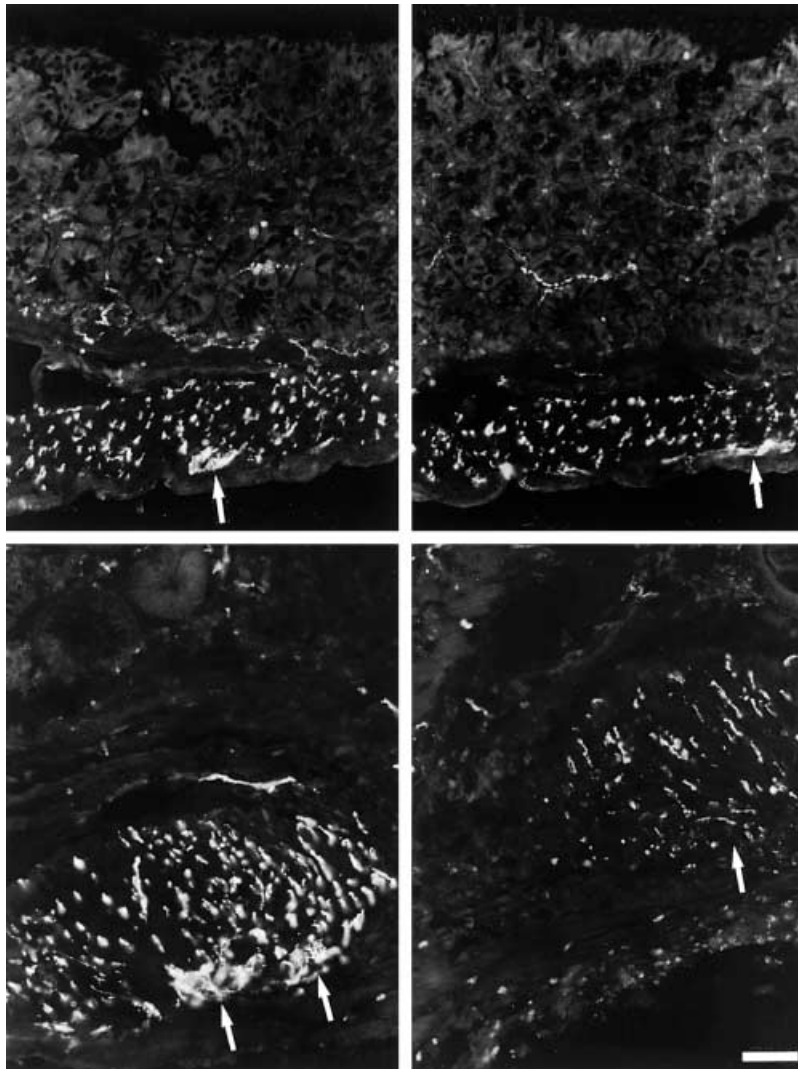
A similar number of CART mRNA-labelled nerve cell bodies was present in hypertrophic as compared with control ileum (Fig. 5A and B). In hypertrophic ileum, the smooth muscle layers were markedly thickened which resulted in a lower density of CART-immunoreactive nerve fibres. In addition, myenteric CART-expressing nerve cell bodies were enlarged.

In the atrophic ileal wall, CART mRNA-labelled nerve cell bodies were increased in number as com-

pared with the control (Fig. 5A and C). In the atrophic ileum, no change in the muscular thickness was noted: the atrophy mainly leads to a reduced circumference.<sup>15</sup> A higher density of CART-immunoreactive fibres was noted in the atrophic intestine, probably reflecting condensation of nerve fibres due to atrophic changes in the intestinal wall.

By neuronal cell counting, the percentage of myenteric neurones expressing CART peptide and CART mRNA was determined in control, hypertrophic and atrophic ileum (Fig. 5D). There were no changes in the relative number of CART-expressing neurones in the hypertrophic gut compared with the control. In the atrophic gut, however, CART-containing nerve cell bodies were fewer ( $P < 0.05$ ), while CART mRNA-expressing neurones increased ( $P < 0.05$ ). In accordance with previous reports,<sup>15,17</sup> the number of myenteric neurones (designated the total number) was, as compared with control ileum ( $4.5 \pm 0.3$  EtBr-stained neurones per visual field), decreased in the





**Figure 4** Cryostat sections from rat large intestine, whole wall. Upper panel shows the normal distribution of cocaine- and amphetamine-regulated transcript (CART)-immunoreactive nerve fibres in control and after extrinsic denervation. No change in the number or topographic distribution of CART-immunoreactive fibres is noted suggesting that the bulk of the fibres is intramural in origin. Lower right panel shows the disappearance of CART-immunoreactive nerve fibres in the circular smooth muscle layer, after local severing of intramural nerves by nerve crush, distal to the lesion while no change in the number of CART-immunoreactive fibres could be noted proximal to the lesion (lower left panel). Arrows indicate myenteric ganglia. The scale bar in E represents 70  $\mu\text{m}$  (upper panel) and 50  $\mu\text{m}$  (lower panel).

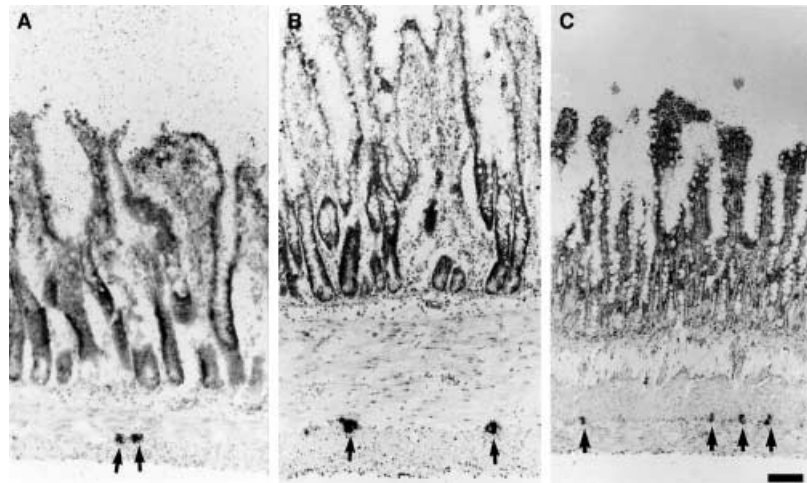
hypertrophic ileum ( $3.6 \pm 0.2$  EtBr-stained neurones per visual field) while it was increased in the atrophic ileum ( $5.9 \pm 0.4$  EtBr-stained neurones per visual field) due to postoperative remodelling of the intestines.

#### CART evoked no gastrointestinal relaxations or contractions

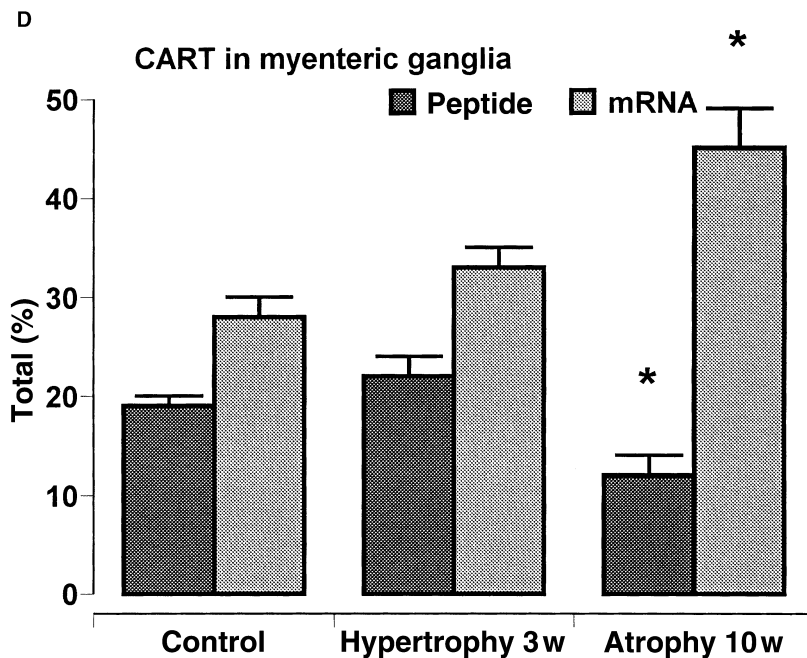
Longitudinal muscle strips with adherent myenteric ganglia from stomach, small and large intestine (at baseline to reveal contractions and precontracted in order to reveal relaxations) were exposed to increasing concentrations ( $10^{-9}$  to  $3 \times 10^{-7}$  mol  $\text{L}^{-1}$ ) of CART, known to be biologically active by other investigators.<sup>27</sup> The peptide fragment, however, induced no motor activity in these preparations.

#### Modulatory effects of CART on NO-, VIP- and PACAP-evoked relaxations

The influence of CART on exogenously applied relaxatory substances was also tested. These experiments were performed on small and large intestinal muscle strips and the amplitudes of relaxations evoked by the NO donor SNAP ( $10^{-8}$  to  $3 \times 10^{-4}$  mol  $\text{L}^{-1}$ ), VIP ( $10^{-6}$  mol  $\text{L}^{-1}$ ) or PACAP ( $10^{-7}$  mol  $\text{L}^{-1}$ ) in the presence and absence of CART ( $10^{-7}$  mol  $\text{L}^{-1}$ ) were registered. Addition of CART caused no change in the amplitude of the VIP- or PACAP-evoked relaxations (Fig. 6). In large intestine, however, addition of CART reduced the amplitude of the SNAP-induced maximal relaxation ( $E_{max}$  expressed as % of maximal relaxation induced by isoprenaline  $10^{-6}$  mol  $\text{L}^{-1}$ ) from  $58.3 \pm 5.9\%$  to  $39.0 \pm 4.6\%$  ( $P < 0.05$ ; Fig. 7). In small intestine,



**Figure 5** Cryostat sections from control (A), hypertrophic (B) and atrophic (C) ileum demonstrating the frequency of autoradiographically labelled cocaine- and amphetamine-regulated transcript (CART) mRNA-expressing myenteric neurones (arrows). In hypertrophic ileum, a marked thickening of the intestinal wall, particularly the smooth muscle layers, can be noted. In atrophic intestine, the thickness of the intestinal wall is similar to control, however, remodelling involving a thickening of the submucosa is readily noted. Neuronal cell counting expressed in % of the total number of myenteric neurones, of CART immunoreactive and CART mRNA-expressing myenteric neurones are presented in (D). In hypertrophic ileum, the numbers of CART peptide and mRNA-expressing neurones are identical to control intestine. In the atrophic ileum, the number of CART-immunoreactive myenteric neurones is reduced while the number of CART mRNA-expressing myenteric neurones is increased as compared with the control (\* $P < 0.05$ ). The scale bar in C represents 100  $\mu\text{m}$  (A–C).



there was no change in the  $E_{max}$  of the SNAP concentration–response curves after addition of CART ( $10^{-7}$  mol  $\text{L}^{-1}$ ). The addition of CART caused no shift in the concentration–response curves constructed by exposure of the small or large intestinal strips to the NO donor SNAP ( $ED_{50}$  values  $1 \times 10^{-5}$  mol  $\text{L}^{-1}$  for ileum and  $2.5 \times 10^{-6}$  mol  $\text{L}^{-1}$  for colon; Fig. 7).

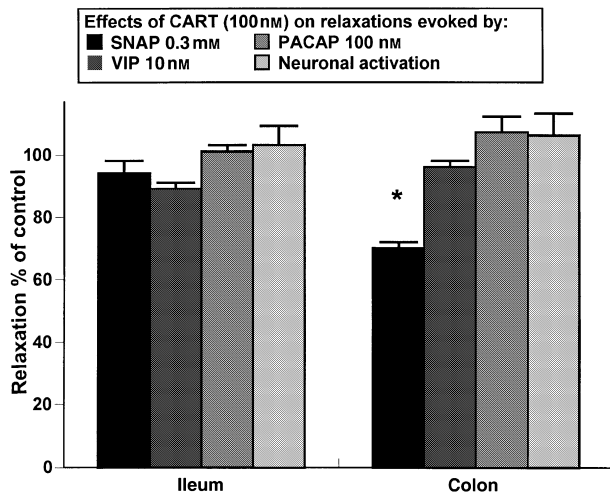
### Modulatory effects of CART on neuronally evoked responses

Possible effects of CART ( $10^{-7}$  mol  $\text{L}^{-1}$ ) on neuronally evoked contractions (induced by EFS) or relaxations (induced by EFS in ileal strips and with high  $\text{K}^+$  in colonic strips) were studied on ileal and colonic muscle

strips. Cocaine- and amphetamine-regulated transcript did not change the amplitudes of the contractile or the relaxatory responses to nerve stimulation in the ileum nor the colon (Figs 6 and 8).

### DISCUSSION

Cocaine- and amphetamine-regulated transcript appears to be a multifunctional peptide neurotransmitter that is involved in feeding and satiety, drug reward and reinforcement, stress responses, endocrine function and sensory processing.<sup>4,7,23,28,29</sup> The evidence that CART is a neurotransmitter has been steadily accumulating.<sup>7</sup> The present study confirms and extends previous observations<sup>4</sup> that CART is

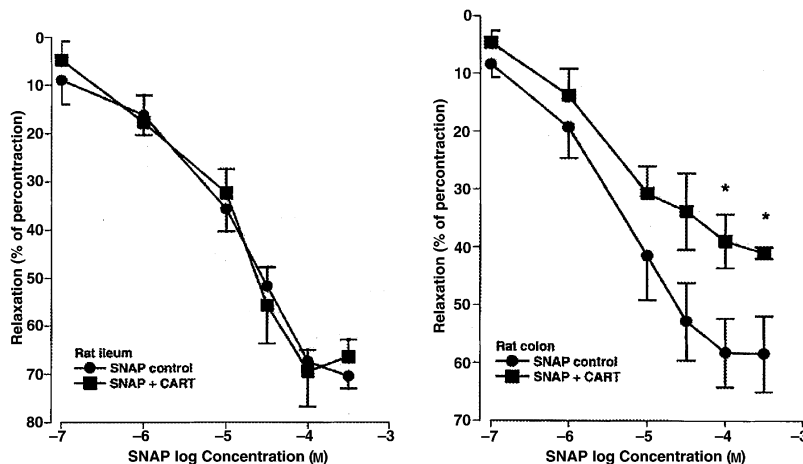


**Figure 6** Relaxatory effects of S-nitro-N-acetyl-D, L-penicillamine (SNAP) ( $3 \times 10^{-4}$  mol L<sup>-1</sup>), vasoactive intestinal peptide (VIP) ( $10^{-6}$  mol L<sup>-1</sup>), pituitary adenylate cyclase-activating peptide (PACAP) ( $10^{-7}$  mol L<sup>-1</sup>) or neuronal activation by electric field stimulation (EFS) (ileum) or high K<sup>+</sup> (colon) in the presence of cocaine- and amphetamine-regulated transcript (CART) ( $10^{-7}$  mol L<sup>-1</sup>) on rat ileal and colonic longitudinal muscle. With the exception of colon presence of CART did not affect any of the relaxatory responses. In colon CART reduced the SNAP evoked relaxation ( $P < 0.05$ ). Each value is the mean of 12–18 experiments. Vertical bars give SEM.

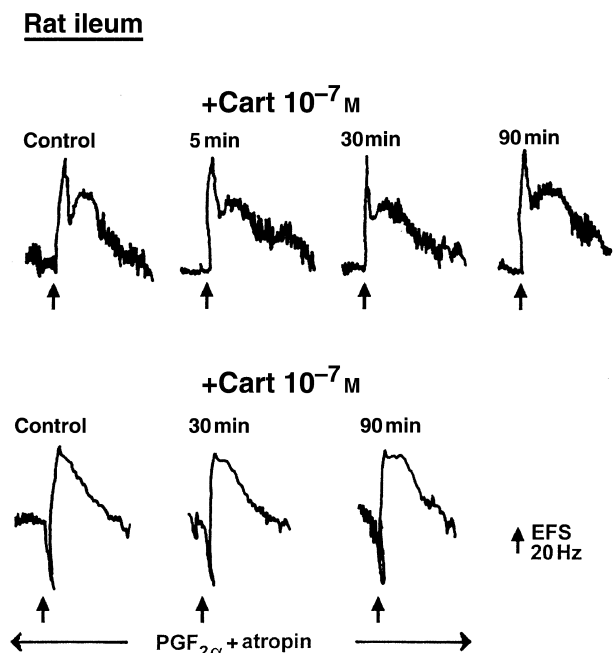
expressed in a widespread system of enteric, preferentially myenteric, neurones. Here we report that the bulk of CART-expressing nerve fibres is intrinsic in origin. This since extrinsic denervation did not cause any loss of CART-immunoreactive nerve fibres in the intestinal wall. We also demonstrate that local severing using nerve crush in both small and large intestine cause a loss of CART-immunoreactive fibres within

the smooth muscle and myenteric ganglia for 1–1.5 mm distally to the lesion indicating that myenteric CART neurones issue ‘short’ descending projections. The designation short is in comparison with those subpopulations of rat enteric neurones that issue ‘long’ projection such as gastrin releasing peptide (GRP)- and galanin-containing neurones which both descend for up to 20 mm.<sup>30,31</sup> Most of the myenteric neuronal subpopulations, for example, VIP-, neuropeptide Y (NPY)-, substance P (SP)-, calcitonin gene-related peptide (CGRP)- and NOS-containing ones issue projections that are short (2–5 mm long) and most of them are descending.<sup>13,14,32</sup> However, ascending myenteric neurones such as enkephalin-containing ones have been described, which, in rat, issue short oral projections in both small and large intestine.<sup>13,14</sup> The finding that myenteric CART-immunoreactive neurones issue short anal projections is not surprising as CART was found in subpopulations of both NOS- and VIP-containing neurones. In fact co-localization of all three transmitters may be anticipated in a subpopulation of myenteric neurones due to the previous finding that VIP and NOS are found to be co-localized in some of these neurones.<sup>32</sup> The submucous CART-immunoreactive neurones were, however, few and contained VIP but not NOS.

The abundance of CART-expressing neurones in the GI tract and the finding of polarized projections strongly suggests a functional role of these neurones in, for example, peristalsis or secretion. Somewhat surprisingly, CART elicited no GI motor responses *per se*, nor did it affect intestinal contractions or relaxations evoked by neuronal stimulation. These results were accomplished on longitudinal GI muscle strips from rat and the possibility that CART elicits motor activity in the circular muscle is still unexplored. Cocaine- and



**Figure 7** Concentration–response curves showing the relaxatory effects of the nitric oxide donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) with or without the presence of cocaine- and amphetamine-regulated transcript (CART) on rat ileal (left panel) and colonic (right panel) longitudinal muscle. Each value is the mean of seven to 18 experiments. Vertical bars give SEM. \* indicates  $P < 0.05$ .



**Figure 8** Tracings of electrically induced responses of rat ileum exposed to cocaine- and amphetamine-regulated transcript (CART) 55–102 ( $10^{-7}$  mol L $^{-1}$ ) for 0 (control), 5, 30 and 90 min. Stimulation (20 Hz, 4 V, 400 mA) was maintained for 5 s (indicated by vertical arrows). Upper panel: The contractile response elicited by electrical stimulation consisted of a fast cholinergic twitch followed by a slower nonadrenergic non-cholinergic (NANC) mediated contraction. No change in the electrically induced contractile responses was noted after CART exposure. Lower panel: After precontraction by prostaglandin F $_2$  (PGF $_{2\alpha}$ ) and in the presence of atropine a relaxation followed by a contraction was elicited by electrical stimulation. The electrically induced relaxatory responses were unaltered by the presence of CART.

amphetamine-regulated transcript did, however, inhibit SNAP-evoked colonic relaxations suggesting that it may modulate NO activity in the colon. This latter observation may be significant and needs further exploration. Taken together it seems, however, that CART does not play any major role in classical neurotransmission or neuromodulation, at least in the set-up tested.

No change in the relative number of CART or CART mRNA containing myenteric neurones could be detected in a model of hypertrophied (partial obstruction) ileum. However, in an experimental model for atrophic ileum (bypass), the relative number of CART mRNA-expressing neurones increased while neurones containing CART peptide decreased. Thus, a marked discrepancy between mRNA expression and peptide content in myenteric neurones from atrophic intestine was noted. Several explanations may be offered: the

most likely is that both synthesis, reflected in an increased expression of mRNA, and secretion, reflected in a lower number of cell bodies containing detectable amounts of the peptide, are increased in this condition. Changes in the expression of several neurotransmitters within the rat ENS have been documented not only in conditions such as axotomy and colchicine treatment<sup>33</sup> but also in intestinal hypertrophy<sup>17</sup> and atrophy.<sup>15</sup> The neurotransmitter most readily up-regulated is VIP, but also PACAP and galanin change their expressions under the conditions above. In a previous study, we have reported that, in rat atrophic ileum, neurones expressing VIP, NPY or PACAP decreased in number while those expressing NOS increased, particularly in the myenteric ganglia.<sup>15</sup> This is in contrast to the rat hypertrophic gut where VIP, PACAP and galanin expressing enteric neurones markedly increased.<sup>17</sup> As shown in the present study, CART behaves somewhat differently in that the number of CART-expressing neurones did not change in the hypertrophic gut while they showed a marked increase during atrophy.

Little is known about the mechanisms regulating cell death or survival, maintenance and adaptation of adult enteric neurones. Changes in neurotransmitter expression have been demonstrated in response to different pathological conditions in human intestine.<sup>34–38</sup> A role for neurotransmitters in mediating neuronal survival and in maintaining the adult integrative ENS may thus be suggested. Changes in the expression of various neurotransmitters probably reflect a functional change from transmission of information to neuroprotection and, eventually, regeneration and reconnecting with the target tissues, or cell death. Based on the findings reported here we suggest that CART peptide may act as a survival factor for myenteric neurones and/or for the smooth muscle. Trophic actions of CART peptides have been reported on various neuronal populations.<sup>39</sup>

Further it is noteworthy that, in the present study, the number of myenteric neurones labelled for CART mRNA slightly exceeded the number of CART peptide-containing. This observation is valid for all three groups studied and, thus, may reflect that the two different methods used (*in situ* hybridization vs immunocytochemistry) have different sensitivity. An alternative interpretation of this observation is that the peptide is rapidly transported out into the nerve terminals leaving amounts below detection limit in some cell somas while the mRNA remains.

In addition to the expression in enteric neurones, CART peptide and CART mRNA were also found in numerous endocrine cells in the antro-pyloric mucosa of the stomach. On the whole, these CART-expressing

cells were identical to the gastrin cells. The production of CART by gastric endocrine cells is of interest in view of the established anorexigenic effect of the CART peptides. Thus, CART emanating from the stomach may, directly or indirectly, affect satiety mechanisms in the hypothalamus possibly in conjunction with vagal afferent neurones.<sup>40</sup> With the exception of a few endocrine cells displaying both CART- and gastrin-immunoreactivity in the duodenum and in the Brunner glands, no CART-expressing endocrine cells were detected in the small and large intestine. An endocrine role for CART peptide in the stomach is supported by similar evidence in other tissues. It is found in abundance in both the anterior and posterior pituitary, various hypothalamic nuclei and in the adrenal medulla<sup>18,41,42</sup> and also in D cells in the pancreas.<sup>5</sup> During development, CART is also expressed in other pancreatic islet cells (unpublished data). It is released from hypothalamic tissue in a calcium-dependent manner,<sup>43</sup> and is found in the circulation.<sup>5</sup> Inevitably, the further development of the field of CART physiology and pharmacology much depends on the finding of CART receptor(s).

In conclusion, CART is extensively expressed in myenteric neurones all along the GI tract and in antral gastrin cells. The addition of CART attenuated the relaxatory response to NO donor in colon, but not in ileum. Cocaine- and amphetamine-regulated transcript seems, however, not to play any major role in classic motor neurotransmission but may be involved in survival and maintenance of enteric neurones and/or in intestinal adaptation and may also be a gastric hormone.

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