

**Immunohistochemical investigations  
of neuropeptides in the brain, corpora cardiaca,  
and corpora allata of an adult lepidopteran insect,  
*Manduca sexta* (L) \***

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**Summary.** In the brain of adult specimens of the tobacco hornworm moth, *Manduca sexta* (L), cells immunoreactive for several kinds of neuropeptides were localized by means of the PAP procedure, by use of antisera raised against mammalian hormones or hormonal peptides. In contrast, no such neurosecretory cells were found in the corpora cardiaca and corpora allata (CC/CA); in the CC/CA, however, immunoreactive nerve fibres were observed, reaching these organs from the brain.

The neurosecretory cells found in the brain were immunoreactive with at least one of the following mammalian antisera, namely those raised against the insulin B-chain, somatostatin, glucagon C-terminal, glucagon N-terminal, pancreatic polypeptide (PP), secretin, vasoactive intestinal polypeptide (VIP), glucose-dependent insulinotropic peptide (GIP), gastrin C-terminus, enkephalin,  $\alpha$ - and  $\beta$ -endorphin, Substance P, and calcitonin. No cells were immunoreactive with antisera specific for detecting neurons containing the insulin A-chain, nerve growth factor, epidermal growth factor, insulin connecting peptide (C-peptide), polypeptide YY (PYY), gastrin mid-portion (sequence 6-13), cholecystokinin (CCK) mid-portion (sequences 9-20 and 9-25), neurotensin C-terminus, bombesin, motilin, ACTH, or serotonin.

All the neuropeptide-immunoreactive cells observed emitted nerve fibers passing through the brain to the CC and in some cases also to the CA. In CC these immunoreactive nerve fibers tended to accumulate near the aorta. It was speculated that neuropeptides are released into the circulating haemolymph and act as neurohormones.

**Key words.** Insect brain – Corpus cardiacum/corpus allatum – Neuropeptides – Immunohistochemistry – Tobacco hornworm

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In insects, several neurosecretory cells, immunoreactive with antisera raised against mammalian neuropeptides, essentially of gastro-entero-pancreatic (GEP) type, have been reported to occur in the brain and other nervous tissues (see Falkmer and Van Noorden 1983; Kramer 1983). Most of these observations have been based on immunohistochemical investigations (e.g., El-Salhy 1981 a; Hansen et al. 1982). Only a few reports include the results of radioimmunochemical assays of extracts prepared from insect nervous tissue (e.g., Gros et al. 1978; Duve et al. 1979, 1981; Kramer 1983) or those of receptor binding studies (Stefano and Scharrer 1981). Only rather few comprehensive investigations of the occurrence of neuropeptides in the nervous system of adult insects have yet been made (Hansen et al. 1982); most immunohistochemical studies of adult insects have comprised rather few neuropeptide antisera (see Falkmer and Van Noorden 1983).

In most insects it is far from clear whether any chemically identified neuropeptides are released by neurosecretory mechanisms to the circulating haemolymph, or whether they act as neurotransmitters or neuromodulators within the nervous system (see Hansen et al. 1982; Kramer 1983). However, in a variety of insects, including lepidopterans (e.g., Lane 1974) the pathways of neurosecretory material from the brain to the neurohemal organs, corpora cardiaca/corpora allata (CC/CA), and to other release sites, are well established.

Against this background, the brain and the CC/CA of the lepidopteran insect *Manduca sexta*, the tobacco hornworm moth, were systematically investigated immunohistochemically for the occurrence of mammalian-type neuropeptides and for their possible transportation and accumulation in CC/CA. This insect has been the object of several investigations of a neuro-endocrine nature (see Kramer 1983).

## Materials and methods

About 400 adult specimens of *Manduca sexta* of both sexes were collected on the first day after eclosion from the stock colony maintained at the Manhattan laboratory. The eggs (a gift from the Metabolism and Radiation Research Laboratory, U.S. Department of Agriculture, Fargo, N.D., USA) were hatched, and the larvae reared at 28°C and 60% relative humidity during a 16 h light/8 h dark photoperiod on a standard diet (Bell and Joachim 1976). The one-day-old adult insects were anaesthetized by cooling to 4°C, the brain and the CC/CA were rapidly microdissected while they were immersed in Ringer's solution and then fixed over night in Bouin's fluid. Specimens were rinsed in 70% ethanol for several days, dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin prior to sectioning.

For routine histological examination the brains and the CC/CA were cut serially at about 5 µm and stained by one of the following techniques: acetaldehyde fuchsin (Buehner et al. 1979), and the Sevier-Munger, the Grimelius, and the Masson-Hamperl procedures for detecting argyrophil and argentaffin cells; the latter indicate the presence of GEP neuroendocrine cells (Grimelius and Wilander 1980).

The neuropeptides were demonstrated by means of the peroxidase-anti-peroxidase (PAP) method of Sternberger (1979). A detailed account of the 44 antisera used is given in Table 1. Organs from 7 to 20 moths were used to test each antiserum. As the number of immunoreactive cells was found to be low (see Results), it was necessary to have all the serial sections from 3 to 5 moths immunostained by only one antiserum to determine the exact localization and incidence of the immunoreactive cells, as well as the precise pathways of the immunoreactive

**Table 1.** Detailed account of the antisera used

Antisera raised against	Working dilutions	Code No.	Specificities	Sources
Porcine insulin <sup>a</sup>	1:100 to 1:2000	912	A-chain of insulin	L. Wide, Dept. Clin. Chem., Univ. Hosp., Uppsala, Sweden
A-chain of bovine insulin <sup>a</sup>	1:100 to 1:2500	Ma 47	A-chain of insulin	P. Westermark, Dept. Pathol., Univ. Uppsala Sweden
B-chain of bovine insulin <sup>a</sup>	1:2000	Ma 37	B-chain of insulin	P. Westermark, Dept. Pathol., Univ., Uppsala Sweden
Mouse nerve growth factor	1:100 to 1:1000	0301	Nerve growth factor	LAREF, Cadempino, Switzerland
Mouse epidermal growth factor	1:100 to 1:1000	1302	Epidermal growth factor	LAREF, Cadempino, Switzerland
Human insulin C-peptide	1:100 to 1:1000		Insulin C-peptide	N. Yanaihara & L. Orci, Inst. Histol. Embryol., Univ. Geneva, Med. School., Switzerland
Synthetic ovine somatostatin	1:1000	213/2	Somatostatin	J.F. Rehfeld, Dept. Med. Biochem., Univ. Aarhus, Denmark
Porcine glucagon	1:1500	1C7	N-terminal of glucagon (Cross reacts with both pancreatic and gut glucagon)	G. Lundqvist, Dept. Clin. Chem., Univ. Hosp., Uppsala, Sweden
Porcine glucagon	1:2000	K 4023	N-terminal of glucagon (Cross reacts with both pancreatic and gut glucagon)	Novo Research Inst., Copenhagen, Denmark
Porcine glucagon	1:1000	AS-1A5	N-terminal of glucagon (Cross reacts with pancreatic and gut glucagon)	G. Lundqvist, Dept. Clin. Chem., Univ. Hosp., Uppsala, Sweden
Glucagon	1:2000	C1-NS	Gut glucagon (glicentin)	H.S. Tager, Dept. Biochem., Univ. Chicago, USA (Tager and Kramer 1976)
Glucagon	1:2500	CTS	C-terminal of glucagon	H.S. Tager, Dept. Biochem., Univ. Chicago, USA (Tager and Kramer, 1976)
Sequence 19-29 of pancreatic bovine/ porcine glucagon	1:100 to 1:2000	GC-5	Sequence 19-29 of pancreatic glucagon. (Contaminated with a subpopulation antibodies against glucagon N-terminal)	K. Imagawa, Res. Div., Otsuka Assay Lab., Otsuka, Japan
Porcine glucagon	1:1000	K 964	C-terminal of glucagon	Novo Research Inst., Copenhagen, Denmark

Table 1 (continued)

Antisera raised against	Working dilutions	Code No.	Specificities	Sources
Porcine glucagon	1:2500	781031	C-terminal of glucagon	Milab Imu-Tek, Malmö, Sweden
Bovine pancreatic polypeptide	1:2000	615-R-110-146-10	Pancreatic polypeptide	R.E. Chance, Lilly Res. Lab., Indianapolis, USA
Polypeptide YY	1:100 to 1:3000	69 D	Polypeptide YY	L. Terenius, Dept. Pharmacol., Biomed. Cent., Univ. Uppsala, Sweden
Synthetic porcine secretin	1:1000	R1-7	Secretin	W.Y. Chey, Rochester, NY, USA
Synthetic porcine secretin	1:1000		Secretin	J. Fahrenkrug, Dept. Clin. Chem., Copenhagen County Hosp., Glostrup, Denmark
Porcine secretin	1:2500	R-787502-1	Secretin	Milab, Imu-Tek, Malmö, Sweden
Porcine vasoactive intestinal polypeptide (VIP)	1:1600	5601-8	VIP	J. Fahrenkrug, Dept. Clin. Chem., Copenhagen, County Hosp., Glostrup, Denmark
Porcine gastric inhibitory peptide (or glucose-dependent insulinotropic polypeptide) (GIP)	1:1600	378	GIP	J.M. Polak, Dept. Histochem., Hammersmith Hosp., London, U.K.
Porcine GIP	1:1000	R 65	GIP	Novo Research Inst., Copenhagen, Denmark
Synthetic human gastrin 17	1:2000	4562	C-terminus of gastrin	J.F. Rehfeld, Dept. Med. Chem., Aarhus Univ., Denmark
Synthetic mid-portion (6-13) of non-sulphated human gastrin-17 <sup>b</sup>	1:100 to 1:800	4710	Sequence 6-13 of gastrin	J.F. Rehfeld, Dept. Med. Chem., Aarhus Univ., Denmark
Porcine CCK-33 <sup>a</sup>	1:100 to 1:2000	4478	Mid-portion of CCK, probably sequence 19-25	J.F. Rehfeld, Dept. Med. Chem., Aarhus Univ., Denmark
Synthetic mid-portion (9-20) of CCK	1:100 to 1:20000	280	Sequence 9-20 of CCK molecule	J.M. Polak, Dept. Histochem., Hammersmith Hosp., London, U.K.
Mixture of synthetic human met- and leu-enkephalin	1:1600	336 K	Enkephalin	L. Terenius, Dept. Pharmacol., Biomed. Cent., Uppsala Univ., Sweden

Table 1 (continued)

Antisera raised against	Working dilutions	Code No.	Specificities	Sources
Synthetic bovine $\alpha$ -endorphin	1:2000	11 B	$\alpha$ -Endorphin	L. Terenius, Dept. Pharmacol., Biomed. Cent., Uppsala Univ., Sweden
Synthetic bovine $\beta$ -endorphin	1:3000	372 B	$\beta$ -Endorphin	L. Terenius, Dept. Pharmacol., Biomed. Cent., Uppsala Univ., Sweden
Synthetic bovine $\beta$ -endorphin	1:1800	7763	$\beta$ -Endorphin	J.I. Thorell, Dept. Nuclear. Med., Malmö Gen. Hosp., Malmö, Sweden
Synthetic human Substance P	1:1600	SP8	Substance P	P.C. Emson, Cambridge Univ., U.K.
Synthetic human Substance P	1:2000	F2SPAb	Substance P	S.E. Leeman, Lab. Hum. Reproduc., Harvard Med. Sch., Boston, USA
Synthetic bovine neurotensin	1:100 to 1:2000	HC 8	C-terminus (sequence (sequence 6-13) of neurotensin molecule	S.E. Leeman, Lab. Hum. Reprod., Harvard Med. Sch., Boston, USA
Synthetic bombesin	1:100 to 1:1500	374	Bombesin	J.M. Polak, Dept. Histochem., Hammersmith Hosp., London, U.K.
Synthetic bombesin	1:10000	R-805101-2	Bombesin	Milab, Imu-Tek, Malmö, Sweden
Synthetic C-terminus of bombesin	1:100 to 1:4000	627	C-terminus of bombesin	J.M. Polak, Dept. Histochem., Hammersmith Hosp., London, U.K.
Porcine motilin	1:100 to 1:1000	326	Motilin	J.M. Polak, Dept. Histochem., Hammersmith Hosp., London, U.K.
Bovine ACTH (1-39)	1:100 to 1000	K 8770601	ACTH	Ferring, Malmö, Sweden
Synthetic ACTH (1-24) <sup>a</sup>	1:250	GPL 54	Sequence 1-24 of ACTH molecule	L.-I. Larsson, Dept. Med. Chem., Aarhus Univ., Denmark
Synthetic ACTH (18-39)	1:250	CLIP	Sequence 18-39 of ACTH molecule	L.-I. Larsson, Dept. Med. Chem., Aarhus Univ., Denmark
Synthetic serotonin	1:100 to 1:2000	6794 A	Serotonin	Coppel Laboratories, Philadelphia, USA

Table 1 (continued)

Antisera raised against	Working dilutions	Code No.	Specificities	Sources
Synthetic serotonin	1:100 to 1:6000	18240	Serotonin	Coppel Laboratories, Philadelphia, USA
Human calcitonin	1:800	A 7	Calcitonin	G. Lundqvist, Dept. Clin. Chem., Univ. Hosp., Uppsala, Sweden

<sup>a</sup> Most of the antisera were raised in rabbits; only a few originated from guinea-pigs (marked with an *asterisk*)

<sup>b</sup> Pretreated with 100 µg of synthetic caerulein per ml diluted antiserum. Caerulein was a gift from Farmitalia Carlo Erba, Lot No. GP/2511/12

nerve fibers. Moreover, in order to adequately control the immunostaining procedure, the serially cut sections from other specimens were stained alternatively with the antiserum and one or another of the negative controls described below. A complete set of sections was stained by one or another of the negative controls, and then immunostained by reactive antiserum (Duve and Thorpe 1981). In addition, the antisera were pre-incubated with rabbit anti-human Clq complement, diluted 1/50 (Dako, Copenhagen; Batch No. 03813) for 24 h at 4° C, as recommended by Buffa et al. (1979). The controls used were as follows:

1. *Positive controls.* Sections of Bouin-fixed mammalian tissue, known to contain cells immunoreactive with the antiserum studied, were processed together with each series of *Manduca* specimen sections.

2. *Negative controls.* a) The first layer antiserum was replaced by normal rabbit or guinea-pig serum;

b) The first layer antiserum was pre-incubated for 24 h at 4° C with the corresponding or related polypeptide(s) (Table 2).

## Results

A diagrammatic representation of the *Manduca* brain, the CC/CA, and their nerves and adjacent structures is given in Fig. 1.

### *Histological background*

In the brain two groups of aldehydefuchsinophil cells were observed in the pars intercerebralis in each half of the protocerebrum, the median neurosecretory cells (MNC) and the lateral neurosecretory cells (LNC). The MNC contained 8 to 10 large round or polygonal (12–20 µm) cell bodies (Fig. 2A). The LNC consisted of 4–5 somewhat smaller (6–10 µm) cells, also round or polyhedral (Fig. 2B). Aldehydefuchsinophil nerve fibers were seen in the nervus corporis cardiaci (NCC), CC, nervus corporis allati (NCA), and CA. None of the three silver staining procedures revealed any argyrophil or argentaffin cells.

**Table 2.** Results of neutralization experiments and sources of antigens used

Antisera	Antigen incubated with 1 ml diluted antiserum	Source	Result <sup>a</sup>
Anti-insulin B-chain (No. Ma 37)	100 µg of porcine insulin	Vitrum, Stockholm, Sweden Lot No. 1800L	+
Anti-insulin B-chain (No. Ma 37)	100 µg of A-chain of bovine insulin	Boehringer Mannheim, F.R.G. Lot No. 1040320	-
Anti-insulin B-chain (No. Ma 37)	100 µg of B-chain of bovine insulin	Sigma Chem., St. Louis, MO, USA, Lot No. 39C-8005	+
Anti-somatostatin (no. 213/3)	100 µg of synthetic human somatostatin	Beckman, Geneva, Switzerland, Lot No. E 1035	+
Anti-glucagon <sup>b</sup>	100 µg of porcine glucagon	Novo Res. Inst., Copenhagen Denmark, Reg. No. 8075	+
Anti-glucagon	100-200 µg of porcine secretin	Kabi Diagnostica, Stockholm Sweden, Lot No. 162904	-
Anti-glucagon	75-125 µg of porcine GIP	J.C. Brown, Univ. Brit. Columbia, Vancouver, Canada	-
Anti-glucagon	50-100 µg of bovine VIP	V. Mutt, Karolinska Inst., Stockholm, Sweden	-
Anti-BPP	100-125 µg of BPP	R.E. Chance, Lilly Res. Lab., Indianapolis, USA	+
Anti-BPP	100-200 µg of porcine insulin	Vitrum, Stockholm, Sweden, Lot No. 18004	-
Anti-BPP	100-150 µg of porcine glucagon	Novo Res. Inst., Copenhagen, Denmark Reg. No. 8075	-
Anti-secretin <sup>b</sup>	100-125 µg of porcine secretin	Kabi Diagnostica, Stockholm, Sweden, Lot No. 162904	+
Anti-secretin	100-300 µg of porcine glucagon	Novo Res. Inst., Copenhagen, Denmark, Reg. No. 8075	-
Anti-secretin	50-100 µg of porcine GIP	J.C. Brown, Univ. Brit. Columbia, Vancouver, Canada	-
Anti-secretin	50-75 µg of bovine VIP	V. Mutt, Karolinska Inst., Stockholm, Sweden	-
Anti-VIP	75-100 µg of porcine VIP	V. Mutt, Karolinska Inst., Stockholm, Sweden	+
Anti-VIP	100-200 µg of porcine glucagon	Novo Res. Inst., Copenhagen, Denmark, Reg. No. 8075	-
Anti-VIP	75 µg of porcine GIP	J.C. Brown, Univ. Brit. Columbia, Vancouver, Canada	-
Anti-VIP	100 µg of porcine secretin	Kabi Diagnostica, Stockholm, Sweden Lot No. 162904	-
Anti-GIP <sup>b</sup>	100 µg of porcine GIP	J.C. Brown, Univ. Brit. Columbia, Vancouver, Canada	+

Table 2 (continued)

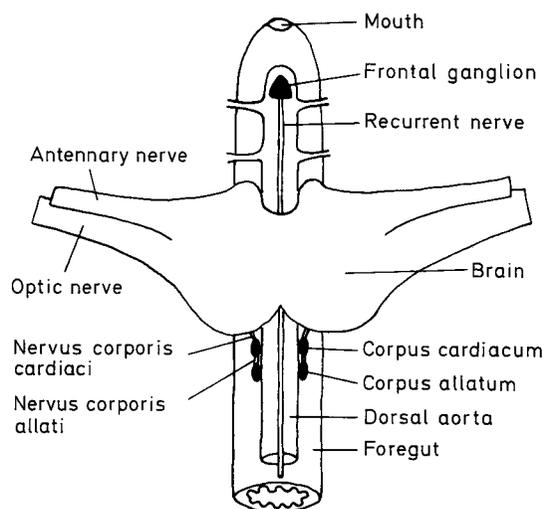
Antisera	Antigen incubated with 1 ml diluted antiserum	Source	Result <sup>a</sup>
Anti-GIP	100–500 µg of porcine glucagon	Novo Res. Inst., Copenhagen, Denmark, Reg. No. 8075	–
Anti-GIP	5–15 µg of glicentin	Novo Res. Inst., Copenhagen, Denmark	–
Anti-GIP	100–300 µg of porcine secretin	Kabi Diagnostica, Stockholm, Sweden, Lot No. 162904	–
Anti-GIP	50–75 µg of porcine VIP	V. Mutt, Karolinska Inst., Stockholm, Sweden	–
Anti-gastrin (No. 4562)	100 µg of synthetic human gastrin I	Beckman, Geneva, Switzerland, Lot No. 0616	+
Anti-gastrin (No. 4562)	100 µg of porcine CCK	V. Mutt, Karolinska Inst., Stockholm, Sweden	+
Anti-gastrin (No. 4562)	100 µg of synthetic caerulein	Farmitalia Carlo Erba, Milano, Italy, Lot No. GP/2511/12	+
Anti-enkephalin	100 µg of synthetic human met-enkephalin	Beckman, Geneva, Switzerland, Lot No. C0422	+
Anti-enkephalin	100 µg of synthetic human β-endorphin	Beckman, Geneva, Switzerland, Lot No. B9034	–
Anti-α-endorphin	100 µg of synthetic α-endorphin	Beckman, Geneva, Switzerland	+
Anti-α-endorphin	100 µg of synthetic human β-endorphin	Beckman, Geneva, Switzerland, Lot No. B9034	–
Anti-β-endorphin	100 µg of synthetic β-endorphin	Beckman, Geneva, Switzerland, Lot No. B9034	+
Anti-β-endorphin	75 µg of synthetic γ-endorphin	Peninsula Lab., San Carlos, CA, USA, Lot No. OD173	+
Anti-β-endorphin	100 µg of synthetic met-enkephalin	Beckman, Geneva, Switzerland, Lot No. CO422	–
Anti-Substance P <sup>b</sup>	100–150 µg of synthetic human Substance P	Beckman, Geneva, Switzerland, Lot No. B90117	+
Anti-calcitonin	100 µg of synthetic human calcitonin	Beckman, Geneva, Switzerland, Lot No. 90828	+

<sup>a</sup> + = Complete inactivation of the antiserum; – = unaffected activity

<sup>b</sup> Includes all the antisera raised against the same antigen, listed in Table 1

#### *Immunohistochemical observations*

In the brain, insulin B-chain, somatostatin, glucagon/glicentin, PP, secretin, VIP, GIP, gastrin C-terminus, enkephalin, α- and β-endorphin, Substance P, and calcitonin immunoreactive cells and nerve fibers were found, as summarized in Fig. 3. The location of the MNC and LNC is the same as for the insulin immunoreactive cells in this figure (see also Fig. 2C, D): Immunoreactive nerve fibers for all these neuropeptides were seen in CC, but



**Fig. 1.** Diagram of brain, corpora cardiaca (CC), and corpora allata (CA) of *Manduca* in relation to surrounding organs

no actual cells. In CA, only some of these nerve fibers were detected. All the other antisera failed to show any immunoreactive nerve cells or fibers.

**Insulin.** Insulin immunoreactive cells were found with the antiserum raised against insulin B-chain (No. Ma 37), but not with that raised against either insulin (No. 912; A-chain specific) or insulin A-chain (No. Ma 47). Two groups of insulin B-chain immunoreactive cells were detected in the pars intercerebralis of each half of the protocerebrum (Fig. 3). The first group consists of 9 large (12–20  $\mu\text{m}$ ) round or polyhedral cell bodies in the MNC area (Fig. 2C). The second group is situated laterally (LNC area) to the first group and consists of 3 to 4 smaller (6–10  $\mu\text{m}$ ) round or sometimes elongated somata (Fig. 2D). The two groups emit nerve fibers, apparently emerging from the brain in the NCC and accumulating in CC, particularly near the aorta (Fig. 2E). From CC, insulin B-chain immunoreactive nerve fibers appear to pass to CA via NCA, where they can be seen among the CA cells (Fig. 2F).

**Somatostatin.** Somatostatin immunoreactive cells are located in the MNC region of the pars intercerebralis (Fig. 3). Four large (20–25  $\mu\text{m}$ ) round cell bodies are found in each half of the protocerebrum (Fig. 4A). Nerve fibers from these cells traverse the brain (Fig. 4B), leave as NCC, and accumulate in CC near the aorta. Somatostatin immunoreactive axons seem to connect CC with CA through NCA.

**Glucagon/glicentin.** Two groups of glucagon/glicentin immunoreactive cells were seen in the calyx cell area of each cerebral hemisphere (Fig. 3). They showed somewhat different immunoreactivities.

The first group is situated in a position mid-anterior to the corpora

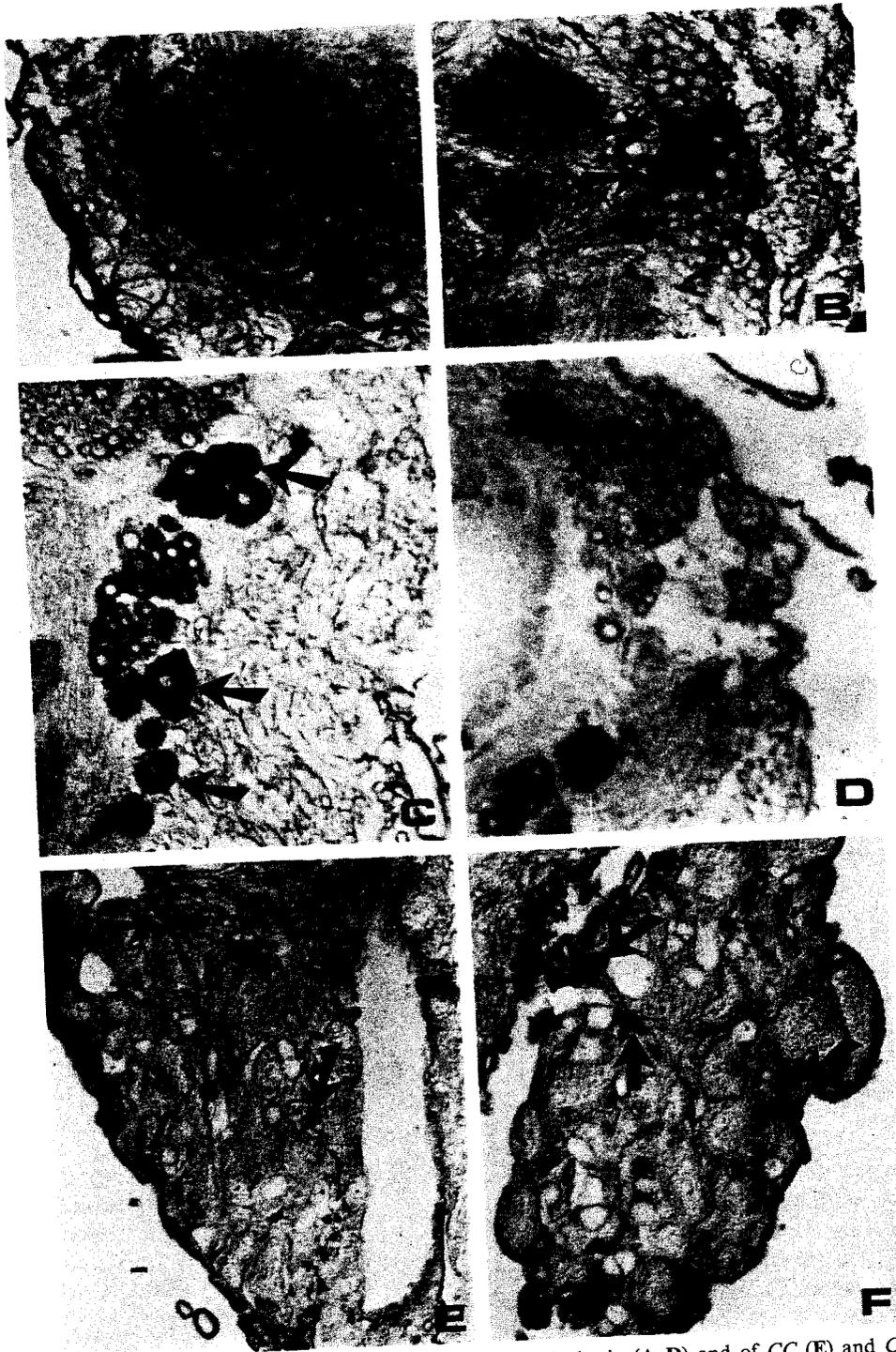


Fig. 2A-F. Photomicrographs of pars intercerebralis in brain (A-D) and of CC (E) and CA (F). A median neurosecretory cells (*MNC*) (arrows); B lateral neurosecretory cells (*LNC*) (arrows); acetaldehydefuchsin staining; C some *MNC* showing insulin B-chain immunoreactivity (arrows); D some *LNC* with insulin B-chain immunoreactivity (top), and in lower part another insulin B-chain immunoreactive cell belonging to *MNC*; E *CC* with fibers (arrows) containing insulin B-chain immunoreactivity; F, *CA* with some fibers showing B-chain reactivity (arrows). A, B, C  $\times 500$ ; D  $\times 400$ ; E, F  $\times 600$

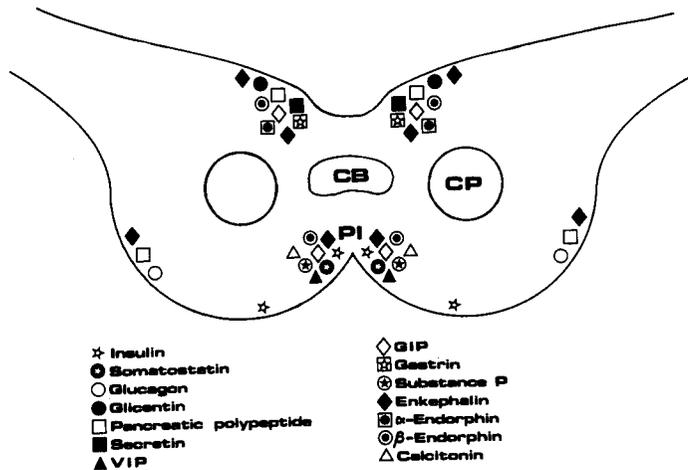


Fig. 3. Diagram of distribution of various neuropeptide immunoreactive cells in brain of *Manduca*; assembly of horizontal sections. *CB* Central body; *CP* corpora pedunculata; *PI* pars intercerebralis. Region around *CP* is calyx-cell area

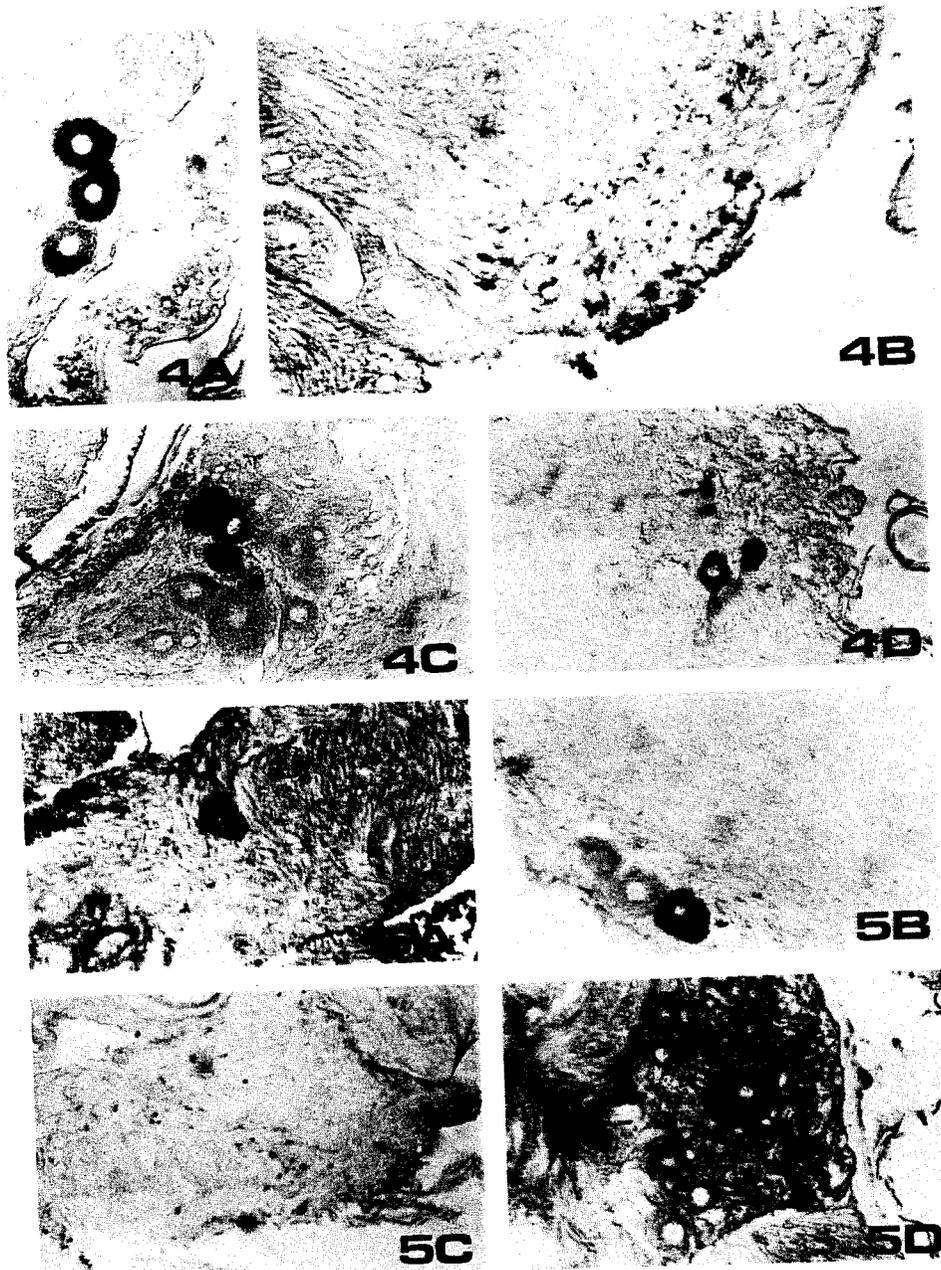
pedunculata in the calyx cell area. It contains 3 to 4 large (15–20  $\mu\text{m}$ ) round or polyhedral somata (Fig. 4C). They reacted with all the antisera specific for the N-terminus of glucagon, both pancreatic glucagon and glicentin-specific (Nos. 1C7, AS-1A5, K 4023), as well as with antiserum No. C1-NS, specific for glicentin. They also reacted with the C-terminal glucagon antiserum (No. GC-5) but only up to a dilution of 1:500.

The second group is also located in the calyx cell area but more ventrolaterally to the aldehydefuchsinophil LNC (Fig. 3). It consists of a pair of small (8–10  $\mu\text{m}$ ) round cells (Fig. 4D). They reacted only with glucagon C-terminus antisera specific for pancreatic glucagon (Nos. GC-5, even at dilutions up to 1:2,000, C, K 964, and 781031).

Axons from the two groups of cells pass through the brain to the CC via NCC. No glucagon/glicentin immunoreactive nerve fibers or cells were observed in NCA or CA.

**PP.** Two groups of PP immunoreactive cells were detected in each protocerebral hemisphere (Fig. 3). The first group is located in the calyx cell area, latero-dorsally to the LNC and consists of 4–5 medium-sized (10–15  $\mu\text{m}$ ) round or pearshaped cells. The second one occurs also in the calyx cell area, in a mid-anterior position to the corpora pedunculata. It contains 3–4 spindle-shaped medium-sized (12–15  $\mu\text{m}$ ) cells. From these two groups of PP immunoreactive nerve cells nerve fibers were seen to pass through the brain, apparently to CC via NCC. In CC, PP immunoreactive nerve fibers accumulate close to the cells lining the aorta. PP immunoreactive nerve fibers are rare in NCA and in CA.

**Secretin.** Secretin immunoreactive cells were detected among the calyx cells, anterior to the corpora pedunculata (Fig. 3). They form a group of 2–3



**Fig. 4A-D.** Photomicrographs of somatostatin immunoreactive cells in pars intercerebralis of *Manduca* brain (A); transverse sections of somatostatin immunoreactive fibers in brain (B); three glucagon N-terminal immunoreactive cells among calyx cells in mid-anterior position to corpora pedunculata (C); and two glucagon C-terminal immunoreactive cells in pars intercerebralis (D). A, C, D  $\times 400$ ; B  $\times 600$

**Fig. 5.** A Secretin immunoreactive cell in calyx cell area, anterior to corpora pedunculata. B, C VIP immunoreactive cell in pars intercerebralis of *Manduca* brain, and VIP immunoreactive fibers in CC. Note that VIP immunoreactive fibers accumulate at nervus corporis cardiaci (arrow). D GIP immunoreactive cell in pars intercerebralis. A-D  $\times 400$

cells in each half of the protocerebrum (Fig. 5A). These cells are large (20–25  $\mu\text{m}$ ), round or polyhedral, and seem to send axons to NCC through the brain to the CC. No secretin-like immunoreactivity was detected in NCA or CA.

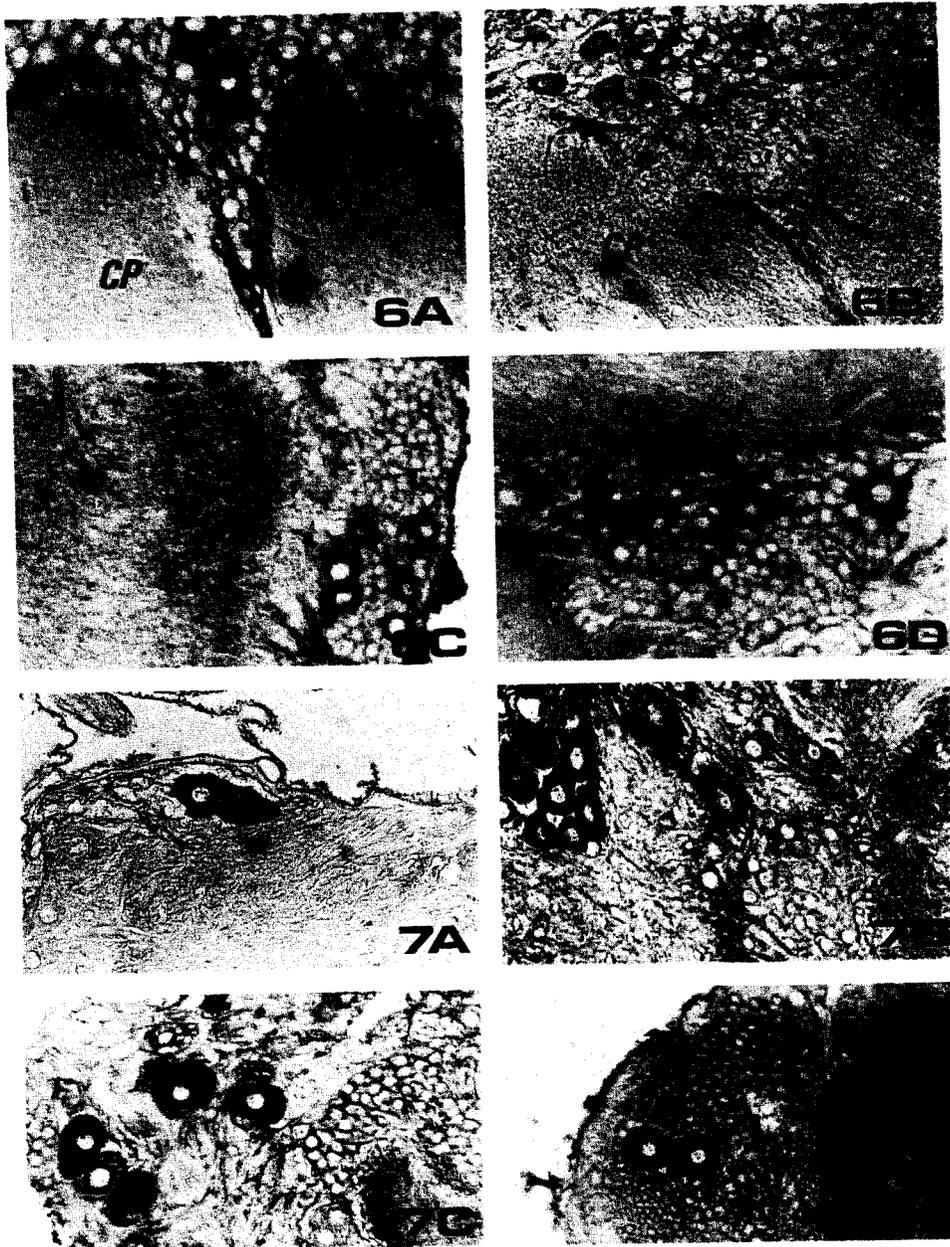
*VIP.* VIP immunoreactive cells are located in the MNC area of the pars intercerebralis (Fig. 3). They form a group of 3–4 large (16–20  $\mu\text{m}$ ) round cells in each protocerebral hemisphere (Fig. 5B). The axons of these cells pass through the brain to NCC, where they accumulate in CC (Fig. 5C). From CC, VIP nerve fibers run to CA via NCA.

*GIP.* Two groups of GIP immunoreactive cells were observed in each half of the protocerebrum, one in the MNC region and the other in the calyx cell area (Fig. 3). The first group is located in the pars intercerebralis and contains a pair of large (16–20  $\mu\text{m}$ ) round cells (Fig. 5D). The second group is situated in a mid-anterior position to the corpora pedunculata and consists of 2 or 3 large (20–25  $\mu\text{m}$ ) round or polyhedral cells. Only a few GIP immunoreactive nerve fibers were detected in the brain, NCC, and CC. Neither in NCA, nor in CA, were there any GIP immunoreactive nerve fibers.

*Gastrin.* Gastrin immunoreactive cells were observed among the calyx cells anterior to the corpora pedunculata (Fig. 3). They form a group of 3 to 4 cells in each half of the protocerebrum. They are medium-sized (12–15  $\mu\text{m}$ ), round, pearshaped, or polyhedral with several processes (Fig. 6A). Gastrin immunoreactive cells and nerve fibers were found with an antiserum (No. 4562) specific for the C-terminus of gastrin, but not with the antiserum (No. 4710) specific to gastrin mid-portion (sequence 6–13). Gastrin C-terminus immunoreactive nerve fibers were detected in the brain, NCC, and CC. NCA and CA did not display any gastrin immunoreactivity.

*Enkephalin.* Enkephalin immunoreactive cells occur in 4 groups in each protocerebral hemisphere (Fig. 3). The first group consists of 5 to 6 small (4–6  $\mu\text{m}$ ) oval or round cells, situated anterior to the corpora pedunculata in the calyx cell area (Fig. 6B). The second group forms 7 to 9 small (5–8  $\mu\text{m}$ ) round cells lateral to the first group (Fig. 6C). The third group consists of 2 or 3 large (18–20  $\mu\text{m}$ ) oval or round cells situated antero-posterior to the second group and posterior to the corpora pedunculata (Fig. 6D). The fourth group is a pair of small (8–12  $\mu\text{m}$ ) round cells in the pars intercerebralis lateral to the MNC. Abundant enkephalin immunoreactive nerve fibers were seen in the brain, NCC, CC, NCA, and CA.

*$\alpha$ -Endorphin.*  $\alpha$ -Endorphin immunoreactive cells are located in the calyx cell area anterior to the corpora pedunculata of each cerebral hemisphere. They are 2 or 3 large (20–25  $\mu\text{m}$ ) polyhedral round or pyramidal cells (Fig. 7A) which emit nerve fibers, passing through the brain to NCC and finally reach the CC. Here, an accumulation of  $\alpha$ -endorphin immunoreactive



**Fig. 6.** A Gastrin C-terminus immunoreactive cell and axons (*arrow*) in calyx cell area, anterior to corpora pedunculata (CP). B Small enkephalin immunoreactive cells anterior to corpora pedunculata (CP). C Enkephalin cells lateral to group in B. D Another group latero-posteriorly to group in C. A  $\times 500$ ; B, C, D  $\times 600$

**Fig. 7.** A  $\alpha$ -Endorphin immunoreactive cell in calyx cell area, anterior to corpora pedunculata. B Group of somewhat smaller  $\beta$ -endorphin immunoreactive cells in same area. C Group of Substance P immunoreactive cells in pars intercerebralis. D Pair of calcitonin immunoreactive cells in same area of brain. A, C, D  $\times 400$ ; B  $\times 600$

nerve fibers was observed. No  $\alpha$ -endorphin immunoreactive nerve fibers or cells could be demonstrated in NCA or CA.

*$\beta$ -Endorphin.* Two groups of  $\beta$ -endorphin immunoreactive cells were observed in each cerebral hemisphere, one in the MNC region and the other in the calyx cell area (Fig. 3). The first group is in the pars intercerebralis and contains 3–5 small (5–8  $\mu$ m) round cells. The second group lies among the calyx cells anterior to corpora pedunculata. It consists of 5 medium-sized (10–15  $\mu$ m) round or oval cells (Fig. 7B).  $\beta$ -Endorphin nerve fibers were observed in the brain, NCC, and CC, but not in NCA or CA.

*Substance P.* Substance P immunoreactive cells were found in the pars intercerebralis dorsal to the MNC (Fig. 3). They constitute a group of 4 large (15–20  $\mu$ m) round or pearshaped cells in each cerebral hemisphere (Fig. 7C). Immunoreactive nerve fibers are seen in the brain and NCA. In CC, Substance P immunoreactive nerve fibers are situated away from the aorta. Substance P immunoreactive nerve fibers also seem to connect CC with CA via NCA.

*Calcitonin.* Calcitonin immunoreactive nerve cells are located in the pars intercerebralis latero-anterior to the LNC. They constitute a group of 2 to 3 large (15–18  $\mu$ m) round cells in each cerebral hemisphere (Fig. 7D). Axons of these cells connect the brain with the CC. No calcitonin immunoreactivity was observed in NCA or in CA.

#### *Specificity controls*

No immunostaining was obtained when the first layer antiserum was replaced by normal rabbit or guinea-pig serum. The pre-incubation of the antisera with rabbit anti-human Clq complement had no effect on the immunostaining obtained. All the antisera giving immunostaining of the nerve cells and fibers were inactivated completely after pre-incubation with the corresponding peptides but not with related ones (Table 2). The staining of serially cut sections by one of the negative controls (normal rabbit serum or guinea-pig serum, or neutralized antisera) and re-staining of the same sections with active antisera proved the specificity of the immunostaining obtained. This latter type of control provided the most precise evidence possible for specificity, as the same cells in the same section could be tested.

The possibility that some of the neurosecretory cells and nerve fibers may contain more than one neuropeptide (see Falkmer and Van Noorden 1983) was not examined in the present investigation. Such a study requires large numbers of additional specimens, preferably embedded in resins, allowing semithin serial sections to be cut. Thus, it must await future investigations.

#### *Frequency and size of various neurosecretory cells*

In terms of frequency, the approximate population of immunoreactive cells in *Manduca* brain ranged as follows: 34 cells – enkephalin; 26 – insulin;

20 -  $\beta$ -endorphin; 18 - PP; 12 - glucagon/glicentin; 8 - gastrin, GIP, somatostatin, Substance P, VIP; and 6 - calcitonin,  $\alpha$ -endorphin, secretin. A total of approximately 170 cells in the brain have been associated with neuropeptide components.

Approximate cell sizes were: 20-25  $\mu\text{m}$  - somatostatin, secretin, GIP (2nd group),  $\alpha$ -endorphin; 15-20  $\mu\text{m}$  - insulin (1st group), glicentin, VIP, GIP (1st group), enkephalin (3rd group), Substance P, calcitonin; 10-15  $\mu\text{m}$  - PP, gastrin, enkephalin (4th group),  $\beta$ -endorphin (2nd group); and 5-10  $\mu\text{m}$  - insulin (2nd group), glucagon, enkephalin (1st and 2nd groups),  $\beta$ -endorphin (1st group).

### Discussion

In general, three groups of neurosecretory cells were demonstrated in the *Manduca* brain. The first group, situated in the pars intercerebralis, contains insulin B-chain, somatostatin, GIP, VIP, enkephalin,  $\beta$ -endorphin, Substance P, and calcitonin immunoreactive cells. The second group, located posterior to the corpora pedunculata, consists of glucagon C-terminal (pancreatic glucagon), PP, and enkephalin immunoreactive cells. The third group, anterior to the corpora pedunculata, includes glucagon N-terminal (glicentin), PP, secretin, GIP, gastrin C-terminus, enkephalin, and  $\alpha$ - and  $\beta$ -endorphin immunoreactive cells. In a previous study (El-Salhy et al. 1980) of the larval brain of a dipteran insect, the hoverfly (*Eristalis aeneus*), the neurosecretory cells also form three groups in similar locations to those of *Manduca sexta*. This observation suggests that neurosecretory cells occur in distinctive locations within the insect brain, viz. in the pars intercerebralis and the calyx cell area, both anteriorly and posteriorly to the corpora pedunculata. Obviously, further investigations are needed in order to test whether this topography, based on two species only, has general validity.

### *The islet hormones*

Insulin-like immunoreactive material has been detected in extracts of heads and of whole bodies of several species of insects (Tager et al. 1976; Duve et al. 1979; Le Roith et al. 1981); the cellular site of this material has been demonstrated in some of the MNC of the brain (Duve and Thorpe 1979; El-Salhy et al. 1980; Yui et al. 1980). The present finding in the brain of *Manduca sexta* of insulin-like immunoreactivity in cells with similar shape and location to those of MNC is thus in agreement with those of previous reports. In *Manduca*, however, insulin immunoreactivity was also observed in cells of similar shape and location as the LNC. The cells containing insulin-like peptide(s) are the second most frequent cell type identified in this study. The present immunohistochemical observations indicate that the insulin immunoreactive material reported to occur in CC/CA of *Manduca* (Tager et al. 1976) is probably that present in nerve fibers of insulin immunoreactive cells coming from the brain to CC/CA.

Insulin-like material, isolated from the head of dipteran insects (Duve

et al. 1979; Le Roith et al. 1981) has been reported to be similar in molecular size, physico-chemical properties and biological activities to those of mammalian insulin. Preliminary characterization of insulin-like material isolated from the nervous tissue and haemolymph of *Manduca sexta* has shown that it also has a molecular weight, an amino-acid composition and biological activity similar to those of mammalian insulins (Tager et al. 1976; Kramer et al. 1980, 1982). Our immunohistochemical findings, however, point to the possibility that the insulin-like material in *Manduca* brain is not wholly identical to mammalian insulin. As a matter of fact, the amino-acid composition of the insulin-like material of *Manduca sexta* differs from that of porcine insulin in three residues, viz. serine, leucine and phenylalanine (Kramer et al. 1982); this difference may be in the A-chain rather than in the B-chain. The absence of C-peptide immunoreactivity in the *Manduca* brain and CC/CA may rather be due to differences in the molecular structure between the *Manduca* C-peptide and human C-peptide than to the absence of this peptide. This assumption gets support from the fact that, even in vertebrates, the C-peptide molecular structure is highly variable (Falkmer and Emdin 1981).

Insulin belongs to a family of polypeptides sharing biological activities (receptor sites) and, to some extent, having also molecular similarities. This family includes insulin, insulin-like growth factors I and II (IGF-I, IGF-II) (somatomedin A and C), nerve growth factor (NGF), and relaxin (see Blundell and Humbel 1980). Insulin and NGF are said to show the closest functional and structural homology (Sabesan 1980). The absence of NGF immunoreactivity in the *Manduca* brain and CC/CA indicates that the insulin immunoreactive material detected is more insulin-like than NGF-like.

The present detection of somatostatin immunoreactive cells and nerve fibers in the *Manduca* brain and that of immunoreactive nerves in CC/CA are both in accordance with earlier reports on the occurrence of somatostatin immunoreactive cells in the nervous system of other protostomian invertebrates (Doerr-Schott et al. 1978; El-Salhy et al. 1980; Martin and Dubois 1981). The same statement applies to our finding of glucagon immunoreactive cells and nerves in the *Manduca* brain (El-Salhy et al. 1980; Schot et al. 1981). The glucagon immunoreactivity detected previously in *Manduca* CC/CA (Tager et al. 1976) is probably localized in nerve fibers coming from the brain (see above). The recent observation that somatostatin-like material is synthesized and stored in cells of the CC of the dictyopteran insect, *Leucophaea maderae* (Hansen et al. 1982) is not paralleled by our data, nor by those made in a locust (Doerr-Schott et al. 1978). As suggested by Hansen et al. (1982), "genus differences may offer a possible explanation for this discrepancy".

The results of the present study show that actually two groups of glucagon immunoreactive cells occur in the *Manduca* brain. The reaction of the group immunoreactive for glucagon N-terminal and glicentin with one of the glucagon C-terminal specific antisera at low dilution is apparently due to the presence of a subpopulation of antibodies cross-reacting with glucagon N-terminal as demonstrated in the characterization of the antiserum

by RIA (Imagawa et al. 1979). On the other hand, the inability of the group of cells immunoreactive to glucagon C-terminus to react with glucagon N-terminal specific antisera shows that these cells do not contain glucagon identical to that of mammals. In the *Manduca* midgut a glucagon-like peptide of approximate molecular weight of 15,000 has previously been described (Tager and Kramer 1980), partly by use of the same antisera as in the present study. Again, no glucagon immunoreactivity identical to mammalian pancreatic glucagon of low-molecular weight (3,500) was discovered.

PP is a member of a newly discovered family of peptides including the polypeptide YY (PYY) and the neuropeptide Y (NPY) (Tatemoto 1982a, b). This family has the same C-terminus and many amino-acid residues in common. PP immunoreactive cells and nerve fibers have been found in the nervous system of an annelid (Sundler et al. 1977), two molluscs (Van Noorden et al. 1980; Schot et al. 1981), and three insects (Duve and Thorpe 1980; El-Salhy et al. 1980; Yui et al. 1980; El-Salhy 1981b). Partial characterization of PP-like material isolated from the head of the blowfly, *Calliphora vomitoria*, suggests that this material has a close structural similarity to the mammalian PP (Duve et al. 1981). Recently, however, it has been questioned whether or not the PP-like material detected in the nervous system of protostomian invertebrates is actually genuine PP, regarding the close structural similarities between PP, PYY, and NPY (Tatemoto 1982a, b). The present findings of cells and fibers immunoreactive to PP but not to PYY antisera indicate that the PP immunoreactivity observed in *Manduca* brain is not due to cross-reaction with PYY-like material. However, the possibility is still open that it may be NPY.

#### *The gastrin family*

Gastrin immunoreactive cells and nerve fibers have been reported to occur in the central nervous system of three species of insects (El-Salhy et al. 1980; Yui et al. 1980; Duve and Thorpe 1981) as well as in a mollusc (Schot et al. 1981). Previous observations with RIA of gastrin-like immunoreactive material in extracts of the *Manduca* brain and CC/CA (Kramer et al. 1977) could now be explained as being due to cells in the brain and their axons in the CC. By means of gastrin/CCK region specific antisera, the gastrin cells in the nervous tissue of other insects have been found to contain only gastrin C-terminus immunoreactivity (El-Salhy et al. 1980; Duve and Thorpe 1981). The gastrin immunoreactivity detected here, as revealed by several region-specific antisera, was also due to the gastrin C-terminus only. This observation is thus in keeping with the assumption that in invertebrates and lower vertebrates the gastrin/CCK family is represented by one cell type only, containing the C-terminus which is common to gastrin and CCK (Falkmer et al. 1980a; El-Salhy 1981a).

#### *The secretin family*

The present observation of secretin immunoreactive cells and fibers in the *Manduca* brain as well as immunoreactive nerve fibers in CC, together with

the previous reports of secretin immunoreactive cells and fibers in molluscs (Van Noorden et al. 1980; Schot et al. 1981) and in an insect larva (El-Salhy et al. 1980), support the assumption that this peptide appeared early in the course of evolution as a neuronal material (El-Salhy 1981 a). Likewise, it has been speculated that VIP represents a good candidate for being closely related to the ancestral molecule of the secretin/glucagon family as it is the only member that has all the biological actions of the group (see Van Noorden and Falkmer 1980). This assumption gains support from the present findings of VIP immunoreactive cells and fibers in the brain and of immunoreactive tracts in CC/CA of the tobacco hornworm moth and the previous observations of VIP immunoreactive cells and axons in the nervous system of two other protostomian invertebrates, viz. an annelid (Sundler et al. 1977) and a mollusc (Schot et al. 1981).

It has been hypothesized that GIP does not appear before the evolutionary stage where the islet organ has been established and only in the mucosa of the alimentary canal (Falkmer et al. 1980 b; Falkmer and Van Noorden 1983). The present localization of GIP immunoreactivity in neurons of the *Manduca* brain, as well as in nerve fibers in CC, together with the recent report of GIP immunoreactive nerve fibers in the nervous system of a mollusc (Schot et al. 1981), do not support this assumption. Instead, these observations show that GIP might well have appeared in the course of evolution as early as the other members of the secretin/glucagon family.

#### *The lipotropin family*

Enkephalin-like immunoreactive cells and fibers have been reported to occur in the nervous system of annelids (Alumets et al. 1979; Zipser 1980), of molluscs (Martin et al. 1979; Schot et al. 1981) and of insects (El-Salhy et al. 1980; Rémy and Dubois 1981; Hansen et al. 1982). By means of RIA, enkephalin-like immunoreactive material has been detected in the brain and CC of another insect, *Locusta migratoria* (Gros et al. 1978). Our observations of enkephalin immunoreactive cells and tracts in the *Manduca* brain agree with all these reports. There were more enkephalin-like peptide containing cells in *Manduca* brain than any other cell type identified. In regard to our finding of enkephalin immunoreactive fibers in CC/CA, however, they rather conform to the RIA results of Gros et al. (1978) than to the immunohistochemical observations of Rémy and Dubois (1981) who found immunoreactive enkephalin in CC/CA of the locust.

$\alpha$ -Endorphin immunoreactive cells have been reported to occur in the suboesophageal ganglion of some lepidopteran insect species, but not in that of a hemipteran or of an orthopteran (Rémy et al. 1978, 1979). In two dipteran insects (El-Salhy et al. 1980; Duvé and Thorpe 1982), as well as in annelids (Alumets et al. 1979; Rémy and Dubois 1979), endorphin immunoreactive cells and nerve fibers have been observed in the brain. Moreover, there is a recent report that in the CC/CA of the dictyopteran insect, *Leucophaea maderae*,  $\beta$ -endorphin immunoreactive nerve fibers are present (Hansen et al. 1982). The results of the present study confirm and extend the reports mentioned above by showing that  $\alpha$ - and  $\beta$ -endorphin

immunoreactivities occur in two different cell populations, and that these cells seem to send axons to CC.

#### *Other neuropeptides*

Our finding of Substance P in the brain of the tobacco hornworm moth gives support to previous observations in insects (El-Salhy et al. 1980; Benedeczy et al. 1982) and molluscs (Schot et al. 1981). Moreover, the results of the present study indicate that Substance P immunoreactive cells within the *Manduca* brain send fibers to the neurohaemal organ, CC. This observation conforms with that recently reported for the dictyopteran *Leucophaea maderae* where Substance P immunoreactive deposits have been demonstrated in CC/CA (Hansen et al. 1982). The observation made in that report that Substance P is synthesized and stored in insect CC, does not, however, parallel that made in the present study. It is, however, well known that the degree of variability among insects is particularly evident with respect to the CC.

The demonstration of calcitonin immunoreactivity in neurons within the *Manduca* brain and their axons in CC, together with that made previously in molluscs (Schot et al. 1981) and in CC of another insect (Hansen et al. 1982), shows that also calcitonin, or calcitonin-related peptide(s), may have a wide distribution in the nervous system of protostomian invertebrates.

The absence of neurotensin and bombesin immunoreactivity in the *Manduca* brain may seem puzzling as these two peptides recently have been observed by Grimmelikhuijzen and co-workers (1981) already in neurons of coelenterates (see Falkmer and Van Noorden 1983) and by Hansen et al. (1982) in CC of an insect, *Leucophaea maderae*. However, the anti-neurotensin antiserum used in the present study (HC8) failed to detect any immunoreactivity in *Hydra* nerve cells. Similarly, bombesin immunoreactivity in coelenterates has been demonstrated only with an antiserum raised against GRP (gastrin releasing peptide) which cross-reacts with both bombesin and GRP (Grimmelikhuijzen et al. 1981). Thus, the absence of neurotensin and bombesin immunoreactivity in the *Manduca* brain may be due to an inability of the antisera used to cross-react with these neuropeptides rather than to their absence, particularly since strong immunoreactions were observed in CC of *Leucophaea* (Hansen et al. 1982).

#### *Hypothetical role of neuropeptides in insect brain*

The present observation that GEP neuropeptide immunoreactive cells observed in the *Manduca* brain emit nerve fibers which seem to reach the neurohaemal organ, CC, suggests that these neuropeptides may be produced in the brain and transferred to the neurohaemal organ for storage and subsequent release into the circulating haemolymph. This assumption gains some support from the fact that two of these peptides, viz. insulin and glucagon, have been detected in the circulating haemolymph of insects

(Kramer et al. 1982) and that they display biological activities comparable to those of mammalian insulin and glucagon (Tager et al. 1976; Duve et al. 1979; Le Roith et al. 1981). The possibility that some of these GEP neuropeptides may act as neurotransmitters and/or neuromodulators and exert their effect on neighbouring cells in the brain or CC/CA cannot, however, be excluded. If the hypothesis that the GEP neuropeptides observed in *Manduca* brain, like the classical neurosecretory products of insects (cf Scharrer 1983), act as neurohormones, has any validity, it strengthens the view that the insect brain exerts not only nervous control but also endocrine functions of the kind that in vertebrates are carried out by the classical GEP endocrine cells. The present data represent one of many evidences supporting the pioneer work of Ernst and Berta Scharrer (1937), showing the commonality between the nervous and endocrine systems (see Scharrer 1983).

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