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Localisation of Pancreatic Polypeptide (PP)-like Immunoreactive Material in Neurones of the Brain of the Blowfly, *Calliphora erythrocephala* (Diptera)

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Summary. The brain of the blowfly, *Calliphora erythrocephala*, has been studied by means of the peroxidase-antiperoxidase immunocytochemical method, with the use of antibodies to bovine pancreatic polypeptide (BPP). A number of immunoreactive neurones have been localised, some corresponding to neurones previously identified tentatively as neurosecretory. This finding is further evidence that biologically active peptides, previously considered to be "vertebrate", also exist in invertebrates. It also supports the concept of their evolutionary origin in nervous tissue.

Key words: Pancreatic polypeptide (PP)-like material – Immunocytochemistry – Neuropeptides – Neurosecretory cells – *Calliphora erythrocephala.*

Many of the biologically active peptides initially isolated from the gastro-entero-pancreatic region of vertebrates have now been demonstrated in the central and peripheral nervous system (Table 1a) and, conversely, neuropeptides have been shown to occur in the gut (Table 1b). Furthermore, there is increasing evidence that several of these and other "vertebrate" peptides are also present in invertebrates, particularly in nervous tissue of the Insecta. Thus, somatostatin-like material has been shown to be present in neurosecretory cells of the pars intercerebralis of the orthopteran *Locusta migratoria* (Doerr-Schott et al. 1978). Moreover, Remy et al. (1977, 1978, 1979) have shown that a neurophysin-vasopressin-like substance occurs in the suboesophageal ganglion of *Locusta migratoria* and the phasmid *Clitumnus extradentatus* and that an α-endorphin-like substance is present in the suboesophageal ganglion of two larval lepidopterans, *Bombyx mori* and

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Table 1a. Gastro-entero-pancreatic peptides also occurring in the nervous system

Peptide	Reference
Cholecystokinin (CCK)	Dockray (1976)
Gastrin	Rehfeld (1978)
Glucagon	Lorén et al. (1979a)
Insulin	Havrankova et al. (1978)
Pancreatic polypeptide (PP)	Lorén et al. (1979b)
Vasoactive intestinal peptide (VIP)	Fahrenkrug (1979)

Table 1b. Neuropeptides also occurring in the gut

Peptide	Reference
Endorphin	Grube et al. (1978)
Enkephalin	Polak et al. (1977)
Neurotensin	Sundler et al. (1977b)
Somatostatin	Luft et al. (1978)
Substance P	Pearse and Polak (1975)
Thyrotropin-releasing hormone (TRH)	Morley et al. (1977)

Thaumetopoea pityocampa. A gastrin-like peptide has been demonstrated in extracts of the neuroendocrine system of the lepidopteran *Manduca sexta* Kramer et al. 1977), and both glucagon-like and insulin-like peptides have been shown to be present in extracts of the corpora cardiaca/corpora allata complex in this species (Tager et al. 1976). Insulin-like material has been isolated from extracts of the brain of the dipteran *Calliphora vomitoria* (Duve et al. 1979) and has been localised to certain of the median neurosecretory cells (Duve and Thorpe 1979). In the present immunocytochemical study of the brain of *Calliphora* we have tested the possibility that pancreatic polypeptide-like material is present.

Materials and Methods

Animals and Tissue Preparation: Female blowflies (*Calliphora erythrocephala*) 3 to 5-day-old and fed *ad libitum* on a diet of sugar and water were anaesthetised with carbon dioxide. Two types of specimens were prepared: (a) the brain minus the outer parts of the optic lobes, and (b) the entire brain with the optic lobes intact. The tissues were fixed overnight in aqueous Bouin's fluid, and the brain pieces were collected in groups of 50 and embedded as a pellet in paraffin wax. Serial sections (6 μm) of such pellets permitted initial screening of antisera and control experiments to be carried out whereby a maximum tissue surface area was exposed to minimum amounts of antisera. The whole brains were embedded singly, and serial sections (6 μm) were used to determine the precise location and numbers of reactive cells.

Antisera and Hormones: Two antisera against bovine pancreatic polypeptide (BPP) were kindly provided by (i) Dr. R.E. Chance, Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, U.S.A. (Lot 615-R 110-146-10) and (ii) Dr. N.R. Lazarus, Wellcome Foundation Research Laboratories, Dartford, Kent, England (Lot WRR-1-28679). BPP hormone (Lot 615-D 63-166-7) was provided by Dr. R.E. Chance.

Peroxidase-Antiperoxidase (PAP) Immunocytochemistry: Sections were stained according to the peroxidase-antiperoxidase method of Sternberger (1974). BPP antiserum was applied at dilutions of from 1 : 10 to 1 : 5000. A suitable working dilution for both antisera, yielding dark-staining cells with a clear background, was found to be in the range of 1 : 1000 to 1 : 2000. Sections were deparaffinized in xylene and treated with a solution of 0.03% H_2O_2 in methanol. They were then washed in Tris-HCl buffer pH 7.2 containing 0.5% human albumin (Behringwerke, FRG) for 1 h followed by Tris-HCl buffer alone for 30 min. Antiserum was applied for 20 h at 4° C and the sections were then treated with antibody against rabbit serum IgG raised in swine (Dako Immunoglobulins, Copenhagen, Denmark) at a concentration of 1 : 20 for 1 h at room temperature (20° C). This was followed by application of rabbit antiperoxidase bound to horseradish peroxidase (PAP) at a concentration of 1 : 50 for 30 min at room temperature. All antisera were diluted with 0.04 M phosphate buffer (pH 7.2) and thorough washing between antisera applications was with 0.05 M Tris-HCl buffer (pH 7.2). Sections were developed for 20 min in a solution of 0.05% 4-Cl-naphthol in Tris-HCl buffer (pH 7.6) containing 0.005% H_2O_2. They were then mounted in 0.2 M glycerine buffer (pH 2.3), in which the purplish-black immunoreaction product remains stable for at least one month. When required, the cover slip was removed and the section destained with xylene and restained with paraldehyde fuchsin, phloxine, or haematoxylin and eosin. For controls, adjacent sections were treated with antigen-inactivated antisera (30 µg BPP, dissolved in phosphate buffer, pH 7.2, per ml diluted antiserum, 1 : 2000; 24 h at 4° C). Complete "absorption-out" of the two antisera was verified in sections of normal human and mouse pancreas. Other controls included the use of normal rabbit serum to replace the specific BPP antisera and omission of either rabbit IgG or PAP in the procedure.

Results

Cells showing PP-like immunoreactive material were found in several clearly defined regions of the brain (Fig. 1). They are most numerous in the suboesophageal ganglion, being found at various levels in the anterior-posterior axis. They are located predominantly in the midline of the ganglion, especially in the thickened area of neurones (Fig. 1 H 1), but are also present laterally where the neuronal layer is very thin (Fig. 1 H 2, H 3). The total number of immunoreactive cells identified in the suboesophageal region was 10–12. In the mass of neurones situated in the intermediate zones between the central body of the brain and the optic lobes 3–4 PP-like positive cells are found dorsally, as well as 3–4 cells ventrally (Fig. 1 A 1, B 1, F, G). In addition, one cell lies close to the optic nerve fibre tracts (Fig. 1 D, E). In the dorsal region of the brain 2 immunoreactive cells are located in a position slightly anterior to the median neurosecretory cells (Fig. 1 C) and occasionally, at the base of the median neurosecretory cell group, 2–4 cells are seen.

Reactive cells are 15–22 µm, with a rounded or elliptically shaped perikaryon containing considerable cytoplasm. An axon hillock, filled with immunoreactive material, was seen in several cells (Fig. 1 A 2, B 2), but it was not possible to follow the axonal pathway beyond this. No clear pattern emerged with regard to the relationship of immunoreactive cells to their neighbouring cells or tissues. They are situated adjacent to nonreactive cells of similar size and cytoplasmic content in the suboesophageal region, but elsewhere they adjoin much smaller cells with little cytoplasm. They frequently occupy a position immediately adjacent to the neuropile.

Removal of the immunoreaction product and subsequent restaining of the sections showed that the reactive cells are very weakly aldehyde-fuchsin positive and that they are also phloxinophil. Restaining with paraldehyde fuchsin proved very useful in visualizing cells in the "absorbed-out" sections which were clearly PP-positive in the adjacent serial section (cf. Fig. 2B with Fig. 2D).

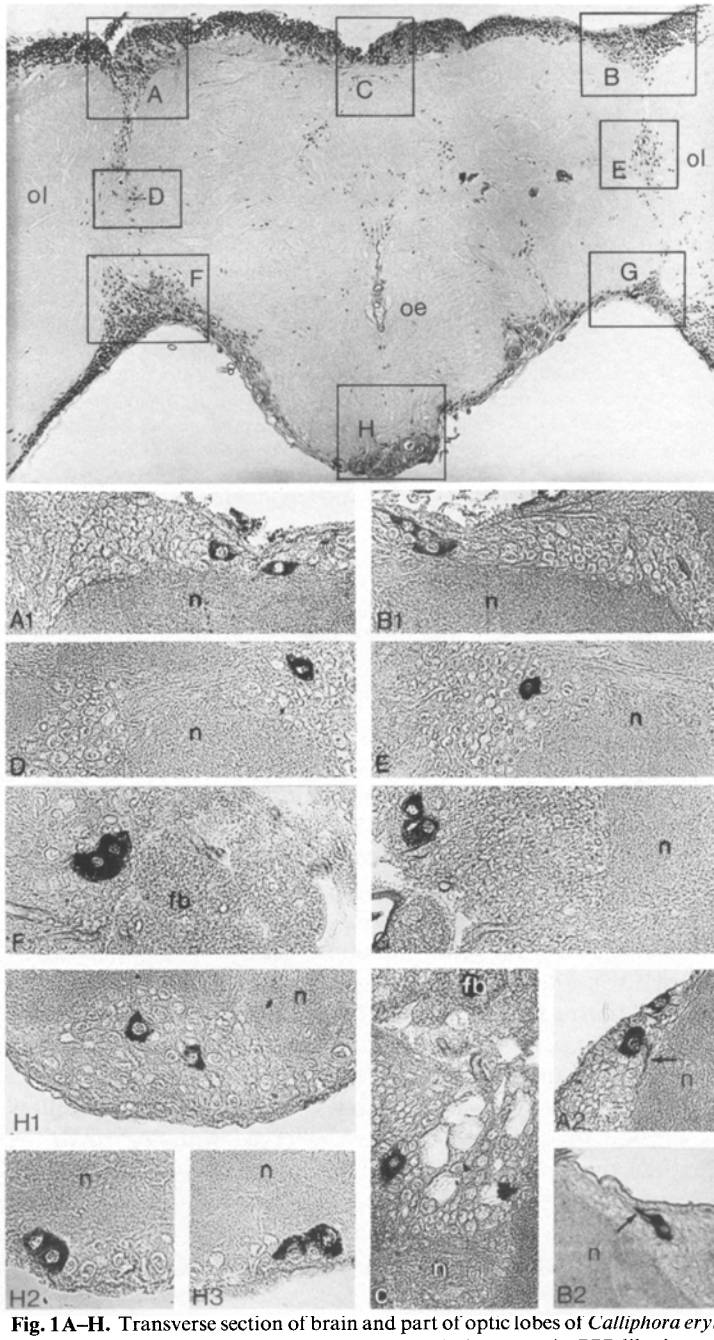

Fig. 1A–H. Transverse section of brain and part of optic lobes of *Calliphora erythrocephala* stained with haematoxylin and eosin to show regions (**A–H**) that contain BPP-like immunoreactive cells. ×140. **A–H** Transverse sections of brain treated with BPP antiserum (1 : 2000 – PAP technique) showing immunoreactive cells; *n* neuropile; *ol* optic lobe; *oe* oesophagus; *fb* fat body. ×200

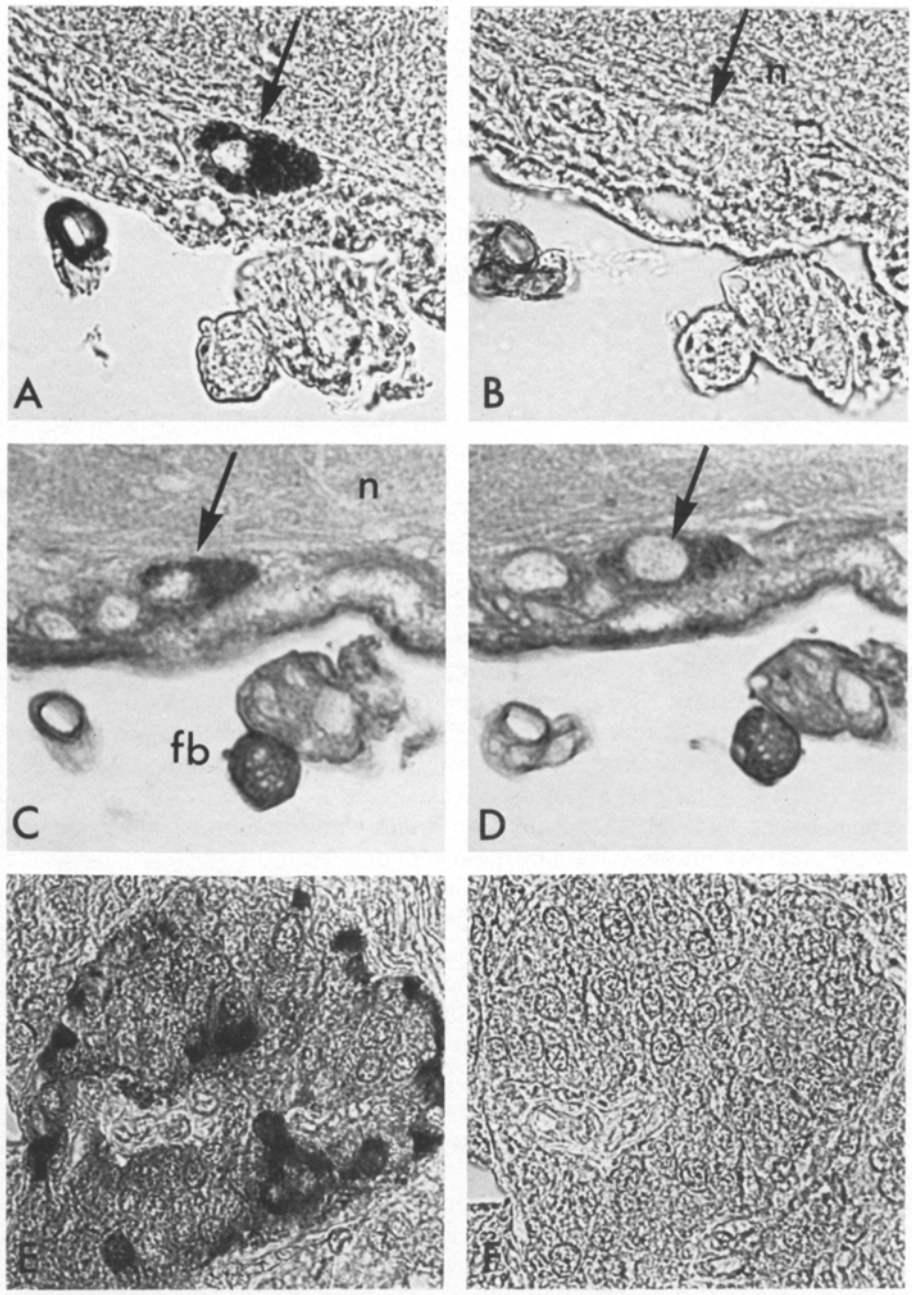

Fig. 2A–F. A–D Transverse sections through part of suboesophageal ganglion of brain. **A** One BPP-like immunoreactive cell (details as in Fig. 1). **B** Adjacent section of A, treated with antigen-inactivated BPP antiserum (30 µg BPP, dissolved in phosphate buffer (pH 7.2), per ml diluted antiserum (1 : 2,000); 24 h at 4° C. **C** Same section as A destained and restained with paraldehyde fuchsin. **D** Same section as B, destained and restained with paraldehyde fuchsin. (Note slight shrinkage following restaining with paraldehyde fuchsin.) ×1,000. **E–F** Transverse sections through mouse pancreas. **E** BPP-immunoreactive cells in islet of Langerhans (details as in Fig. 1). **F** Section adjacent to E treated with antigen-inactivated antiserum. ×950

Sections treated with antigen-inactivated antiserum gave negative results (Fig. 2B), as did control human and mouse pancreas sections treated in the same manner and run in parallel (Fig. 2F). Nonimmune sera and experiments in which certain stages in the procedure were omitted also gave negative results. It should be emphasized that the specificity of the antibodies used has been shown by abolition of the immunoreaction following absorption of the antisera with pure pancreatic polypeptide hormone. Nevertheless, they may cross-react with unknown peptides or protein molecules which contain PP amino acid sequences. It is for this reason that the term PP-like is adopted throughout this paper.

Discussion

An important recent development in vertebrate neurobiology has been the localisation of peptides such as CCK and VIP, previously considered to be gut hormones, within many regions of the central and peripheral nervous system and an increasing amount of evidence that they function there as true neurotransmitters. The widely discussed APUD-concept of Pearse (1969) and Pearse and Takor Takor (1976) suggests, on the basis of common cytochemical and ultrastructural characteristics, that all peptide hormone-producing cells are derived from the neuroectoderm, as are all neurones. If this is accepted, it should perhaps be a natural corollary to find substances such as CCK and VIP in nervous tissue and logical to discover that they have an important role as neurotransmitters.

The whole question of the neuro- and gastro-entero-pancreatic peptides and their interrelationships has been thrown wide open and the recent discoveries that insulin (Havrankova 1978), glucagon (Lorén et al. 1979a), and pancreatic polypeptide (Lorén et al. 1979b) are also found within the brain, illustrates the versatility and ubiquitous nature of these peptides.

If the neuroectoderm is the source in ontogeny of a whole series of highly active peptides, many, if not all of which remain "in situ" and have an important neurophysiological role, it is interesting to consider where they occur in phylogeny. How old are they in evolutionary terms, and are they present in invertebrates as well as in vertebrates? Studies on annelids (Sundler et al. 1977a; Rémy and Dubois 1978; Alumets et al. 1979), and molluscs (Straus et al. 1975; Grimm-Jørgensen 1978; Strambi et al. 1978, 1979), in addition to those on insects referred to in the Introduction, suggest that peptides similar to many of those isolated and purified in vertebrates were present early in evolution. The finding of PP-like immunoreactive material in neurones of the brain of the annelid *Lumbricus terrestris* (Sundler et al. 1977a) and now in the brain of *Calliphora*, strengthens this view.

Since the discovery of PP (Kimmel et al. 1971) in the chicken pancreas, it has been localised to endocrine and exocrine cells within the pancreas itself, as well as to cells within the stomach and small intestine (Larsson et al. 1976; Alumets et al. 1978). The demonstration by Lorén et al. (1979) that it is also present in nerve fibres and cell bodies, widely distributed in the mammalian and avian central and peripheral nervous systems, suggests that it may be a neurotransmitter, in addition to acting as a metabolic hormone of, at present, uncertain function. (The same conclusion has been drawn by Havrankova et al. 1979, with respect to insulin and its presence in the brain of mammals).

The PP-like immunoreactive material of *Calliphora* occurs in neurones, some of which have not been described in any detail previously, presumably because they do not fulfil the exact criteria of neurosecretory neurones with respect to their lack of affinity for the commonly used neurosecretory stains. In a study of the median neurosecretory cells of the pars intercerebralis of *Calliphora* using paraldehyde fuchsin, Thomsen (1965) referred to "other possible neurosecretory cells" and included in these a certain number of lateral (dorso-lateral) cells, optic cells, and a few cells in the suboesophageal ganglion. Almost certainly, these cells which stain very lightly with PAF in conventional techniques and are phloxinophil are included among those we describe here. They, and the other *Calliphora* PP-like immunoreactive cells are different from the neurosecretory cells of the pars intercerebralis in that they occur either singly or in small groups of 3–4. Furthermore, although immunoreactive material can frequently be seen in the axon hillocks, as well as in the perikarya, we have not been able to show that it collects in larger axonal tracts or in neurohaemal organs. On this evidence, it seems possible that the product may perhaps be released and have a function locally within the brain but we cannot rule out the possibility that it is transported to more distant sites, either within the brain or beyond to other organs. In this respect, our preliminary examinations of the gut and the corpora cardiaca/corpora allata complex have not revealed PP-like immunoreactivity either in cells or axons.

In conclusion, it is of interest to reflect on the pioneer work on neurosecretory phenomena carried out by Ernst and Berta Scharrer (cf. 1937) in which they demonstrated the universal occurrence of secretory neurones in the animal kingdom. Our findings, presented here, add to the rapidly growing body of evidence which suggests that at least some of these neurones contain peptides, the configuration of which has remained remarkably immutable during evolution. If the structure of the neuropeptides has remained stable however, it seems unlikely that this will be true of their function. In this respect, it will be of particular interest to compare the function of the PP-like material, identified here in invertebrate neurones, with that of vertebrate PP material.

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