decrease in cyclic AMP levels in late G_1 phase is required for the progression from G_1 to S phase[21]. Reduced activity of adenylate cyclase is also observed after stimulation of lymphocytes with the tumor promoter and T-cell mitogen phorbol myristate acetate[22]. These results suggest that different classes of transforming agents also require a reduction in intracellular cyclic AMP levels as an essential permissive condition in the transformation process. It is tempting to speculate that reduced ability of EBV-transformed cells to respond to hormones that stimulate cyclic AMP synthesis is one step in the progression of normal lymphocytes into immortal cells which have escaped from existing growth control mechanisms.

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Occurrence, distribution and nature of neuropeptide Y in the rat pancreas

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Summary. Significant quantities of a newly discovered peptide, neuropeptide Y, were found in the rat pancreas, where they were localized to nerves in the exocrine parenchyma and around arterial and ductal structures. Although unaffected by surgical parasympathectomy, the periarterial and periductal nerves were abolished by chemical sympathectomy, suggesting that NPY is partially costored with sympathetic transmitters in nerve fibers.
Key words. Immunocytochemistry; neuropeptide Y; radioimmunoassay; rat pancreas.

The characterization of the family of pancreatic polypeptides began in 1974 with the discovery of avian (APP) and bovine (BPP) pancreatic polypeptides[2]. Immunocytochemistry originally localized these peptides to a well-defined endocrine cell type in the islets of Langerhans[3]. However, it was later found that antisera to the PP molecules cross-reacted with central and peripheral nerves[4,5], although the neuronal immunoreactivity could not be detected radioimmunologically[6]. An explanation for this morphologically detectable PP-like substance in nerves came with the recent discovery of NPY (neuropeptide Y), which was isolated from the porcine brain and found to be a member of the PP family of peptides[7,8]. Further studies have revealed a very broad distribution of NPY, not only in the brain[9] but also in most peripheral tissue, including the gut[10-15] and pancreas[10,11,13,16]. NPY-immunoreactive material is frequently found in noradrenaline containing nerve fibers[10,17,18].
Recent studies have demonstrated the existence of a rich nervous network supplying the endocrine as well as the exocrine portion of the pancreas. In addition to the cholinergic and adrenergic nerves, peptide-containing nerves have also been described[19-22]. In view of this, we have carried out a combined (immunocytochemistry-radioimmunoassay) study, to establish the occurrence, distribution, origin and nature of the reported NPY-immunoreactivity of the rat pancreas.
Materials and methods. 50 male Wistar rats weighing 200–250 g each, were divided into five groups (A, B, C, D, E) of 10 animals each. In group A, surgical parasympathectomy of the pancreas was performed by subdiaphragmatic truncal vagotomy. Mickulitz pyloroplasty was also done in this group in order to avoid gastric distension. Group B underwent Mickulitz pyloroplasty alone. Group C had sham-operation (opening and closure of the abdomen under anesthesia). In group D, sympathectomy was performed pharmacologically, by 6-hydroxydopamine (6-OHDA) (200 mg/kg i.p.), a drug that is known to destroy sympathetic nerves[30]. Group E was used as normal controls.
All the animals were kept on a standard diet for 14 days prior to surgery. After 14 days, groups A and B underwent operation (truncal vagotomy and pyloroplasty, or pyloroplasty alone, respectively). After anesthesia (20 mg/kg phenobarbital i.p.) a midline incision was performed and the posterior and anterior branches of the vagus were recognized below the diaphragm, isolated and cut between two ligatures.

Mickulitz pyloroplasty was performed with one layer dexon stitches (5/0). On day 14 group D was injected i.p. with 200 mg/kg 6-OHDA.

All animals were killed on day 21 by ether inhalation after 24 h fasting.

Immunocytochemistry. Five animals from each group were used for immunocytochemical investigation. Benzoquinone solution (BQS)[23] was perfused (500 ml/15 min) immediately via the aorta. The pancreas was collected, washed in 15% sucrose in phosphate buffered saline (PBS pH 7.2) for 2 h and then frozen in melting Arcton 12. Cryostat sections of 7 µm were cut and mounted on poly-L-lysine precoated slides[24].

Indirect immunofluorescence was performed. Antisera to NPY were obtained from New Zealand white rabbits using natural porcine NPY conjugated to bovine serum albumin with bis-diazobenzidine. This antiserum showed insignificant cross-reactivity with either PYY or APP at immunocytochemical dilutions (1:800) (table 1). A rabbit antibody against tyrosine hydroxylase (TH) was used (dil. 1:400) in order to visualize noradrenaline immunoreactivity[25].

After thorough washing in PBS, the second layer of FITC conjugated goat antirabbit serum was applied to the section at 1:200 dilution. The sections were mounted in aqueous medium and observed with a microscope (Leitz Orthoplan) equipped with a UV source.

The controls for immunostaining included the omission of primary antisera or replacement with nonimmune sera. In addition, the specificity of the NPY antiserum was tested by preabsorption with different antigens. Total quenching of immunostaining was observed after addition of as little as 0.1 nmoles of natural porcine NPY per ml of diluted antiserum. Natural porcine peptide tyrosine tyrosine (PYY) quenched the immunostain at a concentration of 10 nmoles/ml of diluted antiserum whereas 20 nmoles of avian pancreatic polypeptide (APP) per ml only partially reduced immunostaining.

Radioimmunoassay. Five animals from each group were used for radioimmunoassay measurement. The pancreas from each animal was dissected and the NPY content extracted by boiling in 0.5 M acetic acid for 10 min.

The concentration of NPY was determined by means of a recently developed radioimmunoassay using a N terminally directed antiserum (YN7) raised in a rabbit to natural porcine NPY conjugated to bovine serum albumin by bisdiazotized benzidine[26]. The antiserum demonstrated no cross-reactivity with the related peptides porcine peptide YY, porcine PP, human PP or avian PP, up to 100 pmoles per tube.

Results. Immunocytochemistry. NPY immunoreactivity was observed in all groups and was confined to nervous structures. No alternations were observed in the occurrence and distribution of NPY in the groups of animals which underwent parasympathec-

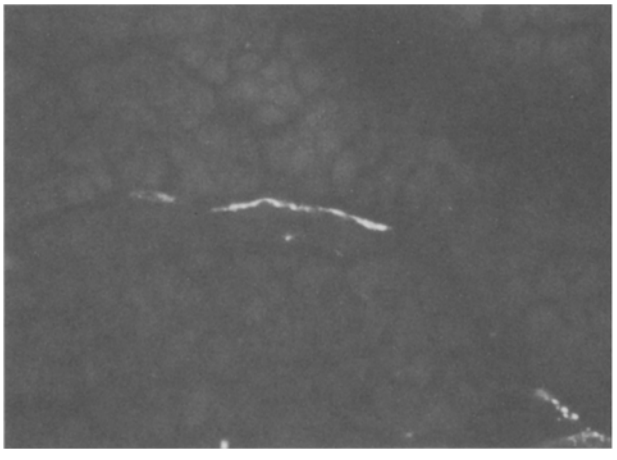

Figure 1. NPY-immunoreactive nerve fibers in the exocrine parenchyma of rat pancreas (× 188).

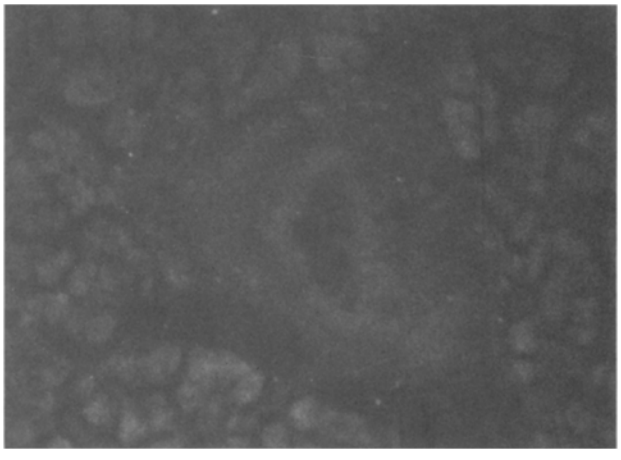

Figure 3. Pancreatic tissue from a 6-OHDA-treated rat immunostained for NPY. No immunoreactive nerves can be seen around the blood vessel (× 263).

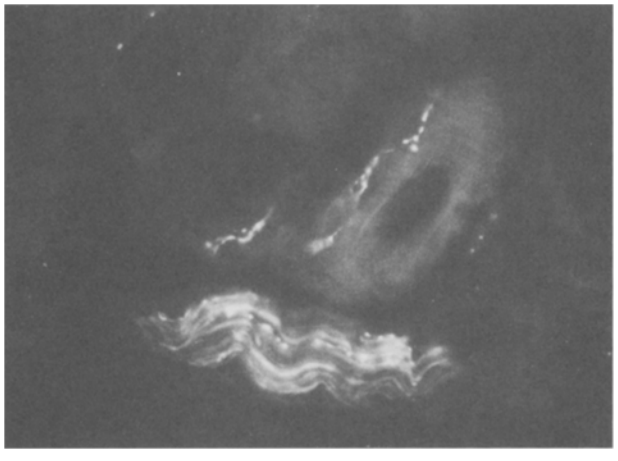

Figure 2. A blood vessel in the connective tissue of rat pancreas with closely associated NPY-immunoreactive nerves. Fibers containing NPY can also be seen in an adjacent nerve bundle (× 263).

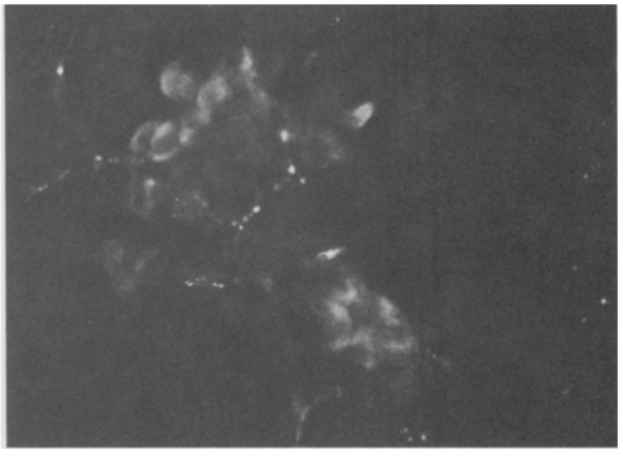

Figure 4. TH-immunoreactive nerves (arrows) in association with islet cells which are visualized by their background fluorescence (× 225).

tomy and pyloroplasty, pyloroplasty alone or sham-operation (groups A, B and C) in comparison with the controls (group E). In these groups, NPY was localized to nerves present within the exocrine parenchyma (fig. 1). These nerves were observed running through interacinar and intraacinar spaces. Occasionally, larger nerve bundles were also immunostained. Abundant NPY immunoreactivity was often found in perivascular nerve endings, particularly around arterioles and larger arterial vessels (fig. 2), while no NPY-immunoreactive fibres were detected surrounding pancreatic veins. A rich network of NPY-containing nerves was also present around pancreatic ducts of various sizes draining the exocrine portion of the gland.

Noticeably, no NPY was found in the endocrine pancreas and particularly within the islets, where PP cells were unstained. Finally, no ganglion cell bodies containing NPY immunoreactivity were observed in any group.

Partial disappearance of NPY immunoreactivity was observed in 6-OHDA-treated animals (group D). In this group, a lack of NPY-immunoreactive nerves was found in periarterial and periductal spaces (fig. 3), while NPY nerves were still detectable in the exocrine parenchyma, mostly among acinar structures.

Tyrosine hydroxylase (TH) immunoreactivity was found in four groups of animals (groups A, B, C and E) but not in any animals of group D (sympathectomized animals). TH immunoreactivity showed a similar distribution in groups A, B, C and E, indicating that vagal denervation and pyloroplasty did not affect the staining pattern and, hence, the noradrenergic innervation. TH positive nerves were observed in the exocrine parenchyma, and were detected more frequently than the NPY-immunoreactive nerves. They were localized to inter- and intraacinar spaces and were also present around arterial vessels and pancreatic ducts. TH-immunoreactive nerves were also found around pancreatic islets (fig. 4), sometimes sending extensions amongst the endocrine cells, although the endocrine cells and intrapancreatic ganglion cell bodies were not immunostained.

Radioimmunoassay. Low concentrations of NPY-immunoreactivity were measured in the control rat pancreas (0.64 ± 0.06 pmoles/g, mean ± SEM, n = 10). After treatment with 6-hydroxydopamine, concentrations of NPY were significantly reduced by 55% to 0.29 ± 0.16 pmoles/g (mean ± SEM, n = 10) ($p < 0.05$). Sham operation, vagotomy and pyloroplasty and pyloroplasty alone resulted in no significant alterations in NPY concentrations of the pancreas in comparison with normal controls (group E) (table 2).

Discussion. In this study we have shown the occurrence, distribution and origin of NPY immunoreactivity in the rat pancreas. This peptide, extracted in significant amounts from four groups of animals (A, B, C and E) with values ranging from 0.64 ± 0.06 pmoles/g (mean ± SEM) (group E) to 1.04 ± 0.17 pmoles/g (mean ± SEM) (group B) and intermediate values (0.94 ± 0.21 pmoles/g in group A and 0.87 ± 0.16 pmoles/g (mean ± SEM) in group C), has been found by immunocytochemistry in nerves located in the exocrine part of the gland in close proximity to acinar structures and the arterial and ductal systems. As in previous studies[13-16], no NPY-immunoreactive endocrine cells were observed.

The anatomical distribution of NPY fibers is distinct from that of the other peptidergic nerves of the pancreas[20,21]. Most of the other peptidergic nerves have been observed in close juxtaposition with the islets in the so-called 'neuroinsular complexes'[19]. In this study no NPY immunoreactivity was found within or around the islets, while NPY nerves were predominantly detected around ductal or arterial structures.

As yet, no studies have demonstrated that NPY can affect the physiological function (exocrine and endocrine) of the pancreas, although NPY has shown other potent biological effects[27]. On the basis of its anatomical distribution, we can hypothesize that NPY may play a role in the regulation of the blood supply to the pancreas and/or in the drainage of pancreatic juice through the ductal system. TH nerves, although present in similar anatomical areas of the pancreas, have also been demonstrated in close anatomical relationship with pancreatic islets, giving morphological evidence for the important role that the sympathetic nervous system plays in the regulation of endocrine and exocrine activities of the pancreas[28,29].

Catecholaminegic nerves frequently contain NPY-immunoreactivity[10,17,18] and a reduction of NPY-containing nerves has been observed, in a number of peripheral organs other than the pancreas, after pharmacological[10] and surgical[11] sympathectomy. In the present study, disappearance of TH-immunoreactive nerves was observed in all animals pharmacologically sympathectomized and this was accompanied by a loss of those NPY-immunoreactive nerves having periarterial and periductal distribution. Similarly, the extraction procedure revealed a significant ($p > 0.01$) decrease of measurable NPY immunoreactivity in this group (group A) with values of 0.29 ± 0.16 pmoles/g (mean ± SEM). Therefore, NPY immunoreactivity, although reduced, was still present in 6-OHDA-treated animals, a fact shown immunocytochemically by the persistence in these rats of NPY-immunoreactive fibers, predominantly localized to intraacinar spaces. This finding of a loss of perivascular NPY-immunoreactive nerves after sympathectomy is in agreement with observations made on nerves in the intestine[10,11]. A clear cut explanation for these results cannot be made. It can only be speculated that a) NPY is stored in two different types of fiber, only one of which contains TH; b) the adrenergic fibers are not completely destroyed by 6-OHDA administration, and partial regeneration has also been described in adult animals[30]; c) since NPY presumably has a different metabolic pathway to that of adrenergic transmitters, it could be less affected by 6-OHDA, which is a selective false mediator interfering with the intracellular synthesis of noradrenaline.

These observations, together with the finding that no ganglion cell bodies containing NPY were observed in any of the groups, support the hypothesis that NPY nerves of the pancreas have an extrinsic origin. They do not reach the pancreas by vagal fibers since vagotomy does not affect their detection. In addition, NPY may have partial colocalization with sympathetic mediators.

Table 1. Absorption controls

Antisera to NPY	Antigens NPY 0.1 nmoles	PYY 10 nmoles	aPP 20 nmoles
	–	–	±

– = negative staining; + = positive staining.

Table 2. Concentrations of NPY in rat pancreas as measured by radioimmunoassay

Group	Procedure	NPY (pmoles/g, mean ± SEM) n = 10 for each group
A	Truncal vagotomy + Mickulitz pyloroplasty	0.94 ± 0.21
B	Mikulitz pyloroplasty	1.04 ± 0.17
C	Sham-operation	0.87 ± 0.16
D	Chemical sympathectomy	0.29 ± 0.16*
E	Normal controls	0.64 ± 0.06*

* Statistically significant difference, $p < 0.05$.

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Amorphous calcium phosphate in the stylets produced by a marine worm (Nemertea)

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Summary. Electron microprobe analyses of the calcified stylets produced by the nemertean worm *Amphiporus formidabilis* reveal large calcium and phosphorus peaks. IR spectroscopy and X-ray diffraction indicate that the calcium and phosphorus in stylets constitute an amorphous calcium phosphate rather than a crystalline mineral phase.
Key words. Amorphous calcium phosphate; calcification; nemerteans; Rhynchocoela; *Amphiporus formidabilis*.

Comparative studies conducted over the past two decades have greatly expanded our knowledge of the various minerals that are produced by organisms[3-6]. Biologically derived calcium phosphates, for example, had been previously considered almost exclusive products of vertebrates, but now it is known that many marine invertebrates form structures containing calcium phosphate[3]. Although crystalline types of calcium phosphate occasionally occur in invertebrate skeletal elements, the crystallographically amorphous form of this mineral appears to be more widespread[3].

As part of an ongoing effort to document the diversity of biologically produced minerals, we have examined the calcified stylets of a marine worm belonging to the phylum Nemertea[7]. Nemertean stylets are nail-shaped stabbing devices that are used in prey capture (figs 1 and 2). Each fully formed stylet measures 50–300 µm long and consists of a relatively thin organic core that is surrounded by an inorganic cortex[8-11]. Preliminary observations reported in the form of an abstract indicate that the inorganic fraction of stylets contains large amounts of calcium and phosphorus, as well as lesser quantities of several other elements[12]. In this paper, we show by means of electron microprobe analysis, IR spectroscopy, and X-ray diffraction that amorphous calcium phosphate (ACP) constitutes the mineral phase of the stylets produced by the nemertean worm *Amphiporus formidabilis*.

Materials and methods. Adult specimens of *Amphiporus formidabilis* Griffin, 1898 (order Hoplonemertea) were collected intertidally on San Juan Island, Washington, USA. Regions of the proboscidial organs that contain stylets were removed from $MgCl_2$-relaxed specimens and subsequently digested in Clorox bleach[10]. The elemental composition of isolated stylets was analyzed with a JEOL JSM-35CF scanning electron microscope equipped with a Tracor Northern TN-2000 microprobe system[13].

About 50 Clorox-digested stylets were also ground into a fine powder with an agate mortar and pestle. The powder was subsequently homogenized in 7 mg of KBr and pressed into a 3-mm diameter pellet. IR spectra were obtained from the stylet-KBr pellet using a Nicolet MX-1 Fourier Transform Infrared spectrometer.

For X-ray diffraction, three or four stylets were glued to the end of a glass fiber and mounted in a Debye-Scherrer powder camera. The sample was continuously rotated for 20 h while an exposure was taken with nickel-filtered copper radiation. Alternatively, a few stylets were placed in a glass crucible and heated to 500 °C for 18 h before being analyzed by X-ray diffraction as described above.

Results. Whole stylets isolated from adult worms display several identifiable peaks of X-rays when examined qualitatively by electron microprobe analysis (fig. 3). Comparatively large calcium and phosphorus peaks are routinely observed along with smaller peaks of barium, strontium, and potassium. The relative heights of the calcium and phosphorus peaks vary considerably depending on the orientation of the X-ray detector to the region of the stylet under analysis. Some specimens also produce a peak corresponding in energy to the K_α X-rays of chlorine; the zinc peak shown in figure 3, however, arises from brass components of the microscope. Contrary to a previous report[12], titanium is not detected in whole stylets or in ground sections analyzed with