Enteroendocrine APUD cells in the digestive tract of larval *Barbus conchonius* (Teleostei, Cyprinidae)

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SUMMARY

The development of *Barbus conchonius* is described with special attention to the differentiation of the gut.

Amine precursor uptake and decarboxylation (APUD)* are present in enteroendocrine cells during development, whereas these processes are lacking in adult specimens. The first APUD cells originate on the fourth day of development in the anterior part of the gut and on the fifth day in the caudal areas. The APUD facility of the cells disappears within 2 days, and after the 6th day APUD cells can no longer be distinguished in the intestinal epithelium.

The first APUD cells were observed when four types of enteroendocrine cells were recognized with the electron microscope. These enteroendocrine cells contain granules of different electron densities, and microtubules and cilia can be observed. Some enteroendocrine-like cells are found below the basement membrane of the intestinal epithelium, indicating a possible extra-endodermal origin.

APUD cells, except melanoblasts, have not been found migrating from the neural crest in ventral direction. The origin of the enteroendocrine cells of *B. conchonius* is discussed.

* Abbreviations: APUD, amine precursor uptake and decarboxylation; FIF, formalin-induced fluorescence; L-DOPA, L-dihydroxyphenylalanine; 5-HTP, 5-hydroxytryptophan.

INTRODUCTION

Enteroendocrine cells and pancreatic islet cells of birds and mammals together with several other endocrine cells belong to the APUD series (reviews: Pearse, 1973; Andrew, 1976a).

APUD cells take up and decarboxylate amine precursors, and most of these cells produce polypeptide hormones. Pearse (1966, 1969) was the first to propose a neural crest origin for the APUD cells; this was based on the ability to store amines and on the regular presence of cholinesterase. These cells have been shown to have a common origin with melanocytes, adrenal medullary cells, ultimobranchial and thyroid C cells, carotid body type I and probably type II cells, and possibly with pituitary ACTH cells (reviews: Pearse, 1973; Andrew,

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Pearse & Polak (1971) described APUD-FIF cells in mouse embryos migrating from neural crest to the future stomach and duodenum and to the dorsal pancreatic bud. For this reason they considered the neural crest as a source of the APUD cells (possibly not of all cell types) in these mentioned places. The presence of polypeptides of hypothalamic origin, e.g. of substance P (Polak, Hertz & Pearse, 1976), somatostatin (Polak et al. 1975), neurotensin (Helmstaedter, Taugner, Feurle & Forssmann, 1977; Sundler et al. 1977) and vasoactive intestinal peptide (Polak, Pearse, Garaud & Bloom, 1974; Buffa et al. 1977) in some enteroendocrine cells is in support of the theory of the neurectoderm origin of these cells. The presence of a gastrin-like peptide in the vertebrate brain (Vanderhaeghen, Signeau & Gepts, 1975) may be additional evidence for this theory. However, Andrew (1963, 1974) cultured parts of blastoderm and primitive gut of chick embryos as chorio-allantoic grafts, and Le Douarin & Teillet (1973) and Fontaine & Le Douarin (1977) transplanted neural primordia or germ layers from quail onto chick embryos (structural differences in the interphase nuclei); they concluded that enteroendocrine cells of birds do not originate in the neural crest. The same was found for pancreatic APUD cells by Andrew (1976b) by applying transplantation techniques.

Enteroendocrine cells are also found in the digestive tract of fish. Recently, four types of enteroendocrine cells have been distinguished in the adult B. conchonius (Rombout, 1977). In this cyprinid, as in other teleosts and cyclostomes, serotonin (Erspamer, 1952) or uptake of L-DOPA (Östberg, Van Noorden, Pearse & Thomas, 1976) was not observed in the digestive tracts of adult specimens. However, in a larval cyclostome (Lampetra) intestinal APUD cells were reported by Van Noorden, Greenberg & Pearse (1972). In order to test the hypothesis of the neural crest origin of the enteroendocrine cells, the distribution of APUD cells has been studied during the early development of B. conchonius, a species in which spawning can easily be induced.

**MATERIALS AND METHODS**

Fertilized eggs of B. conchonius were kept in Petri dishes at 25 °C. Embryos and larvae were fixed in Bouin and Zenker at various developmental stages. After vacuum-embedding in Paraplast plus (Sherwood) the material was serially cross-sectioned at 5 μm and stained with haemalum and eosin. The larvae were fed artemia-nauplii from the 5th day.

*Amine precursor uptake and decarboxylation (APUD)*

*Formalin-induced fluorescence (FIF)*

Embryos and larvae were incubated in 25 mg/1 L-DOPA continuously or during 24 h at different developmental stages and fixed in 4% neutral formalin. Several stages were freeze-dried followed by fixation in formaldehyde vapour.
APUD cells in the larval digestive tract

However, as with respect to fluorescence no difference was found with fixation in formalin solution; the latter method was used routinely. Cross-sections and sagittal sections of 5 \( \mu \)m were examined with a Zeiss fluorescence Standard microscope with an HBO-50 superpressure mercury lamp, and selection filters BP 405:5, BP 405:14, and barrier filter 418 (epi-illumination).

Radioautography

Embryos and larvae were incubated in 50 or 100 \( \mu \)Ci/ml L-3,4-dihydroxy (ring-2,5,6-\( ^3 \)H) phenylalanine (spec. act. 30 Ci/mmol) or in 20 or 100 \( \mu \)Ci/ml DL-5-hydroxy (G-\( ^3 \)H) tryptophan (spec. act. 1·37 Ci/mmol) obtained from the Radiochemical Centre, Amersham, England. After an incubation of at least 4 h, the animals were fixed in 4% neutral formalin or freeze-dried followed by formaldehyde vapour fixation. Cross-sections and sagittal sections were prepared for radioautography with a Kodak NTB-2-emulsion. After an exposure time of 9 (DOPA) or 19 (5-HTP) weeks, the slides were developed with Kodak D-19 developer.

Electron microscopy

Specimens of 3, 4, 5 and 6 days old were fixed for 15 min at 0 °C in a mixture containing 1% OsO\(_4\) and 2% glutaraldehyde buffered with 0·1 M cacodylate, pH 7·2, and postfixed for 1 h at 0 °C in a mixture containing 1% OsO\(_4\), 2% glutaraldehyde and 1% potassium dichromate. Subsequently, the animals were dehydrated and embedded in Epon 812. Ultrathin sections were mounted on pioloform-coated copper grids and stained with saturated uranyl acetate and lead citrate. Photographs were made with a Philips EM 300 electron microscope.

RESULTS

Development

Several developmental stages are shown in Fig. 1.

First day. Within 8 h after fertilization a blastula is formed that consists of blastoderm, blastocoel and periblast. During gastrulation and at 11 h after fertilization, neurulation starts in the anterior area of the embryo. The ectoderm becomes considerably thicker along the central line and forms a wedge-shaped mass of stratified cells, the neural keel. The anterior part of the neural keel forms an enlarged solid mass, the anlage of the fore-brain with lateral enlargements, the prospective optic vesicles (Fig. 2B). The neurulation in the caudal area of the embryo is completed after 19 h. Then the neural crest cells can be recognized (Fig. 2C). At the end of neurulation a cavity is formed in the rostral part of the neural cord (Fig. 2C). During the second day this cavity extends in the caudal direction. During neurulation the notochord, the mesoderm and a thin layer of endoderm become separated from an undifferentiated mass of endochordame-
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soderm, situated above the periblast and yolk. At the end of the first day the rostral part of the notochord becomes vacuolated. At this stage (24 h) the optic cups, lenses, otic vesicles, primary nephric ducts, hearts, blood vessels and all the myotomes are present, and the tail has become separated from the yolk sac (Fig. 1A).

Second day. In the first hours of the second day the heart starts to beat and a few hours later colourless blood begins to circulate. Between 27 and 32 h nearly all larvae have hatched and remain inactive at the bottom of the Petri dishes. Around the time of hatching, pigmentation of the eyes begins, and at the end of the second day the first melanocytes become visible. The anterior endoderm becomes thicker and is elevated at its lateral sides, thus forming the pharyngeal anlage. At the end of the second day the first part of the gut, presumably the bulbus, becomes thicker and a liver diverticulum can be noticed on its lateral side.

The paired primary nephric ducts originate from a glomerular structure immediately behind the heart, between endoderm and myotomes, and join at the caudal end near the presumed anus.

Third day. During the third day a lumen is formed in the digestive tract except in the presumed oesophagus and hindgut. A prospective airbladder originates on the dorsal side of the oesophagus and a pancreas diverticulum is separated from the lateral side of the bulbus immediately caudal to the liver. At the end of the third day a gall-bladder is formed and the first islet of Langerhans can be observed. The head is lifted from the yolk sac and the first extremities become visible.

Fourth day. Gills and pharyngeal teeth are formed in the pharynx and irregular respiration sets in. The whole digestive tract becomes open from mouth to anus. The bulbus becomes very wide while the liver and pancreas proliferate strongly. The first islet of Langerhans is always located near the gall-bladder and close to the place where bile duct and pancreatic duct join and

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**Figures 1 and 2**

Fig. 1. Larvae of *B. conchonius*. (A) 24 h (diameter, 1 mm); (B) 30 h, just hatched (length, 2-5 mm); (C) 2 days (3 mm); (D) 3 days (4 mm; note the decreasing yolk content); (E) 4 days (4 mm; airbladder is filled with air); (F) 5 days (5 mm; after first feeding).

Fig. 2. Cross-sections of larvae of *B. conchonius* as indicated in (A); (B) 13 h, cranial part of the head. Note the neurulation and the prospective optic vesicles; (C) 19 h, same region after neurulation. Neural crest, optic cup, lenses and cavity in the brain are formed; (D) 88 h, the oesophagus is shown near the transition to the bulbus. Note the liver, the pancreas with the principal islet, and the pneumatic duct; (E) 4 days; note the position of the islet of Langerhans (principal islet); (F) 5 days; note the strong proliferation of liver and pancreas, the enlarged airbladder, the position of the spleen, and the pancreatic duct; bar 50 µm. *ab*, airbladder; *b*, bulbus; *bd*, bile duct; *fb*, forebrain; *gb*, gall-bladder; *l*, liver; *le*, lens; *nc*, neural crest; *oc*, optic cup; *oe*, oesophagus; *ov*, optic vesicle; *p*, pancreas; *pd*, pancreatic duct; *pnd*, pneumatic duct; *pi*, principal islet; *s*, spleen; *y*, yolk.
open into the bulbus (Fig. 2E). At the end of the fourth day a spleen is present and contains mainly erythrocytes. The primary nephric ducts join with the narrow hindgut near the anus. The airbladder enlarges and fills with air (Figs. 1E, 2F).

**Fifth day.** At the end of the fifth day, food uptake starts (Fig. 1F), the gut becomes functionally active, and the yolk has almost disappeared. Three zones can now be distinguished in the digestive tract: a fat absorptive zone (75%) is followed by a protein absorptive zone (20%) and a third zone, possibly a water and ion absorptive zone (5%).

At the end of the fifth day the intestinal epithelial cells vary in height, but intestinal folds cannot be observed until the eighth day.

*Amine precursor uptake and decarboxylation*

**Incubation with \(^{3}\text{H}\)l-DOPA**

Around the time of hatching ventrally migrating melanoblasts can be recognized as APUD cells with radioautography; during migration they start to form pigment. Formalin-induced fluorescence, also observed at this developmental stage, must be attributed to the autofluorescence of the blood plasma. With these techniques it was not possible to recognize APUD cells migrating from neural crest to digestive tract. As shown in Table 1, weak greenish-yellow fluorescent or \(^{3}\text{H}\)-labelled APUD cells appear in the bulbus after 3 days. The amine content of these cells increases on the fourth day and again increases after the administration of L-DOPA. After 4 days, APUD cells are present in the first 75% of the gut, the prospective fat absorptive zone (Fig. 3, 4). On the fifth day, APUD cells originate also in the caudal part of the gut, while the APUD facility of these cells disappears in the rostral parts of the gut (Fig. 5). Around the sixth day, APUD cells can be observed only near the anus. As from the seventh day, cells with APUD characteristics are not present anymore. Application of FIF technique and radioautography together to the same sections showed that nearly all the \(^{3}\text{H}\)-labelled cells correspond to fluorescent cells. The weakly fluorescent cells cannot be detected with radioautography and this may be attributed to insufficient exposure time. Radioautographic examination becomes difficult when feeding starts (after 5 days), probably because \(^{3}\text{H}\)l-DOPA is absorbed by the epithelial cells. The APUD cells are distributed between the epithelial cells of the intestine, but most of them are found in the first part of the gut, where the APUD cells are generally of the 'open type' (extending from basement membrane to the lumen), whereas the last part of the gut contains also cells of the 'closed type' (not extending to the lumen; Figs. 3, 4).

Most of the cells of the 'open type' have a flask-like shape (Figs. 3B, 6, 7). After fixation with formalin, many epithelial cells lose the contact with the basement membrane, while APUD cells regularly remain attached to this membrane (Fig. 3B), to which they appear to have a greater affinity. Radio-
Table 1. Amine precursor uptake of enteroendocrine cells in larvae of B. conchonius at different developmental stages

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Location*</th>
<th>FIF (5)</th>
<th>FIF after incubation in L-DOPA (10–20)</th>
<th>Labelling with [3H]L-DOPA (6)</th>
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<tr>
<td>2 days</td>
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<td></td>
<td>II</td>
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<td>3 days</td>
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<td>5 days</td>
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<td>II</td>
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<td>6 days</td>
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<td></td>
<td>II</td>
<td>–/±</td>
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<td>–/+</td>
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<td>7 days</td>
<td>I</td>
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<td></td>
<td>II</td>
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<tr>
<td>8–16 days</td>
<td>I</td>
<td>–</td>
<td>–</td>
<td>–†</td>
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<tr>
<td></td>
<td>II</td>
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</table>

The number of larvae is given in parentheses. –, negative; ±, dubious; +, weak positive; ++, positive; ++++, strong positive.
* I, first 75% of the intestine (fat absorptive zone). II, last 25% of the intestine (protein + water and ion absorptive zone).
† High background, due to the absorption of [3H]L-DOPA.

Autographically several APUD cells can be observed in the submucosa below the basement membrane (Fig. 7).

With the FIF technique these extra-epithelial APUD cells are difficult to observe, because of the autofluorescence of blood plasma (Figs. 3A, 6).

APUD cells cannot be detected in the islets of Langerhans.

Incubation with [3H]5-HTP

No APUD cells have been found by this method in the digestive tract of larval B. conchonius.

Electron microscopy of the intestine

3-day-old larvae. In the bulbus, which has just acquired a lumen, enteroendocrine cells cannot be observed. The epithelial cells (height about 7 μm) contain a basally located nucleus, a small Golgi apparatus, a poorly developed smooth endoplasmatic reticulum, some large mitochondria, many ribosomes and polysomes, and apically some very short microvilli (up to 0.3 μm).

4-day-old larvae. The epithelial cells of the first part of the gut (height about 15 μm) contain microvilli up to 0.7 μm. These cells contain a distinct Golgi apparatus, a swollen smooth endoplasmatic reticulum and a clear terminal web. At this stage goblet cells and enteroendocrine cells of the four types (Rombout,
APUD cells in the larval digestive tract

1977) are present in the rostral part of the gut. Most of the enteroendocrine cells are of type I and II whereas type III and IV are scarce at this stage. The basal part of these cells contain a few too many granules of different electron densities (Fig. 9). The observed narrow apical parts contain microtubules, some cilia, some small pinocytotic (?) vesicles and a few granules (Figs. 10, 12). Cilia are present on the apical side of these cells between the microvilli (Fig. 12) or extending in the intercellular space beneath the junctional complex (Fig. 11).

5- and 6-day-old larvae. The intestinal epithelium seems to be completely differentiated in 5-day larvae, when the absorptive cells have almost reached their maximal size (length about 23 µm; microvilli 1-5 µm).

Enteroendocrine cells are more numerous and contain more electron-dense granules. In the second and third zone nearly all the endocrine cells are of type I. At this stage the food uptake starts and the absorptive cells become functionally active. The absorptive cells of the first zone contain several large lipid vacuoles and many chylomicrons. The absorptive cells of the second zone contain several 'supranuclear bodies' and many pinocytotic vesicles. From the fourth day, cells with granules with an average diameter of 170 nm are found in the submucosa below the basement membrane of the intestinal epithelium (Fig. 8). Possibly these cells represent the enteroendocrine type II cell (Rombout, 1977), as no other cells with granules of this size are known in the digestive tract of B. conchonius.

DISCUSSION

The early development of B. conchonius is very similar to the development of other cyprinids: Cyprinus carpio (Verma, 1971), Labeo gonius (Khan, 1925) and Carassius auratus (Khan, 1929). Differences are only found in the time schedule and the sequence of developmental processes.

During development, APUD cells migrating from neural crest to prospective gut were not observed, contrary to observations in mouse embryos (Pearse & Polak, 1971). Only after administration of [3H]L-DOPA were ventrally migrating ³H-labelled melanoblasts noticed around hatching time.

Figures 3 and 4

Fig. 3. Sagittal section of a 4-day-old larva after L-DOPA administration. (A) The gut is shown from airbladder up to anus. Many APUD cells are seen in the prospective fat absorptive zone, and only a few in the caudal 25% of the gut. The blood vessels dorsal to the gut are also strongly fluorescent. (B) Detail of the rostral part of the gut; bar, 100 µm. a, anus; ab, airbladder; bv, blood vessel; y, yolk.

Fig. 4. Radioautograph of a sagittal section of a 4½-day-old larva after [3H]L-DOPA administration. (A) About 70% of the whole gut is shown. Note the black melanocytes and blood vessels dorsal to the gut. (B) Detail of the rostral part with some clear APUD cells. (C) Detail of the caudal part with some APUD cells of the 'closed type'; bar 100 µm. a, anus; bv, blood vessel; m, musculature; mc, melanocyte; y, yolk.
These melanoblasts can also be recognized by their pigment formation during migration, previously reported by Orton (1953) for fishes. The first APUD cells appear in the intestinal epithelium during the fourth day, when development is rather far advanced.

In contrast to the APUD cells of mammals, the cells of this species lose their characteristics after a few days and these characteristics do not return in later stages. This might be attributed to an increasing monoamine-oxidase activity causing a breakdown of the fluorescent amine. Inhibition of L-DOPA-decarboxylase, preventing the formation of biogenic amines, might be another cause.

Andrew (1975) found APUD cells in the intestinal groove of the chick, concentrated at the site of evagination of the pancreatic bud, at the end of the second day (16–18 somites), disappearing the third day and reappearing in the gut from 12 days onwards. Although she considers the possibility of a temporary loss of biogenic amine-synthesizing ability, she supposes that these early arising APUD cells are the precursors of one or more islet cell types, because APUD cells are found in the pancreas from its formation (27 somites) onwards. In the present study APUD cells have not been observed in the islet of Langerhans (principal islet) of B. conchonius.

The greenish-yellow fluorescence of the intestinal APUD cells of B. conchonius indicates the presence of a catecholamine, possibly of dopamine. This colour, however, does not mean that other catecholamines are absent. The presence of serotonin (yellow fluorescence) in the APUD cells can be excluded, as no uptake of [3H]5-HTP was found. It is very possible that the APUD cells represent the enteroendocrine cells, as their amine storage mechanism is noticeable when four types of enteroendocrine cells can be observed with the electron microscope; moreover, their flask-like shape ('open type') and their occurrence as single cells throughout the intestinal epithelium are normal features of enteroendocrine cells. The amine storage mechanism might be required for the differentiation of the enteroendocrine cells. However, entero-
endocrine cells must also differentiate at later stages and in the gut of adult fishes where amines have not been found in appreciable amounts (Rombout, 1977).

More likely, the amine storage mechanism in the enteroendocrine cells of B. conchonius may be a remnant of their neurulocutaneous origin, in accordance with the hypothesis of Pearse (1966, 1969). The extra-endodermal origin of the enteroendocrine cells is also supported by the presence of APUD cells and enteroendocrine-like cells in the submucosa below the basement membrane. Enterochromaffin cells were also found outside the intestinal epithelium by Kull (1925) and Dias-Amado (1925), who considered them as of mesodermal origin, and by Simard & Van Campenhout (1938) and Monesi (1960), who suggested a migration of these cells from epithelium to connective tissue (endodermal origin).

Contrary to the indication of the neural crest origin of enteroendocrine cells and pancreatic islet cells in mice by Pearse & Polak (1971) are the negative results with the chick obtained by Andrew (1963, 1974, 1976b) and Le Douarin & Teillet (1973). Fontaine & Le Douarin (1977), who combined at an early stage lower and upper germ layers from chick and quail embryos (chimaeras), even excluded the neurulocutaneous as possible origin for enteroendocrine cells and pancreatic islet cells. Consequently, in chick and quail it seems hardly possible that the neural crest or even the neurulocutaneous is the source of these cells.

Andrew (1976a) suggested that 'the common factor sought in the genesis of the APUD cells may be the biochemical rather than the embryological relationship of the progenitor cell types'. Our results show, however, that the amine storage mechanism in fish is an embryological feature that is lacking in adult specimens. This was also found by Fontaine (1974) for the metanephric FIF cells of the chick, which originated in the neural crest. A disappearance of the APUD facility was also suggested by Pearse & Takor Takor (1976) for endo-

**Figures 9-12**

Fig. 9. Electron micrograph of an enteroendocrine type II cell of larval B. conchonius containing granules of about 170 nm; bar 1 \( \mu m \).

Fig. 10. Electron micrograph of the apical side of an enteroendocrine type III cell of larval B. conchonius, containing granules of about 230 nm. Note the presence of pinocytotic vesicles and microtubules; bar 1 \( \mu m \).

Fig. 11. Electron micrograph of the apical side of an enteroendocrine type II cell of an adult B. conchonius. Note the presence of a short cilium, extending in the intercellular space below the junctional complex (arrow), pinocytotic vesicles and microtubules. This type of cilium is also present in the type II cell of fig. 9 (not shown in figure); bar 1 \( \mu m \).

Fig. 12. Electron micrograph of the apical side of an enteroendocrine cell of larval B. conchonius. Note the cilium between the microvilli and the presence of pinocytotic vesicles and microtubules; bar 1 \( \mu m \). bm, basement membrane; bb, basal body; c, cilium; ce, centriole; g, Golgi apparatus; mt, microtubules; mv, microvilli; n, nucleus; pv, pinocytotic vesicle.
crine cells outside the APUD series, e.g. gonadotrophs, thyrotrophs and parathyroid chief cells, which possibly also originate in the neurectoderm or placodal ectoderm. Our results fit the hypothesis of these authors who consider the polypeptide hormone-producing cells as ‘nerve cells whose essential function (neurotransmission) has been transposed into an endocrine one’, with or without loss of the APUD facility. In summary, it may be possible that enterocrine cells of *B. conchonius* have a neurectoderm or placodal ectoderm origin.

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