Tissue effects on the expression of serotonin, tyrosine hydroxylase and GABA in cultures of neurogenic cells from the neuraxis and branchial arches

HARRIS M. MACKEY, ROBERT F. PAYETTE and MICHAEL D. GERSHON

Department of Anatomy and Cell Biology, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, NY 10032, USA

Summary

The phenotypically diverse neurones of the enteric nervous system are developmentally derived from precursors that migrate to the bowel from the vagal and sacral regions of the neuraxis. In order to gain insight into the generation of enteric neuronal diversity, we examined the expression of serotonin (5-HT), tyrosine hydroxylase and GABA in vitro. In the mature avian intestine, intrinsic neurones contain 5-HT or GABA but not tyrosine hydroxylase. These markers were demonstrated immunocytochemically, singly or simultaneously. All three phenotypic markers developed in cultures of cranial, vagal or truncal neural crest when the cultures were grown in enriched medium, containing horse serum and chick embryo extract; however, 5-HT and GABA, but not tyrosine hydroxylase-immunoreactive cells, also developed in cultures that were grown in partially defined medium. Tyrosine hydroxylase immunoreactivity was seen when partially defined medium was supplemented with nerve growth factor (NGF). Cultures of branchial arches (III and IV) contained cells that displayed tyrosine hydroxylase immunoreactivity, but not that of 5-HT- or GABA-; however, 5-HT immunoreactivity was seen when branchial arches were cocultured with aneuronal hindgut (from 4-day chick embryos). Cultures of cells from chick gut dissociated at 7 days contained tyrosine hydroxylase as well as 5-HT and GABA immunoreactivities; however, no cultures of bowel dissociated at 8 days or later expressed tyrosine hydroxylase immunoreactivity. When neuraxial cells were cocultured with branchial arches or heart instead of gut, no 5-HT-immunoreactive cells were seen; nevertheless, the further addition of explants of gut to the heart/crest cocultures did permit the expression of 5-HT immunoreactivity. These results are consistent with the hypotheses that precursors with the potential to give rise to cells that express 5-HT, GABA and tyrosine hydroxylase are found at several levels of the neuraxis; however, the ability to express these phenotypes may be suppressed either while the crest cells are migrating (for example, 5-HT and GABA expression by crest cells passing through the branchial arches) or in their final destination (for example, tyrosine hydroxylase in the gut). This suppression may be transient and reversed by the microenvironment of the target organs.

Key words: serotonin, tyrosine hydroxylase, GABA, neural crest, development, enteric nervous system, catecholamine, branchial arch.

Introduction

The enteric nervous system (ENS) is generally considered to be formed by precursor cells that migrate to the gut from the neural crest (Yntema & Hammond, 1954; Le Douarin, 1982). Recently, however, the possibility has been raised that some enteric neurones may also be derived from the ventral neural tube (Loring et al. 1988). Specifically, experiments with quail–chick interspecies chimeras have revealed that the avian bowel is colonized by precursors that leave the neuraxis from its vagal (corresponding to the axial level of somites 1–7) and sacral (caudal to somite 28) regions (Le Douarin & Teillet, 1973, 1974; Allan & Newgreen, 1980; Newgreen et al. 1980). The migratory pathway followed by neuraxial cells en route to the gut from the vagal level passes through the caudal branchial arches (Payette et al. 1984;
There is evidence that the vagal precursors of enteric neurones may already be committed to the neuronal phenotype before they reach the bowel. For example, neurofilament expression has been detected in cells of the premigratory vagal crest and, later, together with a neurofilament-associated protein in a neurogenic cell population that appears to be migrating through the caudal branchial arches (Ciment & Weston, 1982, 1983; Payette et al. 1984; Ciment et al. 1986). Nevertheless, it is clear that the enteric microenvironment also plays a critical role in enteric neuronal phenotypic expression. Thus, populations of cells derived from levels of the neuraxis that do not normally colonize the gut will give rise to neurones appropriate for the ENS if these cells are experimentally induced to colonize the bowel (Le Douarin, 1982; Rothman et al. 1986b). Consequently, the enteric microenvironment, by instruction or selection, determines which neuronal phenotypes are expressed in the gut by cells of neuraxial origin.

The current study was undertaken in order to investigate interactions between neuraxial and surrounding tissues that help to determine the phenotype of those neuraxial cells that colonize the avian gut. Phenotypic expression was examined in vitro. Neuraxial cells for these experiments were obtained from different regions of the premigratory neural tube and crest, from within the pathway that leads to the gut from the vagal level of the neuraxis (the caudal branchial arches on day 3 of incubation) and, at different stages, from within the bowel itself. In addition, experiments were done in which enteric or cardiac cells were cocultured with neuraxial cells in order to determine the effects of non-neuraxial components of gut or heart on the phenotypic expression of neuraxially-derived precursor cells. Serotonin (5-HT) or $\gamma$-aminobutyric acid (GABA) immunoreactivity served as markers of a neural phenotype appropriate for the bowel, while the immunocytochemical detection of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis (Nagatsu et al. 1964), was used to identify cells displaying a phenotype inappropriate for the ENS. 5-HT is an enteric neurotransmitter (Gershon, 1982a,b) and is present in adult and developing enteric neurones and processes (Costa et al. 1982; Furness & Costa, 1982; Rothman & Gershon, 1982; Rothman et al. 1986b). GABA also has been found in adult and developing enteric neurones (Baetge & Gershon, 1986; Jessen et al. 1986). Tyrosine hydroxylase is not found in intrinsic enteric neurones of the small intestine, where the enzyme is present only in the terminals of sympathetic axons (Furness & Costa, 1979), or in the adult stomach (Costa et al. 1987). Catecholaminergic neurones have been reported in some enteric regions (Furness & Costa, 1971; Bennett et al. 1973), although tyrosine hydroxylase has only been found in the adult mammalian colon (Costa et al. 1987). Tyrosine hydroxylase also appears transiently in some cells in the enteric mesenchyme during development of the mammalian, but not the avian gut (Cochard et al. 1978; Teitelman et al. 1984; Gershon et al. 1984). In several experiments the monoclonal antibody, NC-1 (Vincent et al. 1983; Vincent & Thiery, 1984; Tucker et al. 1986, 1988), was used as a marker for crest cells differentiating along neural or glial lineages. NC-1 immunoreactivity was demonstrated simultaneously with that of the other markers. A preliminary report of some of these observations has been presented (Mackey et al. 1986).

Materials and methods

Animals

Fertile eggs from White Leghorn chickens (Gallus gallus) or Japanese quail (Coturnix coturnix japonica, T.S.) were obtained from Truslow Farms, Chestertown, MD, and incubated at 37-5°C in a humidified, forced-draft incubator. Chick embryos were staged according to the criteria of Hamburger & Hamilton (1951; H-H), and quail embryos according to the criteria of Zacchei (1961; Z).

Source of tissue for explants

Neural tubes from stage 10 (Z) quail embryos were used as a source of neuraxial cells (Cohen & Konigsberg, 1975). They were enzymically freed of adhering somites, notochord and ectoderm by incubation with Dispase™ (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 20 min at room temperature. Branchial arches were obtained from stage-22 (H-H) chick embryos. Only the ventral portions of the caudal branchial arches were used to avoid the inclusion of cranial ganglia or placodes in the explants. Hearts and foreguts were obtained from stage-34 (H-H) chick embryos, and aneural hindguts from stage-23 (H-H) chick embryos.

Source of cells for cultures of dispersed cells

Bowel or heart were dissected from stage-34 (H-H) chick embryos and minced into small pieces. They were dissociated into single cell suspensions by incubation in a solution of 0-25 % trypsin (GIBCO, Grand Island, NY) in Ca$^{2+}$,-Mg$^{2+}$-free saline (HBSS, GIBCO) for 20–30 min at 37°C. The dispersed cells (2 x 10$^6$) were then seeded onto collagen-coated glass coverslips in 16 mm NUNC tissue culture dishes (Thomas Scientific, Swedesboro, NJ) and allowed to grow for 2–4 days until a confluent monolayer was formed.

Tissue culture media

Cultures were grown either in "enriched" medium containing 20 % horse serum (GIBCO), or in a "partially defined" medium containing 10 % Nu-Serum (Collaborative Research, Bedford, MA) (equivalent to 2-5 % horse...
buffer (pH 7-4). The cells were permeabilized with 80% embryo extract (prepared from 10-day chick embryos) and primary antisera overnight: a rat monoclonal antibody to 5-HT (prepared from paraformaldehyde) in a buffer composed of equal volumes of Ham's F-12 (GIBCO) and alpha-MEM. Nerve growth factor (7 S; NGF; Collaborative Research; 50 ng ml^{-1}) was added to some cultures grown in the partially defined medium.

**Immunocytochemistry**

For demonstration of 5-HT, TH or NC-1 immunoreactivities cultures were fixed for 2 h in 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M-phosphate buffer (pH 7.4). The cells were permeabilized with 80% ethanol (10 changes for 10 min each; Costa et al. 1982), followed by a 30 min incubation in 20% horse serum in 0.1 M-phosphate-buffered saline (PBS) to diminish background staining. The coverslips were then exposed to primary antisera overnight: a rat monoclonal antibody to 5-HT (1:200, Seralabs, Accurate Chemical and Scientific, Corp., Westbury, NY), a polyclonal rabbit antisera to TH (1:1000, Eugene Tech. International, Inc., Allendale, NJ) or monoclonal antibody NC-1 (diluted 1:10; provided by Dr Jean-Paul Thiery, Ecole Normale Supérieure, Paris, France). The antisera were diluted in 0.1 M-PBS (pH 7.4) containing 4% horse serum to block nonspecific immunoreactivity. Affinity-purified, species-specific, secondary antisera coupled to fluorophores were used to visualize sites of primary antibody binding. In order to block any possible 5-HT uptake by catecholaminergic cells, some cultures were exposed to desmethylimipramine (0.1 μM) for 30 min prior to fixation for immunocytochemistry. To demonstrate GABA immunoreactivity, cultures were fixed for 20 min at 4°C in a solution of 4% formaldehyde (freshly prepared from paraformaldehyde) and 0.5% glutaraldehyde in 0.1 M-PBS (pH 9.5). The cultures were then washed for 30 min with 0.15 M-glycine in 0.1 M-PBS to quench aldehyde groups, then permeabilized with 0.1% saponin (Sigma Chemical Co., St. Louis, MO). Following a 30 min incubation with 20% horse serum, the coverslips were exposed to the primary antibody overnight at 4°C. A monospecific rabbit polyclonal antiserum against GABA (Immunonuclear Corp., Stillwater, MN) was used at a dilution of 1:5000 in 0.1 M-PBS, containing 4% horse serum. The coverslips were inverted and mounted over glycerol (80% in 0.1 M-borate buffer, pH 8.5) and viewed under vertical fluorescence on a Leitz orthoplan microscope. Appropriate barrier filters permitted the separate visualization of fluorescein or Texas Red (or rhodamine) fluorescence on the same cultures immunostained with primary antibodies to 5-HT and tyrosine hydroxylase.

**Results**

**Primary neuraxial cultures**

Neuraxial cells were obtained from explants of neural tube and crest taken from cranial, vaginal and truncal axial levels. Cranial cells were considered to be those originating from the neural tube anterior to the first visible somite, vaginal cells those originating from the level of somites 1–7, and truncal cells those originating caudal to somite 7, but rostral to somite 28. Crest cells were allowed to migrate away from each of the explants for 48 h, after which time the neural tubes were removed from the culture dishes with a needle (Cohen & Konigsberg, 1975). Although the bulk of the neural tubes were thus removed, it is possible that some of these cultures contained cells that migrated away from the ventral as well as the dorsal surfaces of the neural tubes. Cultures were grown either in enriched or partially defined media (see Materials and methods for composition of the two types of culture medium). Cultures from all three levels of the neuraxis were found to contain many cells that had a mesenchymal morphology, some cells that had a neuronal appearance because they extended neurites and formed small aggregates that resembled ganglia, and melanocytes (Fig. 1A). Fewer cells, and far fewer melanocytes, were found in the cultures grown in partially defined than in enriched media, although cells with a neuronal appearance could be recognized in both. Cells expressing 5-HT, GABA or tyrosine hydroxylase immunoreactivity appeared in roughly equal numbers in cultures derived from the cranial, vaginal and truncal levels of the crest when these cultures were grown in enriched medium (Fig. 1B–D; Table 1). On the other hand, cultures grown in the partially defined medium (without added NGF) contained cells that expressed 5-HT or GABA immunoreactivity in proportions that were not significantly different from those observed in cultures grown in enriched medium, but none of the cultures grown in partially defined medium contained any cells that expressed tyrosine hydroxylase (Table 2). Again, however, no consistent differences were found in the cellular expression of 5-HT or GABA immunoreactivities between cultures derived from different levels of the neuraxis. Addition of 30 units ml^{-1} of 7 S NGF to the partially defined medium did not result in the expression of tyrosine hydroxylase in some of the cultures of neuraxial cells (Table 2; P < 0.01 vs no NGF); however, although the proportion of these NGF-supplemented cultures in which tyrosine hydroxylase expression was detected did not differ significantly from that of cultures grown in enriched medium, the appearance of the tyrosine hydroxylase-immunoreactive elements was very different. Tyrosine hydroxylase immunoreactive cell bodies in the NGF-supplemented partially defined medium tended to be bipolar, rather than multipolar, and cell contours were more rounded (Fig. 1E).

Since it is known that catecholaminergic neurons can take up 5-HT nonspecifically (Thoa et al. 1964), 5-HT and tyrosine hydroxylase immunoreactivities
Fig. 1. Primary neuraxial cell cultures (trunk).
(A) Phase-contrast micrograph of a culture of neuraxial cells grown for 10 days in enriched medium. Neurons (*) and black, phase-dark melanocytes can be distinguished. Background cells have a mesenchymal appearance. ×70. (B) A 5-HT-immunoreactive bipolar cell with a neuronal appearance from a culture grown for 10 days in enriched medium. The underlying cells are not immunoreactive. ×475. (C) Several multipolar GABA-immunoreactive cells with varicose processes from a culture grown for 10 days in enriched medium. The underlying cells are not immunoreactive. ×225. (D) A cluster of cells immunostained for tyrosine hydroxylase from a culture grown for 10 days in enriched medium. The neuritic processes, which may be varicose (→), are often wide and flat (A) near their junction with the perikaryon. They have a very different morphology from the serotoninergic cells also grown in enriched medium (compare with Fig. 1B). ×215. (E) A cluster of tyrosine hydroxylase-immunoreactive cells from a culture grown in partially defined (low serum) medium to which 7S NGF has been added. The cell bodies are more round with smoother contours and the processes are longer and more cable-like than those of the tyrosine hydroxylase-immunoreactive cells grown in enriched medium (compare with Fig. 1D). ×215.

were located simultaneously in order to determine whether the 5-HT immunoreactive neurones in the cultures were catecholaminergic cells that had taken up 5-HT from the ambient medium. This possibility must be considered from cells grown in the enriched medium, which contains 5-HT (Agrez et al. 1984; Saffrey et al. 1984). Since tyrosine hydroxylase is not expressed by cells in cultures grown in the partially defined medium, these cultures presumably do not contain catecholaminergic neurones; thus, nonspecific uptake of 5-HT by catecholaminergic neurones does not complicate identification of serotoninergic neurones in cultures grown in partially defined media. Although some cells grown in enriched medium showed colocalization of tyrosine hydroxylase and 5-HT (supporting the idea that nonspecific uptake of 5-HT by catecholaminergic neurones in the cultures does occur), the majority of 5-HT-immunoreactive neurones, even in the enriched medium, did not contain tyrosine hydroxylase. Moreover, the 5-HT-immunoreactive cells in which no tyrosine hydroxylase could be found differed in morphology from those cells that contained tyrosine hydroxylase immunoreactivity (compare Fig. 1B and 1D). These observations indicate that precursors of cells able to express 5-HT, GABA, and tyrosine hydroxylase are present at cranial, vagal and truncal levels of the neuraxis. At least part of the 5-HT-immunoreactive cell population is not catecholaminergic and, in contrast to catecholaminergic cells, 5-HT- and GABA-immunoreactive cells are able to differentiate in partially defined media.

Cultures of crest cells from the branchial arches

The third and fourth branchial arches, which are visible on the surface of the embryo, were dissected and grown in vitro in either enriched or partially defined media. In both cases, cells were found in the explants that expressed tyrosine hydroxylase immunoreactivity (Fig. 2A; Tables 1 and 2; the proportion of cultures containing tyrosine hydroxylase-immunoreactive cells in the two types of media was not significantly different); however, neither GABA nor 5-HT expression could be detected in cells in the branchial arch cultures. In three experiments, the branchial arches were dissociated into single cell suspensions and plated as dispersed cells in enriched medium. Again, cells manifesting tyrosine hydroxylase immunoreactivity were found, but none expressed the immunoreactivities of 5-HT or GABA. In order to determine whether cells able to express 5-HT immunoreactivity are present in the caudal branchial arches, but fail to do so under the culture conditions used, the branchial arches were cocultured with explants of aneuronal gut. For this purpose hindgut from 4-day chick embryos was used. The chick hindgut at 4 days has not yet been colonized by crest cells (Smith et al. 1977; Allan & Newgreen, 1980; Le Douarin, 1982; Payette et al. 1984) and no 5-HT, TH or GABA expression could be detected in cultures of 4-day hindgut in either enriched or partially defined media (Tables 1 and 2). 5-HT-, but not GABA-, immunoreactive neurones were found in a significant proportion of the branchial arch-aneuronal gut cocultures (Fig. 2B; Table 1). These experiments
Table 1. Phenotypic expression in cultures of neuraxial cells with explants of branchial arch or gut

<table>
<thead>
<tr>
<th>Marker</th>
<th>NCb</th>
<th>BrArch</th>
<th>NCb &amp; BrArch</th>
<th>E4 Gut</th>
<th>NC &amp; Gutd</th>
<th>E4 Gut &amp; BrArch</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>24/30</td>
<td>0/13</td>
<td>0/5e</td>
<td>0/7</td>
<td>8/10</td>
<td>3/15</td>
</tr>
<tr>
<td>TH</td>
<td>20/24</td>
<td>6/10</td>
<td>0/7</td>
<td>8/10</td>
<td>6/11</td>
<td></td>
</tr>
<tr>
<td>GABA</td>
<td>6/10</td>
<td>0/11</td>
<td>0/6</td>
<td>6/8</td>
<td>0/9</td>
<td></td>
</tr>
</tbody>
</table>

*a Number of cultures expressing marker ÷ total number of cultures examined.

b Cells migrating from neural tube.

c Neural tube explanted onto branchial arch.

d E4 hindgut explanted onto cells migrating away from neural tube (tube removed).

e Cells that express 5-HT were all within the confines of the neural tube and did not coexpress NC-1 immunoreactivity.

Table 2. Phenotypic expression in cultures of neuraxial cells and explants of branchial arch or gut

<table>
<thead>
<tr>
<th>Marker</th>
<th>NCb</th>
<th>NC (NGF)c</th>
<th>BrArch</th>
<th>E4 Gut</th>
<th>NC &amp; Gutd</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5/8</td>
<td>4/7</td>
<td>0/11</td>
<td>0/5</td>
<td>5/11</td>
</tr>
<tr>
<td>TH</td>
<td>0/10</td>
<td>3/5</td>
<td>3/8</td>
<td>0/6</td>
<td>0/11</td>
</tr>
<tr>
<td>GABA</td>
<td>4/8</td>
<td>3/5</td>
<td>0/9</td>
<td>0/4</td>
<td>2/5</td>
</tr>
</tbody>
</table>

*a Number of cultures expressing marker ÷ total number of cultures examined.

b Cells migrating from neural tube.

c 7S NGF added to culture medium.

d E4 hindgut explanted onto cells migrating away from neural tube (tube removed).

Fig. 2. (A) A cluster of tyrosine hydroxylase-immunoreactive cells from a branchial arch explant grown in vitro for 7 days. x240. (B) 5-HT-immunoreactive cells in a culture of dispersed branchial arch cells to which was added an explant of aneuronal hindgut from a 4-day chick. Similar cultures grown without added gut never contain serotoninergic cells. x215.

Indicate that the branchial arches contain cells able to express 5-HT immunoreactivity, but this capacity is not expressed in vitro unless a factor or substrate, which can be supplied by the gut, is added to these cultures. On the other hand, the branchial arches do support the development of catecholaminergic neurones even in partially defined media (Tables 1 and 2).

Cultures of dissociated gut

Single cell suspensions were prepared from the entire bowel of 5- to 10-day chick embryos in order to determine what phenotypes are expressed in vitro by crest-derived cells that have completed their migration and have colonized the bowel and/or the ganglion of Remak, which was included in these suspensions. Additional experiments were done in which only the ganglion of Remak was cultured. Cells that expressed tyrosine hydroxylase (Fig. 3A) or GABA immunoreactivity (Fig. 3B) were found in cultures of dissociated 7-day gut grown in enriched medium; however, while GABA and 5-HT could be detected in cells from dissociated 7-day gut cultured in partially defined media (Table 3) tyrosine hydroxylase could not (Table 3). No 5-HT, tyrosine hydroxylase, or GABA immunoreactivity was observed in

Fig. 3. Dispersed cells from the alimentary canals pooled from four 7-day embryos grown in enriched medium. (A) Cells show tyrosine hydroxylase immunoreactivity. The underlying enteric mesenchyme is not immunostained. x1400. (B) Two GABA-immunoreactive cells and varicose neuritic processes are illustrated. x215.
Table 3. Phenotypic expression in cultures of dissociated gut or heart

<table>
<thead>
<tr>
<th>Marker</th>
<th>E5 gut</th>
<th>E7 gut</th>
<th>E8 gut</th>
<th>E9-10 gut</th>
<th>E5-7 gut + NC</th>
<th>E7 heart + NC</th>
<th>Ganglion of Remak</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>0/9</td>
<td>2/12</td>
<td>4/13</td>
<td>8/23*</td>
<td>5/8†</td>
<td>0/11</td>
<td>0/7</td>
</tr>
<tr>
<td>TH</td>
<td>0/9</td>
<td>4/10*</td>
<td>0/5</td>
<td>0/23*</td>
<td>0/11</td>
<td>9/14*</td>
<td>0/7</td>
</tr>
<tr>
<td>GABA</td>
<td>—</td>
<td>9/14</td>
<td>2/6</td>
<td>—</td>
<td>4/7</td>
<td>—</td>
<td>0/7</td>
</tr>
</tbody>
</table>

* Numbers of cultures expressing marker / total number of cultures examined; partially defined medium except as otherwise noted.

† Cells migrating from neural tube.

* *P < 0.004 vs number of cultures of dissociated gut expressing 5-HT at E8.

† In enriched medium only (0/7 cultures expressed TH in partially defined medium).

Fig. 4. Dissociated cells from a 7-day chick bowel grown in culture for 7 days. The cultures were doubly immunostained with antibodies to 5-HT (A,C) and NC-1 (B,D). 5-HT-immunoreactive cells are illustrated. These cells occurred as groups (A,B) or as individual cells with numerous branching neurites (C,D). X215.

Cells in cultures of the ganglion of Remak, even when grown in enriched medium (Table 3). The cells that expressed tyrosine hydroxylase, 5-HT, or GABA immunoreactivity in the cultures of dissociated 7-day bowel, therefore, probably develop from cells of neuraxial origin within the gut and not from the ganglion of Remak.

Cells that expressed 5-HT or GABA immunoreactivity were present in some cultures derived from the gut dissociated at 8 days of incubation as they were present in the cultures from the dissociated bowel of 7-day embryos. Cultures of dissociated 8- or 9-day bowel continued to contain cells that expressed the immunoreactivity of 5-HT (Fig. 4; Table 3). These serotoninergic cells were found in cultures grown either in enriched or partially defined media, coexpressed NC-1 immunoreactivity (Fig. 4), and occurred either as clusters (Fig. 4A,B) or as individual highly branched individual cells (Fig. 4C,D). The coexpression of 5-HT and NC-1 immunoreactivities in the enteric cultures suggests that the 5-HT-immunoreactive cells are neurones (Vincent et al. 1983). On the other hand, no cells expressing tyrosine hydroxylase immunoreactivity were found in cultures of dissociated 9-day bowel, even when grown in enriched medium. The gut of 7- and 8- to 9-day chick embryos, therefore, is substantially different with respect to the neurones that develop in vitro from the neuraxial cells they contain. Neuraxial cells from bowel at both time points give rise to serotoninergic and GABAergic neurones, but the ability to express tyrosine hydroxylase appears to be lost between days 7 and 8 of incubation.

Cocultures of gut with primary neuraxial cells

Primary neuraxial cells, obtained by migration in vitro from explants of vagal or truncal neural tube, were allowed to interact with explants of aneuronal 4-day chick hindgut (Table 2), or with cells dissociated from 5- to 10-day chick bowel (Table 3). This was done to investigate the potential action of enteric factors on the development of crest cells. In order to minimize interference from factors that might be present in complex media, these experiments were generally done on cultures grown in partially defined media. Cells that expressed 5-HT immunoreactivity were seen in all of these cocultures (Fig. 5A,B; Tables 2 and 3). In the cocultures of crest cells with explants of bowel, most of the serotoninergic neurones appeared at the interface where the crest cells grew up to, and merged with, the outgrowth of cells coming from the intestine. Since neither explants of aneuronal chick hindgut (Tables 1 and 2), nor cultures of chick bowel dissociated prior to day 8 of development (Table 3), themselves contain 5-HT-immunoreactive cells when grown alone, the serotoninergic neurones present in these cocultures were probably derived from the...
Tissue effects on phenotypic expression of crest-derived cells

Fig. 5. Primary neuraxial cells cocultured with gut. (A) Numerous 5-HT-immunoreactive cells can be seen in the radial outgrowth of neuraxial cells cultured along with an explant of aneuronal hindgut from 4-day chick embryo. The explant of bowel (G) was not immunostained, but is visible because of the autofluorescence of the thick tissue. x140. (B) Four serotoninergic cells from a coculture of neural crest with dissociated gut from a 7-day embryo are illustrated. x300.

Fig. 6. (A & B) Primary neuraxial cells cocultured in partially defined medium with cells dissociated from 7-day chick heart. (A) Tyrosine hydroxylase-immunoreactive cells appear in these cultures, even without added NGF. x200. (B) A GABA-immunoreactive cell with a long process displays prominent varicosities (→). x400. (C & D) Primary neuraxial cells cocultured in partially defined medium with cells dissociated from 7-day chick heart to which was added an explant of 7-day foregut (esophagus and proventriculus). The culture was doubly immunostained with antibodies to 5-HT and tyrosine hydroxylase. (C) 5-HT-immunoreactive cells appear. These small bipolar cells did not contain tyrosine hydroxylase immunoreactivity. x150. (D) Tyrosine hydroxylase-immunoreactive cells appear in a different region of the same culture illustrated in (C). These cells did not contain 5-HT immunoreactivity. x210. (E & F) Primary neuraxial cells cocultured in partially defined medium with cells dissociated from 7-day chick heart. (E) Tyrosine hydroxylase immunoreactivity; (F) Simultaneous demonstration of NC-1 immunoreactivity. The tyrosine hydroxylase-immunoreactive cell shown in A coexpresses NC-1 immunoreactivity. x200.

primary neuraxial cells and not the bowel. Nevertheless, serotoninergic expression by these neuraxial cells shows that enteric cells do not prevent serotoninergic expression even though neuraxial cells derived from within the gut, up to and including 7 days of development, appear to be unable to express the serotoninergic phenotype under similar conditions in vitro.

In contrast to 5-HT, no cells in cocultures of neuraxial cells with 4-day aneuronal hindgut in partially defined media were found to express tyrosine hydroxylase immunoreactivity (Tables 2 and 3). In the absence of added NGF, catecholaminergic neurones do not develop from primary neuraxial cells in the partially defined media (Table 2); thus, explants of 4-day hindgut are not able to supply factors missing from the partially defined media that are needed for catecholaminergic neuronal development. On the other hand, tyrosine hydroxylase was expressed by cells developing in cocultures of primary neuraxial cells with enteric cells dissociated from the entire bowel after day 5 of development (Table 3); thus, enteric cells do not, in and of themselves, prevent expression of tyrosine hydroxylase.

Cocultures of heart with primary neuraxial cells

Primary neuraxial cells, again obtained by migration in vitro from explants of vagal or truncal neural tube, were allowed to interact in cocultures with cells dissociated from 7-day chick heart (Table 3). This was done to compare the effects of the gut on the development of crest cells with those of another organ at the same age. Once more, in order to minimize interference from factors present in complex media, these experiments were done on cultures grown in partially defined medium. Neuraxial cells cultured with cardiac cells were found to express tyrosine hydroxylase immunoreactivity, even though they were grown in partially defined medium without added NGF (Fig. 6A; Table 3). Similar expression of tyrosine hydroxylase immunoreactivity was found in two out of five cocultures of primary neuraxial cells with cells from dissociated 8-day liver. Some cells in the cocultures of neuraxial and cardiac cells expressed GABA immunoreactivity (Fig. 6B; Table 3); however, no cells could be found in any of the cultures with heart cells that displayed 5-HT immunoreactivity. Heart cells, therefore, differ from cells dis-
associated from gut at 7 days of development in their effect on the in vitro phenotypic expression of neuraxially derived neurones. Cardiac cells support the expression of a catecholaminergic, but not a serotonergic neural phenotype, while gut cells do the reverse and support the development of 5-HT but not tyrosine hydroxylase. In three out of five cocultures that contained all three elements, an explant of 7-day chick foregut (esophagus and proventriculus), cells from dissociated 7-day chick heart and primary neuraxial cells, both 5-HT and tyrosine hydroxylase immunoreactive neurones appeared (Fig. 6C,D). In most of these neurones, 5-HT and tyrosine hydroxylase immunoreactivities were not colocalized. Many, but not all, of the cells that expressed the immunoreactivity of tyrosine hydroxylase coexpressed that of NC-1 (Fig. 6E,F) and thus were probably developing along a neuronal lineage.

An attempt was made to determine whether the effects of heart cells on neuraxial cell development could be mimicked by heart-conditioned medium. Heart cells were dissociated and cultured as above for 4 days in the absence of neuraxial cells. The medium was then withdrawn and added to primary neuraxial cell cultures at the time the neural tube was explanted. The neuraxial cells thus were exposed to heart-conditioned medium throughout their period in culture. Cells that expressed tyrosine hydroxylase (Fig. 7) were found in three out of seven of these cultures; therefore, the factor(s) supplied by cardiac cells that permit catecholaminergic phenotypic expression to occur in partially defined media are evidently soluble and can sometimes be released into the ambient medium. On the other hand, heart-conditioned medium, unlike heart cells in cocultures, did not totally prevent expression of 5-HT immunoreactivity, which also was found in three out of seven cultures. Tyrosine hydroxylase immunoreactivity was found to be colocalized with that of 5-HT in most, but not all, of the cells in which 5-HT immunoreactivity could be found (Fig. 7A,B); therefore, nonspecific uptake of 5-HT by catecholaminergic neurones evidently can occur in cells grown in heart-conditioned medium. On the other hand, the observation that some cells exposed to heart-conditioned medium contained 5-HT, but not tyrosine hydroxylase immunoreactivity, indicates that heart-conditioned medium does not, like heart cells, fully prevent expression of a serotonergic phenotype (Fig. 7C).

Discussion

Considerable evidence has accumulated to support the idea that the specific pattern of development that cells derived from the neuraxis manifest in the bowel following their migration is not entirely due to the existence of a separate and unique cohort of enteric precursors in the premigratory vagal and sacral regions of the neuraxis. Experiments in which cells from inappropriate levels of the neuraxis have been induced to colonize the gut suggest that precursors with the capacity to express correctly enteric neural phenotypes do exist in other than vagal regions of the neuraxis (Le Douarin et al. 1975; Le Douarin, 1986; Rothman et al. 1986). The observation that cells that can give rise to an ENS are found in populations of neuraxially derived cells that normally colonize other organs raises the question of why enteric phenotypes are not normally expressed outside the bowel. One possible explanation is that the induction or selection of an enteric phenotype is a function of a critical interaction between neuraxially derived precursor cells and a microenvironment that is only found in the developing gut. On the other hand, cells that migrate away from the neural tube in primary cultures and thus have never been exposed to the bowel have nevertheless been found to express neurotransmitters characteristic of enteric neurones, such as 5-HT (Sieber-Blum et al. 1983); therefore, migration to the developing gut or exposure to an enteric microenvironment is not an absolute requirement for the expression of at least some of the phenotypes found...
in the ENS. Alternatively, extraenteric tissues may be able to prevent an enteric pattern of phenotypic expression by neuraxially derived cells from developing outside the bowel.

Conceivably, every level of the migratory neuraxis (neural crest plus cells derived from the ventral neural tube if these latter cells do indeed migrate in situ [Loring et al. 1988]) may contain a pluripotential population of cells. Such a pluripotential population could be comprised either of precursor cells that are themselves multipotential or of many different sets of unipotential progenitor cells (Le Douarin, 1986). The number of sets of unipotential progenitors would, in this case, have to be large enough to encompass all of the derivatives of the migratory neuraxis. The initial population of neuraxial cells beginning migration at any level may therefore have the capacity to express many neural phenotypes, some appropriate and some inappropriate for their ultimate destination. The final pattern of phenotypic expression in the target organ may then be governed by an interplay of positive factors promoting the expression of appropriate neural phenotypes and negative factors inhibiting the expression of those neural phenotypes that are inappropriate. In the absence of any external influence, cells may follow a default program of differentiation. With respect to the bowel, the development of inappropriate cells, such as those containing tyrosine hydroxylase, may be suppressed by the enteric microenvironment, while the expression of appropriate phenotypes, such as GABA- and 5-HT-containing cells, is fostered. In parallel, enteric phenotypes may be prevented from being expressed in neuraxial cells that migrate to extraenteric destinations. Since neuraxial cells in migrating to the bowel pass through nonenteric tissues, it is possible that these tissues have effects on the developmental potential of the neuraxial cells. The ability to express enteric phenotypes, for example, may be temporarily repressed while the neuraxially derived cells are exposed to factors or substrates that are not found in the gut. The final microenvironment of the bowel may thus be needed, not only to select, induce or inhibit patterns of phenotypic expression, but to derepress the ability to produce some types of cell as well. Such complex interactions of the microenvironments established by the migratory pathway and the target organ may thus determine the final composition of the ENS. Data derived from the present experiments support the idea that many of these types of interaction participate in enteric neuronal development.

Results obtained by culturing crest cells from different axial levels provided evidence that cells with ability to express neural phenotypes found in the gut (serotonergic and GABAergic neurones) exist at more than just the vagal level of the neuraxis. Moreover, catecholaminergic cells were seen in cultures derived from the vagal as well as the truncal level of the neuraxis (the origin of enteric neuronal and glial precursors); thus, the lack of enteric catecholaminergic neurones in the intestine cannot be attributed to an absence of cells with a catecholaminergic potential in the neuraxial population that emigrates to the bowel. The differentiative behavior in culture of cells removed from the migratory pathway (caudal branchial arches) between the vagal neuraxis and the gut was quite different from that of the starting population of neuraxial cells. Tyrosine hydroxylase immunoreactivity, but not that of GABA or 5-HT, developed in branchial arch cultures. The absence of GABA and 5-HT expression might possibly be explained by a failure of neuraxial cells programmed to express these phenotypes to enter (or survive in) the branchial arches. Such an explanation, however, would make it difficult to account for the expression of GABA and 5-HT in the gut by neuraxial cells that have travelled through the branchial arches and completed their migration to the bowel. An alternative explanation is that the ability to express a GABAergic or a serotonergic phenotype, present in the premigratory neuraxial population, is temporarily suppressed in the branchial arches. This explanation would require that the neuraxially derived cells be released from this branchial arch-related inhibition upon reaching their destination in the gut, enabling them to recover the ability to produce GABA or 5-HT. Since serotoninergic cells developed in cocultures of branchial arches with aneuronal hindgut, the idea that neuraxial cells with the capacity to express a serotoninergic phenotype do not enter the branchial arches can be excluded. The observation is also consistent with the hypothesis that serotoninergic expression is inhibited in the branchial arches and that the inhibition can be overcome by exposure to a factor or factors provided by the non-neuronal cells of the bowel. Also consistent with the notion that the branchial arches inhibit serotoninergic expression is the observation that primary neuraxial cells failed to express 5-HT immunoreactivity in cocultures when they developed in the branchial arch tissue.

The phenotypic expression of neuraxially derived cells obtained from within the bowel itself appeared to depend on the age of the gut at the time it was dissociated. When the bowel was dissociated at 7 days, GABA, 5-HT and tyrosine hydroxylase were expressed by cells in the cultures. The coexpression of 5-HT and NC-1 immunoreactivities suggests that the 5-HT-immunoreactive cells are derived from a lineage different from that of the ventral neural tube 5-HT-immunoreactive cells described by Loring et al. (1988). These serotoninergic cells of neural tube
origin do not stain with HNK-1, a monoclonal antibody that reacts with the same epitope as NC-1 (Tucker et al. 1984). The enteric 5-HT-immunoreactive cells, therefore, may be of crest origin or they may have acquired NC-1 immunoreactivity at a later stage of development. Serotoninergic phenotypic expression by chick enteric neurones cannot be detected in situ until day 9 of incubation (Epstein et al. 1980; Gershon et al. 1980) even though the gut is colonized much earlier by cells from the neuraxis; therefore, it is possible that the neuraxially derived precursors have to reside in the enteric microenvironment for a period of time before they acquire the ability to express a serotoninergic phenotype. Since cells initially migrating away from the neural tube evidently have this ability, while those in the process of migrating to the gut in the branchial arches do not, the time spent in the enteric microenvironment may be required in order for the precursors to recover from branchial-arch-related inhibition of serotoninergic phenotypic expression. The ability of enteric explants in cocultures to ‘rescue’ serotoninergic cells from branchial arches supports this idea.

The hypothesis that some tissues of an embryo are temporarily able to suppress the expression of the serotoninergic phenotype was tested by coculturing neuraxial cells with explants of branchial arches or with cardiac cells. 5-HT-containing cells appeared in cultures of neuraxial cells alone, but not in these cocultures. The effect of the cardiac cells was 5-HT-specific. Expression of tyrosine hydroxylase and GABA was not prevented by adding heart cells to neuraxial cell cultures. As was also true of crest cells grown with branchial arch tissue, the further addition of enteric cells could restore the ability to express 5-HT to neuraxial precursors grown with cells from the heart; therefore, the cardiac effect was not to eliminate a subset of precursors capable of serotoninergic expression. 5-HT is not normally expressed by neuraxially derived cells developing in the heart or the branchial arches. Thus, it is an inappropriate phenotype for these locations. The observations thus support the idea that precursors leaving the premigratory neuraxis may be subject to inhibitory influences of the tissues through which they migrate and be able to recover from this inhibition upon entering the microenvironment of their target organ.

The ability of branchial arch tissue to suppress phenotypic expression of cells from the neural crest has also been seen with respect to melanocyte development (Ciment & Weston, 1985). Crest cells derived from within the branchial arches do not give rise to melanocytes in situ or in vitro. On the other hand, exposure of crest cells within the branchial arches to phorbol esters induces melanocytic expression; therefore, cells with the ability to give rise to melanocytes, like cells with the ability to give rise to serotoninergic neurones, enter the branchial arches and can be ‘rescued’ from branchial-arch-related inhibition. Another example of inhibition of melanocytic development by an embryonic microenvironment is seen in the chick cornea (Campbell & Bard, 1985). The acellular stroma of the cornea suppresses melanogenesis by the neural crest cells that colonize it. Crest cells from the cornea, however, are released from this inhibition and develop into melanocytes in vitro if they are permitted to migrate out of corneal explants shortly after the cornea is colonized.

The regulation of expression of tyrosine hydroxylase by cells of neuraxial origin appears to be quite different from that of 5-HT or GABA. In contrast to these enteric markers, tyrosine hydroxylase is not expressed by neuraxially derived cells grown in partially defined medium. Expression of tyrosine hydroxylase did occur when these cells were cultured in enriched medium or when partially defined medium was supplemented with NGF. Chick embryo extract, which was present in the enriched media (10%), is known to be very effective in supporting the development of tyrosine-hydroxylase-containing neurones (Howard & Bronner-Fraser, 1985); such cells, for example, appear in cultures of dissociated sensory ganglia when chick embryo extract is present in the medium (Xue et al. 1985), even though tyrosine hydroxylase is not found in cells of sensory ganglia in situ. Extracts made from embryonic tissues, such as the neural tube, can substitute for chick embryo extract to permit the expression of tyrosine hydroxylase by cultured neuraxial cells (Howard & Bronner-Fraser, 1986). Similar effects on expression of tyrosine hydroxylase were found in the current experiments for cocultures of heart, heart-conditioned medium, cocultures of gut, gut-conditioned medium and cocultures of liver. These observations support the idea that diffusible substances provided by other tissues of an embryonic organ express development by crest cells of the adrenergic phenotype (Cohen, 1972; Norr, 1973). In contrast to the expression of tyrosine hydroxylase, neither NGF nor chick embryo extract appears to be needed for the expression of 5-HT or GABA by neuraxially derived cells in culture.

Although inappropriate, tyrosine-hydroxylase-immunoreactive cells did develop in cultures of bowel dissociated prior to day 8 of incubation. This observation suggests that the failure of neuraxial cells to express tyrosine hydroxylase in the gut in situ cannot be explained by a failure of neuraxial cells with the ability to express an adrenergic phenotype to colonize the bowel. Cells with this potential must have been present in the explants of gut in order for tyrosine-hydroxylase-immunoreactive cells to develop in the cultures. The presence of precursors with an adren-
ergic potential in the bowel has also been shown by experiments in which segments of foregut containing neuraxial cells were back-transplanted into truncal neural crest migration pathways of younger embryos (Rothman et al. 1986a). Neuraxially derived cells from the backgrafts recovered the ability of their progenitors to migrate; some left the grafts and travelled to the adrenal medulla of the host embryos where they gave rise to pheochromocytes, which are adrenergic. Moreover, in the current experiments tyrosine-hydroxylase-immunoreactive neurons were also found in cultures of branchial arches, which contain neuraxially derived cells migrating to the bowel. It thus seems plausible that the intact gut suppresses tyrosine hydroxylase expression by the neuraxially derived cells that colonize it, but that these cells retain the ability to synthesize tyrosine hydroxylase and do so once they are freed in dissociated cell culture from the restraining influence of the enteric microenvironment. The ability of neuraxial cells derived from within the bowel to express tyrosine hydroxylase appears to be permanently lost after day 8 of development. Since extracts prepared from the 8-day gut or dissociated enteric cells will substitute for chick embryo extract to support tyrosine hydroxylase expression by crest cells grown in partially defined medium, enteric inhibition of tyrosine hydroxylase expression cannot be explained simply as due to the absence in the gut of a promoting factor, such as NGF. Instead, the intact structure of the bowel appears to be important for the inhibition of tyrosine hydroxylase expression. The enteric microenvironment thus appears to promote the expression of 5-HT by crest cells, while inhibiting that of tyrosine hydroxylase.

This investigation was supported by NIH grants NS 15547 and HD 17736, the Council for Tobacco Research, and the Robert Wood Johnson Foundation.

References


(Accepted 14 June 1988)