Peptidergic properties expressed in vitro by embryonic neuroblasts after neural induction

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Summary

As an immediate consequence of neural induction, some neuroectodermal cells acquire the ability to develop a number of characteristic neuronal features, without requiring any subsequent embryonic cues (Duprat et al. 1987). Thus, adrenergic, cholinergic and gabaergic traits are expressed in cultures of neural fold and neural plate isolated from amphibian embryos immediately after induction and grown in a defined medium.

The aim of the present study was to determine, using the same in vitro model, their abilities to develop peptidergic phenotypes.

Using immunocytochemical techniques, we show that substance P-, enkephalin- (leu-enkephalin, met-enkephalin), and somatostatin-like immunoreactivities are expressed in subpopulations of neurones grown in vitro, whereas VIP (vasoactive intestinal polypeptide) is not detected under the same conditions. The appearance and development of the somatostatinergic phenotype has been quantified by RIA both in cell extracts and in the culture medium. Somatostatin-like immunoreactivity (SLI) undetectable at the late gastrula stage, can be measured in cells after 4 days of culture and continues to increase over the next 10 days. In culture medium, SLI is present at a constant level from day 4 up to day 14.

These data reveal that some neuronal precursor cells acquire, during neural induction, the potentiality to biosynthesize, store and release neuropeptides. Furthermore, the expression of these peptidergic phenotypes in distinct subpopulations of neurones suggests that certain neuronal precursors become committed to different metabolic pathways at the earliest steps of neurogenesis.

Key words: neurogenesis, somatostatin, enkephalins, substance P, vasoactive intestinal polypeptide, neural induction.

Introduction

The central and peripheral nervous systems are derived, respectively, from neural plate (NP) and neural fold (NF), which appear in the gastrulated embryo immediately following neural induction. As a result of this initial step of neurogenesis, precursor cells present in these two morphologically distinct areas become determined to undergo neural differentiation, although they do not immediately show any recognizable signs of such differentiation. Many previous studies have provided detailed informations on the subsequent development of derivatives of these two territories, particularly that of the neural crest, which forms from the NF (for review see Le Douarin, 1982). Neural-crest-derived cells express definitive adult phenotypes only when they have reached their peripheral destinations after a characteristic migration pattern, and it is now clear that the terminal phenotype of these cells is greatly influenced by the cellular environment they encounter during migration and by their ultimate localization (Cohen, 1972; Patterson, 1978; Le Douarin, 1982). On the other hand, little is known about the initial potentialities that these neuronal precursor cells possess just after the first inductive step of nervous system ontogenesis.

In order to evaluate the differentiation capacities of neuronal precursors, we have isolated NP and/or NF from their embryonic environment immediately after neural induction, dissociated the cells and cultured them in a defined medium. In vitro, they differentiate, among other cell types, into morphologically recognizable neurones developing specific neuronal traits including the presence of neurofilament polypeptides, tetanus-toxin-binding sites and N-CAMs (neural cell adhesion molecules) (Duprat et al. 1985a, 1986). In addition, they express 'classical' neurotransmitters such as acetylcholine, catecholamines and GABA (Duprat et
It is now well established in a number of species that other neuroactive molecules, in particular neuropeptides, are also present in adult neurones of the peripheral (PNS) and central (CNS) nervous systems, often in addition to a 'classical' neurotransmitter (Hökfelt et al. 1984). Furthermore, experiments on mammalian and avian embryos have demonstrated that some peptidergic phenotypes can be expressed very early during embryonic life, both in the CNS (Palmer et al. 1982; Pickel et al. 1982; Charnay et al. 1985; Nobou et al. 1985; Strittmatter et al. 1986) and in the PNS (New & Mudge, 1986; Fontaine-Pérus et al. 1982; Hayashi et al. 1983; Fontaine-Pérus, 1984; Garcia-Arraras et al. 1984; Fontaine-Pérus et al. 1985; Garcia-Arraras et al. 1987).

Consequently, it would be of interest to know whether neuronal precursor cells acquire, as a direct consequence of neural induction, the ability to develop peptidergic phenotypes. We have therefore used radioimmunoassay (RIA) and immunocytochemistry to investigate the expression of somatostatin-like immunoreactivity (SLI) in neuronal precursor cells isolated at the late gastrula stage from further embryonic environment cues and differentiating in vitro. We began by focusing on cocultures in which the whole neurectoderm (NP+NF) was associated with the underlying chordamesoderm. Such cocultures best reproduce the in vivo conditions obtaining at the end of neural induction. In this model system, we have characterized the neuronal subpopulation expressing SLI and have studied its appearance and development. Experiments were also performed to study the expression in these cocultures of other peptidergic phenotypes, in particular those associated with the production of enkephalins (met- and leu-enkephalins), substance P (SP) and VIP (vasoactive intestinal polypeptide). In addition, we have determined the ability of precursor cells to express SLI without the continuous influence of the chordamesoderm by culturing, separately, cells from isolated NP and NF. Finally, we have compared the in vitro expression of these molecules with the main outlines of their development in vivo.

Materials and methods

_Pleurodeles waltl_ embryos were used for experiments, staged according to Gallien & Durocher, (1957).

Cell cultures

Cell cultures were performed with embryos at the late gastrula–early neurula stage (stage 13; Fig. 1).

After removal of the jelly coat and vitelline membrane, the NP and/or the NF, excised with or without the underlying chordamesoderm, were then dissociated in Ca2+/Mg2+-free Barth’s solution (88 mM-NaCl, 1 mM-KCl, 24 mM-NaHCO3, 2 mM-Na2HPO4, 0.1 mM-KH2PO4, 0.5 mM-EDTA, pH 8.7). The isolated cells were cultured at 20 °C as previously described (Duprat et al. 1985a), in Barth’s solution (Barth & Barth, 1959) supplemented with 1 mg ml−1 bovine serum albumin (BSA; Sigma), 100 IU ml−1 penicillin and 100 µg ml−1 streptomycin, on rat-tail-collagen-coated glass coverslips or directly on 16 mm plastic dishes (Nunclon).

After attachment and spreading of the cells (2 or 3 days), the culture medium was removed and replaced by Barth’s saline solution without BSA. Cells were then maintained in vitro without further medium change for 2 or 3 weeks.

Three types of culture were performed: (1) the isolated cells from whole neurectoderm (NP and NF) were cocultured with the underlying chordamesoderm; (2) the isolated cells from NP were cultured separately; (3) the isolated cells from NF were cultured separately.

Immunoassays

Antibodies

The presence of somatostatin was visualized in the cells using two polyclonal antibodies that react with the cyclic form of the peptide (antibody 6-23-203, from Dr Dubois, (1975); antibody anti-somatostatin, from Dr Brazeau, University of Montreal, Canada). Both immune sera were used at the same dilution of 1/500. Somatostatin radioimmunoassay was performed with Dubois' antibody at a final dilution of 1/35×104.

Enkephalins were evidenced using two immune sera, respectively, directed against leu-enkephalin and met-enkephalin, (from Dr Tramu, INSERM, U 156, Lille, France; for details see Fontaine-Pérus et al. 1988) used at a dilution of 1/250. Cells containing SP were detected with rat monoclonal anti-SP purchased from Sera-Lab (France), diluted 1/100. For VIP immunoreactivity, we used the antiserum 4-151 raised in...
as described above.

sucrose solution before treatment for immunocytochemistry

4°C and then washed 24 h in Barth-Tris solution. The
Tyr-somatostatin was separated from other components by
occurred at a concentration of 0-2 pM. Radioactivity of
counter. Results are expressed as pg of SLI per explant.

Counter. Results are expressed as pg of SLI per explant.

by fixing the excess of free iodine. The radiolabelled 125 I-11-
aqueous chloramine-T solution were added in four steps at
10 s intervals followed by 120 μl of tyrosine to stop the reaction
d by fixing the excess of free iodine. The radiolabelled 125 I-11-
3-5% paraformaldehyde in Barth's solution. After washing, they were permeabilized by treating with methanol at −10°C for 6 min and then with 0.25% Triton X-100 in Barth's solution for 2 min. After three thorough washings in Barth's solution, the cultures were incubated for 30 min at room temperature with the appropriate anti-serum or monoclonal antibody, washed three times and incubated in the dark with the corresponding secondary antibody coupled with fluorescein isothiocyanate for 30 min. Cultures were then rinsed and mounted in Mowiol 4-88 for observation.

Results

Characterization of peptide-like immunoreactivities

In order to verify the specificity of the antibodies used to identify neuropeptide-producing cells in vitro, we performed a series of tests on adult amphibian.

The antisera directed against somatostatin were tested on pancreas, which is known to be an abundant source of somatostatin in various species, including amphibians (Arimura et al. 1975; Alumets et al. 1977; Falkmer et al. 1978). With both the antibodies used, a similar pattern of SLI was observed (Fig. 2A), in agreement with that previously described (Alumets et al. 1977; Forssmann et al. 1978).

The antibodies directed respectively against leu-enkephalin, met-enkephalin, SP and VIP were all controlled on frozen sections and whole-mount preparations of intestine. Enkephalin-like immunoreactivity (Enk-LI), SP-like immunoreactivity (SP-LI) and VIP-like immunoreactivity (VIP-LI) were recognizable in both systems and agreed with the observations of Furness et al. (1980a) and Furness & Costa (1980b). Thus, with the immune serum directed against met-enkephalin as well as with that directed against leu-enkephalin, an intense staining was observed in ganglia of the myenteric plexuses (Fig. 2B), in axons throughout the thickness of the circular muscle (Fig. 2C) and in endocrine cells of the mucosa. A similar, but weaker, pattern of fluorescence was obtained with the monoclonal anti-SP (Fig. 2D). Finally, with the two anti-VIP sera, a particularly strong labelling was seen in ganglia of the myenteric plexus, in axons of the circular muscle layer and in the submucosa and mucosal plexus (Fig. 2E).

In all these cases, immunoreactivity was abolished, when the primary antisera was either replaced with normal rabbit serum or left out altogether.

Whole-mount preparations

Immunohistochemistry on cell cultures

After pretreatment of the cells with colchicine (5x10⁻³ M for 18 h), the cultures were carefully washed and fixed for 30 min at room temperature using 3.5% paraformaldehyde in Barth's solution. After washing, they were permeabilized by treating with methanol at −10°C for 6 min and then with 0.25% Triton X-100 in Barth's solution for 2 min. After three thorough washings in Barth's solution, the cultures were incubated for 30 min at room temperature with the appropriate anti-serum or monoclonal antibody, washed three times and incubated in the dark with the corresponding secondary antibody coupled with fluorescein isothiocyanate for 30 min. Cultures were then rinsed and mounted in Mowiol 4-88 for observation.

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Development of SLI in differentiating neuroblasts

The development of SLI was studied by RIA in cocultures of neurectoderm associated with the under-
lying chordamesoderm. SLI was undetectable in extracts of uncultured cells at the early neurula stage (minimum concentration detectable by RIA: 0.01 pmol ml⁻¹). It was first clearly measurable in 4-day-old cocultures; thereafter the amount increased up to day 14 in culture, which was the latest time examined (Fig. 3). The amount of SLI rose from 5.51 ± 1.76 pg per explant at 4 days of coculture to 10.71 ± 1.85 pg per explant at 7 days, whereas the quantity measured at 14 days was 12.27 ± 2.36 pg per
Initial peptidergic expression

Fig. 3. Quantitative development of SLI expression in cocultures. Each result represents the mean ± SEM of 18 RIA carried out at each time point, on sister cultures. The lower limit of detection is 0.010 pmol ml⁻¹.

Explant.

In contrast, SLI in the culture medium did not increase with the age of the cocultures (Fig. 3). During the period of study, from day 4 to day 14, the level of SLI detected by RIA remained constant, at around 2.0 ± 0.055 pg per explant.

Characterization of the cells displaying SLI

To identify the cell types that contained the SLI detected by RIA, we carried out immunocytochemistry on 14-day-old cocultures in which well-differentiated neurones were present. SLI was only detected in a small proportion of neurones, approximately 10/1000. Non-neuronal cells were always negative. Immunoreactive cells were found both singly and in small clusters (Fig. 4A,B); a few positive cells were also present in large neuronal aggregates. SLI-containing cells displayed fluorescence both throughout the cell body and in neurites, with particularly intense staining on varicose structures. These cells were easier to detect after pretreatment with colchicine.

The subpopulation expressing SLI showed no special morphology and could not be distinguished from the rest of the neuronal population without the use of this immunomarker.

It must be noted that brilliant specific SLI staining was also observed on the extracellular matrix located on the surface of the dish (Fig. 4E,F). This result suggested that the immunoreactive somatostatin-like peptide released by neuronal cells was trapped on the matrix network.

Embryonic origin of the immunoreactive neurones

Subsequently, we performed immunocytochemistry on cultures of cells from separately isolated NP and NF. Our aims were to determine, first, whether the SLI-positive neurones found in the cocultures originated from NP, from NF or both and, second, whether the neuronal precursor cells were able to express SLI without further chordamesodermal influences after gastrulation.

It must be noted that neuronal cells were present in NP cultures only in the form of large aggregates. A few non-neuronal cells were also observed by phase-contrast microscopy. After 14 days in vitro, NP cell cultures contained some neuronal cells displaying SLI (about 3/1000). The latter were most often seen as single immunoreactive cells in the midst of the large neuronal aggregates (Fig. 4C).

Similarly, a small population of neurones (approximately 5–6/1000) also expressed SLI in NF cultures. Immunoreactive cells were found alone or in small groups (Fig. 4D).

In both systems, cell bodies and neurites were highly fluorescent, particularly after pretreatment with colchicine. These neurones possessed no distinguishing morphological characteristics allowing them to be identified by phase-contrast microscopy alone. All of the cells with a non-neuronal morphology were negative.

Expression of other peptides in neuroblasts differentiated in vitro

We were interested in documenting, by immunocytochemistry, the ability of neuroblasts to express other peptidergic phenotypes, particularly those associated with enkephalins, SP and VIP. Differentiated 14-day-old cocultures were used for this investigation.

Enkephalin-like immunoreactivity

After several days in vitro, cocultures possessed a small population of cells that contained Enk-LI (Fig. 5A–C). Both Enk-LI antisera gave similar patterns of immunoreactivity. Fluorescence was seen in a few isolated cells (Fig. 5A) and extended to a part of the neurite network. In the fibres, the immune reaction was more intense in varicose structures (Fig. 5C).

SP-like immunoreactivity

With a monoclonal antibody directed against SP, a faint, but clearly discernible, immunoreactivity was detected in long, neurite fibres (Fig. 5E). These weakly fluorescent processes were often very thin and could not be visualized by phase-contrast microscopy alone. No staining was observed either in grouped or in isolated nerve cell bodies.

VIP-like immunoreactivity

Experiments were performed with two different antisera raised against VIP. No staining was observed either in neurones or in non-neuronal cells.

Expression of peptidergic properties in vivo

The data obtained in vitro demonstrated that some neuronal precursors expressed SLI, Enk-LI and SP-LI after 14 days in culture. What was the point during
Fig. 5. For legend see p. 536
Fig. 4. Expression of SLI in neuroblasts differentiated in vitro. (A) 14-day-old cocultures. Immunoreactive cells in a small cluster (arrowhead). Cell bodies and neurites are positive. Note the presence of negative neurones (thin arrow). (B) Phase-contrast. ax, axons; cb, cell bodies. Bar, 20 μm. (C) 14-day-old neural plate cell cultures. In a large neuronal aggregate only one cell expresses SLI (thick arrow). Bar, 10 μm. (D) SLI visualized in 14-day-old neural fold cell cultures. A nerve cell body and its neurite are immunopositive (thin arrows) whereas the other neurones of the cluster are negative (arrowhead). Bar, 10 μm. (E) Immunostaining of the extracellular matrix. (F) Phase-contrast. n, nuclei of non-neuronal cells. Bar, 20 μm.

Fig. 5. Peptide biosynthesis visualized in 14-day-old cocultures. (A) Leu-enkephalin is expressed by an isolated neurone in culture. Both the nerve cell body (thin arrow) and the neurites (arrowhead) are fluorescent. (B) Phase-contrast. (C) Met-enkephalin is visualized on neurites; the varicosities are particularly well stained (arrowhead). (D) Phase-contrast. (E) Weak fluorescence of neurites (thin arrow) observed after treatment with the monoclonal antibody directed against SP. Note the presence of negative axons (thick arrow). Non-neuronal cells are also negative (arrowhead). (F) Phase-contrast. n, nucleus; nb, nerve cell bodies; my, myoblast; cb, non-neuronal cell bodies; y, yolk. Bar, 20 μm.

normal development of embryos? Experiments were carried out by immunohistochemistry on sagittal and transversal frozen sections of equivalent embryos (14 days after gastrulation, stage 38). At this stage of development, SLI, Enk-LI and SP-LI were visualized in some neurones both in nerve cell bodies (thin arrow) and in neuritic fibres of the CNS and PNS (Fig. 6A–C). It must be noted that some endocrine cells particularly in the intestine were also positive (data not shown). Similar studies performed on embryos at stages 22, 28 and 32 (respectively 2, 4 and 7 days after gastrulation), showed that these peptidergic phenotypes began to be detected at stage 22 (early tailbud stage) and were clearly observed at stage 28 (corresponding to 4-day-old cultures).

Discussion

Previous studies have shown that neuronal precursor cells, isolated from the embryonic environment immediately after neural induction, can develop in vitro well-defined neuronal characteristics including the presence of neurofilament polypeptides, tetanus-toxin-binding sites, N-CAMs and the expression of phenotypes related to the neurotransmitters acetylcholine, dopamine, norepinephrine and GABA (Duprat et al. 1985a; Duprat et al. 1985b, 1986, 1987). The data reported here clearly demonstrate that some of them are also able to express certain peptidergic phenotypes.

Initial expression of SLI in neuronal precursor cells differentiating in vitro

SLI was detected in cocultures, the model system which is the most representative of the situation in vivo by virtue of the fact that dissociated cells from neural primordium (NP and NF) are subjected to the influence of chordamesodermal cells throughout the period of culture.

As revealed by immunodetection in situ on 14-day-old cocultures, only a small subpopulation of neurones grown in vitro express SLI. These positive cells, with no distinguishing morphological traits, usually occur singly or are located in small ganglion-like structures, while a few positive neurones can also be detected in larger neuronal aggregates.

Therefore, at the earliest step of neurogenesis some neuroblasts have acquired the potentiality to differentiate into cells that biosynthesize and store somatostatin. Furthermore, prior treatment with colchicine increases the peptide content in the positive cells, suggesting that axonal transport is also effective.

The appearance and development of SLI, analysed by RIA both in cell extracts and in culture medium, seem to parallel the differentiation of neuronal cells. In cell extracts, SLI is undetectable at the late gastrula stage. It must be emphasized that, at this time, neuronal precursor cells are determined to follow the neural ontogenetic pathway but do not yet show any signs of phenotypic differentiation (Duprat et al. 1985b). SLI is clearly detectable at 4 days in vitro, i.e. at the same time as characteristic morphological neuronal features, including neurite outgrowth, are observed in cocultures (Duprat et al. 1985b). Subsequently, the amount of SLI increases significantly over the period of 4 to 14 days. A similar pattern of SLI development has been reported for quail neural crest cultures. There also, SLI appears soon after differentiation of neurone-like cells, increases during the following days, and subsequently reaches a constant level (Garcia-Arraras et al. 1984).

As previously seen, using the same amphibian system, neuronal precursor cells are also able to differentiate into neurones with ‘classical’ neurotransmitter-related phenotypes (Duprat et al. 1985b, 1987). It is particularly interesting to compare the time course of SLI development with that of nonpeptidergic neurotransmitters. The development of SLI follows a pattern very similar to that characterizing the development of cholinergic, adrenergic or GABAergic traits between day 4 and day 7; from then on, the quantitative rise in SLI falls off, while the quantitative expression of the other phenotypes continues to increase steeply (Duprat et al. 1985b, 1987; Boudannaoui et al. unpublished data).

This discordance between the expression of SLI and other neurotransmitters might mean that peptidergic neurones are subjected to regulatory mechanisms earlier than are neurones expressing acetylcholine, dopamine, norepinephrine or GABA. Indeed, after 7 days in vitro, neurones have acquired a high degree of differentiation, so factors including innervation pattern, electrical activity and molecules produced by non-neuronal cells might modulate somatostatin expression. Examples of phenomena of this kind have been observed for this neuropeptide and others in different model systems (Kessler et al. 1981; Mudge, 1981;
Fig. 6. Expression of peptidergic phenotypes in *Pleurodeles* embryos at stage-38 (sagittal sections). (A) Met-enkephalin expressed in a ganglion neighbouring the spinal cord. Note the intense fluorescence visualized in cytoplasm of the positive nerve cells (arrowhead), nuclei are negative (thin arrow). Some cells present in this ganglion are totally negative (thick arrow). (B) Corresponding phase-contrast. (C) Somatostatin-like immunoreactivity in neuritic fibres of the spinal cord (arrowhead). (D) Phase-contrast. *sp*, spinal cord; *gm*, grey matter; *wm*, white matter; *gg*, ganglion; *n*, nuclei; *mf*, muscle fibres. Bar, 20 μm.

We have also analysed the kinetics of appearance of SLI in the culture medium. As assessed by RIA, SLI is present at a constant level from day 4 in vitro. The equilibrium between release and degradation seems to be rapidly reached in the medium. It must be noted that the release of SLI is also confirmed by immunocytochemistry as an intensive staining of the extracellular matrix. This matrix, essentially biosynthesized by non-neuronal cells, is rich in fibronectin, laminin and various glycoconjugates (Huang S. *et al.* unpublished results). Somatostatin, released by neurones, is most likely trapped on it.

These data show that, in the presence of the chordamesoderm, some neuronal precursor cells, isolated at the earliest step of neurogenesis, can differentiate *in vitro* into neurones expressing SLI. However, it is now well established that chordamesodermal cells are capable of stimulating neurotransmitter biosynthesis, as
has previously been shown for cholinergic or catecholaminergic metabolism (Duprat et al. 1985b, 1987; Boudanaoui et al. unpublished results). Consequently, in order to analyse further the ability of neuroblasts to express a peptidergic phenotype without a continuing influence of the chordamesoderm, immunocytochemistry was carried out on cultures of cells from isolated NP and isolated NF. After 14 days in vitro, we observed subpopulations of neurones containing SLI in both systems.

It must be concluded that the positive cells found in cocultures originate both from NP and NF. Furthermore, the findings reported here show that, during neural induction, some neuronal precursor cells have already acquired the ability to express the somatostatin-related phenotype without requiring, subsequently, further cues from the chordamesoderm.

Expression of other peptidergic phenotypes

After differentiating for 14 days in coculture, some neuronal precursor cells display enkephalin- and SP-like immunoreactivities. Enkephalin-positive neurones occur either singly or in clusters, which give out a neurite network displaying particularly fluorescent varicosities. SP-LI is also detected in such cocultures as a weak fluorescence restricted to long thin fibres. However, the cells exhibiting SP-LI seem more often to occur in ganglion-like structures that can be seen by phase-contrast microscopy. It must be emphasized that enkephalins, like SP, are expressed by a minor subpopulation of neurones, suggesting that certain precursors become engaged along different metabolic pathways at the earliest step of neurogenesis. These observations are in agreement with the recent analysis by Buse (1987) of mouse neural plate ventricular cells. In vitro, the neurones deriving from early ventricular cells can be categorized into different phenotypic classes according to their morphological characteristics and immunoreactivity for neuropeptides or serotonin (Buse, 1987).

In contrast to the other neuropeptides, VIP is not detectable in 14-day-old cocultures. It has been demonstrated in quail neural crest cultures that precursor of VIP-producing cells have no specific medium requirements, unlike cells producing other peptides, SP for example (Garcia-Arraras et al. 1987). Taken together, these observations suggest that neuronal precursor cells have not acquired the potentiality to express VIP at this early stage. However, other RIA determinations on cell extracts obtained from a pool of cocultures will be necessary to ensure that the content of VIP is not below the minimum threshold detectable by immunocytochemistry in situ.

All these data indicate that some neuronal precursors acquire, during neural induction, the ability to differentiate in vitro into neurones expressing SLI, Enk-LI and SP-LI. The question now is, when do these peptides appear in vivo? Although the time course of peptidergic differentiation during embryonic life of Pleurodeles is still under investigation, some preliminary results can be considered. SLI, Enk-LI and SP-LI, slightly detected first in embryos at stage 22 (corresponding to 2-day-old cultures), increase with age to be clearly observed at stage 28 (corresponding to 4-day-old cultures) and are particularly well expressed at stages 32 and 38 (corresponding to 7- and 14-day-old cultures) both in the CNS and PNS as well as in endocrine cells. These preliminary studies on in vivo expression are now under extensive investigation.

On other models, many studies have been carried out to investigate the in vivo appearance and development of these neuroactive molecules, particularly in the mammalian central nervous system and in avian neural crest derivatives. Thus, in the developing brain of the rat, somatostatin, enkephalins and SP are found before birth, when the neurones are still immature, while VIP appears only during the first few postnatal days (Palmer et al. 1982; Pickel et al. 1982; Nobou et al. 1985; Strittmatter et al. 1986). In the peripheral nervous system of avian embryos, somatostatin and SP are detected, respectively, in sympathetic and sensory ganglia from embryonic days 4-5, soon after the end of neural crest cell migration when the ganglionic rudiments first appear (Hayashi et al. 1983; Garcia-Arraras et al. 1984; New & Mudge, 1986). Unlike somatostatin, enkephalins and SP, VIP is only detectable later in development (12 days), both by immunocytochemistry and RIA (Hayashi et al. 1983; New & Mudge, 1986). However, more recently Garcia-Arraras et al. (1987) have found VIP-LI from the 6th day of embryonic life in the quail sympathetic chain. This discrepancy is most likely due to a difference of sensitivity between methods or antibodies. It must be noted that, with our cocultures, the results were always negative, despite the fact that we used the same antibody as that used by Garcia-Arraras et al. (1987). Subsequent development of expression varies according to the neuropeptide considered (Hökfelt et al. 1982; Hayashi et al. 1983; New & Mudge, 1986; Strittmatter et al. 1986). Furthermore, for a given peptide the development can be different according to the regions of the CNS or PNS considered (Hayashi et al. 1983; Ayer-Lelièvre & Seiger, 1984; Fontaine-Pérus, 1984; Garcia-Arraras et al. 1980; Strittmatter et al. 1986).

More recent is the finding that some neurones can coexpress a neuropeptide with a 'classical' neurotransmitter very early in embryonic development. In this field, it is now well documented that catecholaminergic properties and SLI can coexist in the same cell (Maxwell et al. 1984; Garcia-Arraras et al. 1986). Likewise, VIP and cholinergic traits develop similarly in cultures of neural crest derivatives (Garcia-Arraras et al. 1987). Moreover, such coexpression can be regulated in vitro by the same factors.

This observation, together with the findings reported here, i.e. that some neuronal precursor cells have acquired the ability to express peptidergic phenotypes in addition to other neurotransmitters at the earliest step of neurogenesis, provides the basis for new investigations now in progress. For instance, it will be of great interest to determine whether a cell has already acquired, during neural induction, the aptitude to

...
coexpress a neuropeptide with a classical neurotransmitter. It will be also particularly interesting to examine the early behaviour of CNS and PNS phenotypes in cultures of isolated NP and NF in order to compare the early behaviour of CNS and PNS neuronal precursor cells.

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