In vitro differentiation of neuronal precursor cells from amphibian late gastrulae: morphological, immunocytochemical studies, biosynthesis, accumulation and uptake of neurotransmitters

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SUMMARY
Neuronal differentiation has been studied in dissociated cell cultures from early neurulae of *Pleurodeles waltl* and *Ambystoma mexicanum*. Cocultures were prepared from the neural primordium and underlying chordamesoderm. NP and NF cultures were prepared from isolated neural plate and neural folds, respectively.

Neuronal precursors in NP and NF cultures had distinctive aggregation properties already evident after 1–2 days in culture. After 10–15 days, mature neurones and synapses were observed by electron microscopy in the three culture types. The expression of neurofilament polypeptides and tetanus-toxin-binding sites was also present in these cultures. A small percentage of neurones contained cytochemically detectable catecholamine. Many neurones took up tritiated dopamine with a high affinity.

Quantitative measurement of [3H]acetylcholine synthesis and storage from [3H]choline were negative at the early neurula stage and in 5 to 15-day-old NF cultures, and remained low in 5 to 15-day-old NP cultures. Acetylcholine production in cocultures increased linearly with time and was always much higher than in NP cultures.

These results suggest that, at the early neurula stage, some neuronal precursors have acquired the capacity to express a high degree of morphological and biochemical differentiation even in the absence of further chordamesoderm influence. However, the chordamesodermal cells in the cultures increased acetylcholine synthesis.

INTRODUCTION
During ontogenesis, the embryonic environment greatly influences the pattern of differentiation of neural cells. In particular, a remarkable plasticity has been demonstrated in immature neurones from the autonomic nervous system of birds and mammals, both *in vivo* and *in vitro* (for reviews, see Patterson, 1978; Black, 1978).

Key words: Neuronal differentiation, neurotransmitters, neuronal markers, amphibian, molecular embryology.
These experiments have shown that relatively late steps in neuronal development are influenced by interactions between embryonic tissues or cells. However, little is known on the neuronal differentiation capabilities acquired by ectodermal cells during neural induction, and on the role played by the chordamesoderm in neuronal differentiation after neural induction.

Some information on this topic has been gained from the study of the developmental capacities of cells cultured from the neural crest of the avian embryo. In particular, Sieber-Blum & Cohen (1980) demonstrated that a certain degree of adrenergic differentiation could be observed among neural crest cells cultured at clonal density, but that adrenergic differentiation was stimulated by extracellular material from somitic and ectodermal cells. These experiments thus suggested that, at the level of the early neural crest, some cells had already acquired the potential for independent differentiation, but that the full expression of these capabilities required an interaction with adjacent tissues.

In fact, the avian neural crest contains neuronal precursor cells which have already withdrawn from the cell cycle and can differentiate in serum-free cultures (Ziller et al. 1983). On the other hand, immature precursor cells of neural crest origin exist in autonomic ganglia even at late embryonic stages and can express various neuronal or glial phenotypes when back-transplanted in a suitable environment (Ayer-Le-Lievre & Le Douarin, 1982). The differentiation of various neural crest derivatives thus appears to extend over a long period of embryonic development.

The amphibian embryo is a suitable model for the study of still earlier steps in neurogenesis. Neuronal differentiation has been observed in cultures of cells dissociated from the neural primordium with underlying chordamesoderm at the neurula stage (Jones & Elsdale, 1963; Duprat, Zalta & Beetschen, 1966; Messenger & Warner, 1979). When the competent presumptive ectoderm and the blastoporal lip are dissected from the gastrula of Pleurodeles waltl and associated in vitro, a 4 h contact between the two tissues is sufficient for the neural induction to occur, as revealed by the differentiation of neurones in cultures of cells dissociated from the re-isolated ectoderm (Gualandris & Duprat, 1981). Whether glial cells also differentiate under these conditions remains to be studied with appropriate cell markers.

The aim of the present study was to delineate the information acquired by ectodermal target cells during neural induction by culturing ‘induced’ neuroectodermal cells either with or without the underlying chordamesoderm. Three types of cultures were used 1) cocultures were prepared from the totality of the neural primordium plus chordamesoderm, dissociated from early neurulae. 2) NP cultures were dissociated from the isolated neural plate and 3) NF cultures were dissociated from the isolated neural folds. We have first focused on neuronal differentiation in these cultures, although presumably glial cells in addition to neurones differentiate under these conditions. By using a variety of biochemical and morphological criteria, we have demonstrated that neurones differentiate in cultures prepared from the isolated neural plate or folds, but that coculture with the chordamesoderm exerts a beneficial influence on this differentiation.
MATERIAL AND METHODS

Dissociated cell cultures

_Pleurodeles waltl_ and _Ambystoma mexicanum_ early neurulae were used in this study (stage 13 of Gallien & Durocher (1957) for _P. waltl_ and stage 15 of Harrison (1969) for _A. mexicanum_). The jelly coat and the vitelline membrane were removed with fine forceps and the neural plate and/or neural fold were excised with platinum threads mounted on stalk (Fig. 1). Optimal separation of the ectodermal and chordamesodermal sheets was obtained by bathing the embryos in 2-fold concentrated Holtfreter saline solution (120 mM-NaCl, 1-3 mM-KCl, 1-8 mM-CaCl₂). The tissues were then transferred in Ca²⁺-, Mg²⁺-free Barth solution (88 mM-NaCl, 1 mM-KCl, 2-4 mM-NaHCO₃, 2 mM-Na₂HPO₄, 0-1 mM-KH₂PO₄, 0-5 mM-EDTA) and dissociated by gentle pipetting. Cell counts were performed with a haemocytometer monitored under a phase-contrast microscope. Trypan-blue-dye exclusion was used as the criterion for viable cells. The isolated cells were cultured as described (Duprat, 1970; Duprat et al. 1966) in Barth balanced solution (1959) supplemented with 1 mg ml⁻¹ bovine serum albumin (Sigma), 100 i.u. ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin, in modified 35 mm Petri dishes (Hawrot & Patterson, 1979) on plastic or on a u.v. irradiated dried film of rat tail tendon collagen (Bornstein, 1958). After a 48 h culture in air atmosphere at 20°C, the medium was removed and replaced by Barth saline solution without BSA (Barth & Barth, 1959). The cultures were then maintained at 20°C for 2–3 weeks without medium change. Individual cultures contained 3-75 × 10⁴ cells;

Fig. 1. Experimental procedure.
384 cocultures, 369 isolated neural plate cultures and 350 isolated neural fold cultures were used in this study.

**Neurotransmitter synthesis and accumulation**

\[^{3}H\]acetylcholine synthesis and accumulation from \[^{3}H\]choline was measured by the method of Mains & Patterson (1973) adapted for amphibian cell cultures. The cultures were incubated for 6 h at 20 °C in Barth saline solution supplemented with 69 \mu m\[^{3}H\]choline (5-6-7-5 Ci/mmole) and 25 \mu g ml\(^{-1}\) ascorbate. Fresh ascorbate was added every second hour as a 100-fold concentrated solution. At the end of the incubation, the cultures were extensively washed and dissolved in 100 \mu l formate-acetate pH 2 buffer containing 1 % sodium dodecyl sulphate and carrier choline (1 mg ml\(^{-1}\)) and acetylcholine (1 mg ml\(^{-1}\)). \[^{3}H\]acetylcholine was then purified by high voltage paper electrophoresis at pH 2 (Hildebrand, Barker, Herbert & Kravitz, 1971), eluted with 0.1 M HCl and counted with 2 ml Picofluor 15 (Packard) with a 30 % counting efficiency. The significance of the data was assessed with Student's test with d.f. = 2(n – 1).

**Cytochemical staining for acetylcholinesterase activity**

Staining for acetylcholinesterase activity was performed by the method of Karnovsky & Roots (1964) in the presence of 10 \mu M-tetraisopropyl pyrophosphoramide (iso-OMP, Sigma), an inhibitor of pseudocholinesterase. The substrate used was either acetylthiocholine (1-7 mM) or butyrylthiocholine (1-9 mM). Incubation was carried out at 4 °C for 4–12 h. The staining was abolished by performing the reaction in the presence of 10 \mu M-BW 284 C 51, a specific inhibitor of acetylcholinesterase.

**Cytofluorescence of catecholamines**

Catecholamine (CA) visualization was performed by a modification of the method of Furness & Costa (1975). The cultures were washed and incubated first for 10 min at 20 °C in Barth solution supplemented with 0.1 mm-pargylin and 1.1 mm-ascorbic acid (solution A) and then for 10 min in solution A containing 10 \mu M-norepinephrine. This last step was omitted in some experiments. The cells were fixed for 5 min at 4 °C with 0.22 M-sodium glyoxylate in 50 mM-sodium phosphate buffer pH 8-4, gently dried with an air stream and incubated for 6 min at 100 °C in the dark. Observation was performed with a Leitz Dialux microscope with D filters (BP 355-425, LP 460).

\[^{3}H\]dopamine uptake and radioautography

\[2,5,6-^{3}H\]-dopamine (18 Ci/mmole, Amersham) was purified by HPLC on a Bondapak C\(_{18}\) column (Waters Associate) equilibrated in 0.25 % acetic acid, 5 % methanol, lyophilized and redissolved in H\(_{2}\)O. For uptake experiments, cultures were incubated for 4 h at 20 °C in Barth solution containing 0.1 mm-pargylin and 0.5 \mu M-purified \[^{3}H\]dopamine.

After washing with Barth saline, the cultures were fixed for 1 h at 4 °C with 3 % glutaraldehyde in 50 mm sodium phosphate buffer pH 7-4, rinsed, coated with Kodak AR\(_{10}\) emulsion and developed 7 days later using Kodak Dektol.

**Electron microscopy**

Cultures were fixed for 1 h at 4 °C with 3 % glutaraldehyde in 50 mm-sodium phosphate buffer pH 7-4, washed and postfixed for 30 min at 20 °C in 2 % OsO\(_{4}\), 50 mm-phosphate buffer. Inclusion in Epon was performed \textit{in situ} on the coverslip. Tangential or transverse sections were contrasted with 7 % uranyl acetate in methanol, lead citrate according to Venable & Coggeshall (1965) and examined with a Hitachi HS\(_{9}\) microscope.

**Immunocytochemical methods**

\textit{Neurofilament component 200 kDa and 70 kDa polypeptides:}

Cells on coverslips were fixed with 3 % formaldehyde in Barth solution for 30 min at 20 °C and then at −10 °C with methanol for 6 min followed by Triton X-100 (0.25 % in Barth solution) for
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2 min at 4 °C. After washings, anti-200 kDa or 70 kDa antibodies (a gift from Prof. D. Paulin, Inst. Pasteur Paris; see Prochiantz, Delacourte, Daguete & Paulin, 1982) were added and the cells were then incubated for 30 min at 20 °C. After washings, FITC-conjugated goat antirabbit antiserum (Nordic) was added and the cells incubated for a further 30 min. After further washings, the coverslips were mounted in Mowiol 4-88 and viewed with epifluorescent Leitz Dialux microscope with an incident source HBO 50, filters I2 (BP 450-490, LP 515). Controls with non-immune rabbit serum and FITC-GAR were negative.

Tetanus-toxin (Tt)-binding molecules

Cultures were incubated at 20 °C for 30 mn at a time, first with 10 μg ml⁻¹ Tt in Barth solution, then with rabbit anti-Tt antibodies (1/500) and then with goat anti-rabbit antiserum conjugated with FITC (Nordic). Washings before each incubation were in Barth solution. Toxin and anti-Tt antibodies were a gift from Dr. B. Bizzini; Inst. Pasteur Paris (Bizzini, Grob & Akert, 1981; Koulakoff, Bizzini & Berwald-Netter, 1983). Cultures were then extensively washed, fixed in 3 % paraformaldehyde in Barth solution at 20 °C for 20 min and mounted in Mowiol 4-88.

RESULTS

I Morphological events

1. Behaviour and growth of isolated embryonic cells

   a) Control cultures. The control cultures were cocultures including neural primordium cells (neural plate + neural fold) plus underlying chordamesodermal cells (Fig. 1A) in a serum-free, unconditioned Barth saline medium (Jones & Elsdale, 1963; Duprat et al. 1966). In pilot experiments, we first separated the two layers which were then dissociated and cocultured. The behaviour of these cultures appeared identical to those prepared by dissociating the neural primordium with attached chordamesoderm. The latter method was thus used for simplicity in all subsequent experiments.

   At the beginning of the culture, the cells were filled with yolk platelets, without any phenotypical features (Fig. 2). After 48 h at 20 °C, they had attached and spread out over the culture substratum. Clear signs of neuronal differentiation, including neurite outgrowth, were observed after 3–4 days (10–15 % of the total population). At the same time, melanoblasts (20 %), epidermal cells, chordal cells, myoblasts (20 %) were easily identified in the cultures, as well as flat cells which have not been identified but may include immature glial cells. All these cells progressively differentiated in culture over 10–12 days (Fig. 3).

   Neuroblasts presented two different behavioural patterns after only 24 h in these cocultures. Some of them soon reaggregated to form large clusters out of which grew thick fascicules of neuronal processes. The others remained either isolated or in small aggregates and formed thin neurites giving rise after 10–12 days to a fine fasciculated network.

   Preliminary experiments were performed to see if neuronal differentiation occurred in these cultures in the absence of cell division. Cytosine arabinoside (50 μM) added to the culture medium from day 0 on effectively suppressed mitotic figures among non-neuronal cells, but neurones clearly differentiated under these conditions. This preliminary experiment suggests that at least a subpopulation of
neuronal precursors in the early neurula are already postmitotic. In fact, it is known that Rohon-Beard neurones originate during the gastrula stage (Lamborghini, 1980).

b) **Isolated neural plate cells** (Fig. 1B). Only neuroblasts and a few unidentified flat cells were observed in these cultures. After 24–48 h at 20°C, neuroblasts reaggregated and formed very large clusters. The attachment to the substratum was delayed and took place only after 5–6 days. Isolated neurones were not observed (Fig. 4).

c) **Isolated neural fold cells** (Fig. 1C). After a time course for attachment and spreading identical to that observed in cocultures (48 h), different cell types differentiated: neuroblasts, pigment cells, epidermal cells as well as unidentified flat cells. Neuroblasts remained isolated with thin neurites or gathered only in small aggregates (Fig. 5).

2. **Ultrastructural observations.**

At the ultrastructural level, the differentiation in vitro of all cultured embryonic cells was quite normal (Figs 6, 7). After 10–12 days, cultured neurones contained characteristic organelles (neurotubules, neurofilaments, clear- and dense-cored vesicles, ...) and established synapses with each other and with other cell types.

II **Immunocytochemical detection of early markers of differentiation**

Neurofilament polypeptides (Hoffman & Lasek, 1975; for review see Lazarides, 1980) and tetanus-toxin-binding sites (Dimpfel, Huang & Habermann, 1977; Mirsky et al. 1978) are reliable markers for the identification of neurones in vertebrate tissues. We have used these two markers in amphibian cultures.

1. **Neuronal intermediate filaments.** Neurofilaments (NIF) are composed of three polypeptides of molecular masses 200, 160 and 70 kDa. Our data were based on the immunofluorescent detection of NIF proteins with two antisera raised against the 200 and 70 kDa components (cf. Material and Methods), either in cocultures or in NP or NF cultures.

   In the three types of cultures, neuronal cell bodies as well as neurites show intense fluorescence (Fig. 8). The non-neuronal cells did not exhibit any specific labelling with these two antineurofilament antisera.

   2. **Tetanus toxin** (Tt) binds with high affinity to GD1b and GT1b gangliosides (Van Heyningen, 1963) which are presumed to be components of the toxin receptor

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Fig. 2. Dissociated cells from the neural primordium and chordamesoderm of early neurulae are round, filled by yolk platelets without any phenotypical features. Phase contrast. Bar equals 50 μm.

Fig. 3. Differentiation of neurones (star), muscle cells (arrow), unidentified flat cells (arrowhead) in a 12-day coculture. Phase contrast. Bar equals 300 μm.

Fig. 4. 12-day culture from neural plate: neuroblasts reaggregate in large clusters. Phase contrast. Bar equals 150 μm.

Fig. 5. 8-day culture from neural fold: neuroblasts gather in small aggregates. Phase contrast. Bar equals 50 μm.
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site in the neural plasma membrane (Dimpfel et al. 1977). The use of antibodies against Tt on 10-day cultures previously incubated with Tt showed the following (Fig. 9): in the three types of cultures (Fig. 1) immunostaining of the neurones was very distinctive and bright. Intricate networks were labelled by the toxin, including many fine neurites that could not be seen by phase contrast. Both the cell bodies and the processes were stained and growth cones were also distinct. As for NIF polypeptides, no immunofluorescence could be observed in non-neuronal cultured cells.

III Synthesis and uptake of neurotransmitters

In these still preliminary experiments, we have focused on the metabolism of acetylcholine and catecholamines, although other neurotransmitters, as well as neuropeptides may also be present in these cultures (Duprat, unpublished data).

1. Visualization of catecholamines by glyoxylic acid fluorescence

The intrinsic catecholamine pools in neurones were visualized by the fluorescence induced by glyoxylic acid in cultures not preincubated with norepinephrine (Furness & Costa, 1975). In 3 to 4-day NP and NF cultures as well as in cocultures, scarce fluorescent cells, representing 1–2% of the neuronal population were observed (Fig. 10). Due to the cell aggregation occurring in these cultures, it was not possible to decide if this percentage increased with further time in culture, but it remained small. Basically identical results were obtained with cultures preincubated with norepinephrine, although small quantitative differences may have been overlooked.

2. Uptake of tritiated dopamine

We then evaluated the capacity of cultured cells to take up exogenous [3H]dopamine. The high-affinity uptake of [3H]dopamine in neurones was detected in situ by autoradiography, in NP and NF cultures as well as in cocultures (Fig. 11). In the presence of desmethylimipramine (5 × 10^{-6} M), an inhibitor of the

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Fig. 6. Electron micrograph showing neurotubules (arrow), neurofilaments (double arrow), clear- (arrowhead) and dense-cored vesicles (curved arrow), synapsis (star) in neuronal processes. (OsO4 fixation). Bar equals 0.5 μm.

Fig. 7. Electron micrograph showing characteristic neuronal organelles in neuroblasts differentiated from isolated neural fold cells. (Glutaraldehyde fixation and OsO4 post fixation). Bar equals 0.5 μm.

Fig. 8. Immunocytochemical detection of 200 kDa neurofilament polypeptide in 12-day coculture. Bar equals 20 μm.

Fig. 9. Immunocytochemical detection of tetanus-toxin-binding molecules in 10-day culture from neural plate. Bar equals 20 μm.

Fig. 10. Catecholamine synthesis and storage visualized in situ in 5-day cultured cells, by the fluorescence induced after glyoxylic acid treatment. In a large cluster only some neuroblasts are GIF positive. Bar equals 75 μm.

Fig. 11. Autoradiography showing the uptake of [3H]dopamine by neurones differentiated in culture. 14-day culture. Bar equals 40 μm.

Fig. 12. Presence of acetylcholinesterase in differentiated neurones from isolated neural fold (Karnovsky & Roots method). 12-day culture. Bar equals 25 μm.
Figs 8, 9 for legend see p. 79
Figs 10–12 for legend see p. 79
uptake process in noradrenergic neurones, no labelling was observed suggesting the presence of noradrenergic neurones in the cultures.

3. Acetylcholine (ACh)

ACh synthesis and storage was measured by incubating the cultures with $[^3]$H]choline for 4 h and then isolating the $[^3]$H]ACh accumulated in the cultures (Hildebrand et al. 1971).

To make the results comparable, this experiment was performed with eggs from a single laying; each result was the mean from five sister-cultures. Four series of cultures were used to determine the amount of ACh synthesized and stored, at the beginning of the culture, as well as after 5, 11 and 15 days of culture. Moreover, this experiment was performed simultaneously for cocultures, for isolated NP and for isolated NF with eggs of the same laying. The results of one experiment are presented on Fig. 13 and are representative of five similar experiments performed over a 2-year period.

No ACh synthesis could be detected in any of the samples examined at the beginning of the culture (early neurula stage). The presumptive ectoderm (neural target cells) from early gastrula (st. 8) and the ventral ectodermal cells from late gastrula (st. 13) did not synthesize detectable amounts of ACh.

In isolated NF cultures, no significant ACh synthesis was detectable up to 2–3 weeks, whereas 5-day-old NP cultures synthesized and accumulated about 0.5 pmole $[^3]$H]ACh/explant. This figure stayed approximately constant up to 15

Fig. 13. Synthesis and storage of $[^3]$H]acetylcholine from exogenous $[^3]$H]choline. This experiment was performed with eggs from a single laying. Data are means ± s.e.m. for five sister-cultures.
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days in culture. On the other hand, the synthesis and accumulation of $[^3\text{H}]\text{ACh}$ in
cocultures increased linearly with time in culture to reach 8–15 pmoles/explant at
day 15. In control experiments, no $[^3\text{H}]\text{ACh}$ synthesis was measured in 15-day-old
cultures of chordamesodermal cells, suggesting that the excess of $[^3\text{H}]\text{ACh}$
synthesis observed in cocultures over both NF and NP cultures was not due to
chordamesodermal cells.

4. **Acetylcholinesterase (AChE) detection.** We visualized AChE using the
Karnovsky & Roots (1964) cytoenzymological method in the presence of iso-
OMPA ($10\mu\text{M}$) an inhibitor of pseudocholinesterase (Fig. 12). This enzyme was
detected in neurones isolated from NP or NF and in cocultures. As expected,
muscle cells which differentiated in coculture also reacted positively.

The staining was abolished by performing the reaction in the presence of a
specific inhibitor of acetylcholinesterase (BW 284, C 51, 10$\mu\text{M}$).

**DISCUSSION**

The aim of this study was to evaluate, at the late gastrula stage in amphibians,
the early differentiating potential of neuronal precursor cells. We were also
interested to study whether the presence of chordamesodermal cells influenced the
differentiation pattern of microsurgically excised and cultured neural plate (NP) or
neural fold (NF) cells. We showed that, in the absence of chordamesodermal
influence, such cultured cells exhibited a high degree of morphological and
biochemical differentiation, as revealed by the expression of neurofilament
polypeptides, tetanus-toxin-binding sites and by the metabolism of neurotrans-
mitters. Before these various neuronal phenotypic characters could be detected in
the cultures, remarkable differences in the cell aggregation properties were ob-
served in the different culture types.

*Aggregation properties of neuronal precursor cells*

In a previous study (Gualandris & Duprat, 1981), it was found that, at the early
gastrula stage, non-induced ectodermal cells strongly reaggregated in culture and
formed an epithelial sheet, whereas induced neural cells remained in isolation or
formed clusters. This difference constituted the most precocious indicator of the
occurrence of neural induction. Similarly, cells in NP and NF cultures displayed
different aggregation properties which were evident after 1–2 days. Neuroblasts
dissociated from the NP rapidly formed large clusters out of which thick neurite
fascicules emerged after 3–4 days. On the other hand, neuroblasts from the NF
remained isolated or gathered in small clusters. In these cultures, the neurite net-
work was finely reticulated without tendency to form large cables.

These adhesive differences were the earliest manifestation of neural induction
and may reflect the modulation of tissue-specific adhesive molecules at the cell
surface. Cell adhesion molecules have been demonstrated to play a crucial role in
cytodifferentiation and morphogenesis (for reviews, see Edelman, 1984a,b;
Rutishauser, 1984). Such molecules may be differentially expressed on non-induced and induced ectodermal cells and also on NP and NF cells.

**Development of neurone-specific markers**

Neurofilament proteins and tetanus-toxin-binding sites were detected after 3-4 days in the three culture types. Although no quantitative study was performed, it was clear that the labelling increased during time in culture concomitant with neurite outgrowth. All the cells labelled in these cultures with either tetanus toxin or anti-neurofilament antibodies displayed a clear neuronal morphology, whereas cells classified as non-neuronal by phase-contrast microscopy were not labelled. In agreement with previous studies by Vulliamy & Messenger (1981), tetanus toxin is thus a marker for neuronal differentiation in amphibian cultures.

**Development of neurotransmitter metabolism in culture**

The development of both catecholamine and acetylcholine metabolism has been evidenced in the cultures, whereas the synthesis of neither transmitter could be detected at the early neurula stage.

a) **Catecholamines**

About 1-2% of the neuronal population synthesized catecholamines, as revealed by glyoxylic-acid-induced fluorescence. The percentage of fluorescent cells was not significantly modified by a preincubation with norepinephrine. On the other hand, about 50% of the neurones took up $[^3]$H]dopamine by a desmethyl-imipramine-sensitive, high-affinity process. The difference in these two figures might simply reflect differences in the sensitivity of the autoradiography and glyoxylic-acid-induced fluorescence, but different neurone populations may have been revealed by the two methods. An answer to this question would first necessitate a better knowledge of the specificity of uptake inhibitors for dopaminergic and noradrenergic neurones in amphibians.

b) **Acetylcholine**

ACh synthesis and storage in the cultures was measured quantitatively by the isotopic metabolic method of Hildebrand *et al.* (1971). With this sensitive method, no ACh metabolism could be detected in the neural primordium before culture. Moreover, it was not detectable in NF cultures up to 15 days. On the other hand, a small but significant ACh synthesis and storage was observed in NP cultures, without variation between days 5-15. Only in cocultures did ACh synthesis and storage develop to a large extent; control experiments demonstrated that chordamesodermal cells themselves did not synthesize ACh. Similar results have been obtained by measuring choline acetyltransferase activities in these cultures (Kan *et al.* in preparation). Thus the cholinergic trait is differentially expressed in NF and NP cultures and appears to be controlled by chordamesodermal cues. The influence of chordamesoderm may require direct cell contacts or may be mediated by diffusible factor(s). A variety of rat non-neuronal cells release in culture a macromolecule which stimulates ACh synthesis in cultured sympathetic neurones.
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(Patterson & Chun, 1977; Weber, 1981; Swerts, Le Van Thai, Vigny & Weber, 1983) as well as in cultures from rat spinal cord (Giess & Weber, 1984) and from the nodose ganglia (Mathieu, Moisand & Weber, 1984). Experiments are in progress to determine if conditioned medium by chordamesodermal cells stimulates ACh synthesis in NP and NF cultures.

From these data, it can obviously not be concluded that the chordamesoderm specifically exerts a cholinergic influence on neur ectodermal cells, since ACh synthesis is the only neuronal differentiation marker which has been quantitatively studied. Although catecholamine synthesis was visualized in approximately the same percentage of neurones in the three types of cultures, quantitative differences may have been overlooked.

It would be interesting to know how the time course of neuronal differentiation in culture compares to that observed in vivo. Although a lot of information is available on the development of amphibian nervous system at the cellular level (for reviews see Jacobson, 1978; Spitzer, 1984; Warner, 1984), little is known on the development of the molecular markers used in this study, as compared to higher vertebrates (Vogel & Model, 1977). A comparison between in vivo and culture data is thus difficult for the time being. Moreover, such a comparison may have inherent limitations, since the neuronal population which develop in culture may not be representative of the overall population. In the case of rat hippocampal neurones, Banker & Cowan (1977) have shown 'a clear relationship between the stage of cells' development and their growth in culture', and a similar phenomenon may occur in amphibian cultures as well. On the other hand, the neurotransmitter sensitivity of Rohon-Beard neurones has been shown to develop with a similar time course in vivo and in culture (Spitzer, 1984). Such a detailed comparison on an identified neurone population remains to be made with the biochemical markers used in this study. In addition, amphibian cultures may be useful for the study of the differentiation of glial cells and of the segregation of the neuronal and glial cell lineages.

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