

## Neural induction: embryonic determination elicits full expression of specific neuronal traits

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### SUMMARY

In *Pleurodeles waltl*, the early neuronal differentiation of precursor cells from late gastrula stage has been studied by culture *in vitro* from either isolated neural plate (NP) or isolated neural fold (NF).

The aim of this study was to delineate the information acquired by ectodermal target cells during neural induction. By culturing these cells *in vitro* either with or without the underlying chordamesoderm, we showed that in the absence of chordamesodermal influence such NP or NF cells exhibited a high degree of biochemical and morphological differentiation as revealed by the synthesis and the storage of neurotransmitters, the activity of specific enzymes, as well as by the expression of neuronal markers: specific changes in cell surface carbohydrates, tetanus toxin binding sites and neurofilament polypeptides. Remarkable changes in the cell adhesive properties were the first events observed in the different central (NP) and peripheral (NF) types.

In cocultures the chordamesodermal cells exert a beneficial influence on this differentiation, specially increasing acetylcholine synthesis. There are some differences between central (NP) or peripheral (NF) neuroblast response to this further notochord or mesodermal influence.

### INTRODUCTION

During amphibian gastrulation, morphogenetic movements lead to inductive neural interactions between the chordamesoderm (dorsal blastoporal lip) and the ectodermal target tissue (presumptive neural ectoderm). The molecular mechanism of ectodermal cell neuralization is still an unsolved problem of neuroembryology. Several authors have shown the key role played by glycoconjugates and/or the structural organization of the plasmalemma of target cells in the onset of the molecular events in the neural inductive process which ultimately lead to neural commitment (Tiedemann & Born, 1978; Grunz & Staubach, 1979; Takata, Yamamoto & Ozawa, 1981; Takata, Yamamoto, Ishii & Takahashi, 1984a; Takata,

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Yamamoto & Takahashi, 1984b; Duprat, Gualandris & Rouge, 1982; Duprat, Gualandris, Kan & Foulquier, 1984; Gualandris, Rouge & Duprat, 1983, 1985). Neuronal differentiation has been observed in cultures of cells dissociated from the neural primordium and underlying chordamesoderm at the neurula stage (Jones & Elsdale, 1963). Glial cells may also differentiate under these conditions and remain to be studied with specific glial markers. In the same way, when the competent presumptive ectoderm and the blastoporal lip were dissected from the gastrula of *Pleurodeles waltl* and associated *in vitro*, a 4 h contact between the two tissues was sufficient for the neural inductive process to occur, as revealed by neuron differentiation in cultures of cells dissociated from the re-isolated ectoderm (Gualandris & Duprat, 1981).

Thus, after *in vivo* or *in vitro* association with chordamesoderm, isolated presumptive ectodermal cells are able to differentiate into neurons in culture.

In Vertebrates, neuronal potentialities acquired during neural induction are still poorly understood. Amphibian gastrulae and early neurulae are suitable material for such a study by culturing isolated induced neural target cells, either alone or associated with chordamesoderm. Moreover, the first molecular events that occur after neural induction and the molecular mechanisms generating neuronal differentiation, in terms of expression of specific molecules, have been little studied in Amphibians (Vulliamy & Messenger, 1981; cf. Spitzer, 1984; Warner, 1984; Duprat *et al.*, 1984; Duprat, Kan, Foulquier & Weber, 1985a; Duprat, Rouge, Flottes & Bleys, 1985b). The present study aimed to delineate the neuronal properties acquired by the precursor cells during neural induction and to focus on the expression of cholinergic metabolism with regard to further influence from either notochord or mesoderm.

## MATERIAL AND METHODS

### *Cell cultures*

Neural primordia were isolated from late gastrulae – early neurulae of *Pleurodeles waltl* (st. 13) staged according to Gallien & Durocher, 1957. Neurulae were manually dejellied and the vitelline membrane removed with fine forceps.

The neural plate and neural fold were excised with or without the underlying chordamesoderm with a platinum thread; optimal separation of ectodermal and chordamesodermal sheets was obtained by immersing the embryos in two-fold concentrated Holtfreter saline solution. After dissociation of explants in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Barth medium (88 mM-NaCl, 1 mM-KCl, 2.4 mM- $\text{NaHCO}_3$ , 2 mM- $\text{Na}_2\text{HPO}_4$ , 0.1 mM- $\text{KH}_2\text{PO}_4$ , 0.5 mM-EDTA), the isolated cells were cultured as previously described (Duprat, Zalta & Beetschen, 1966) in Barth's balanced solution (Barth & Barth, 1959) supplemented with 1 mg ml<sup>-1</sup> bovine serum albumin (Sigma), 100 i.u. ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> 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). Cell counts were performed with a

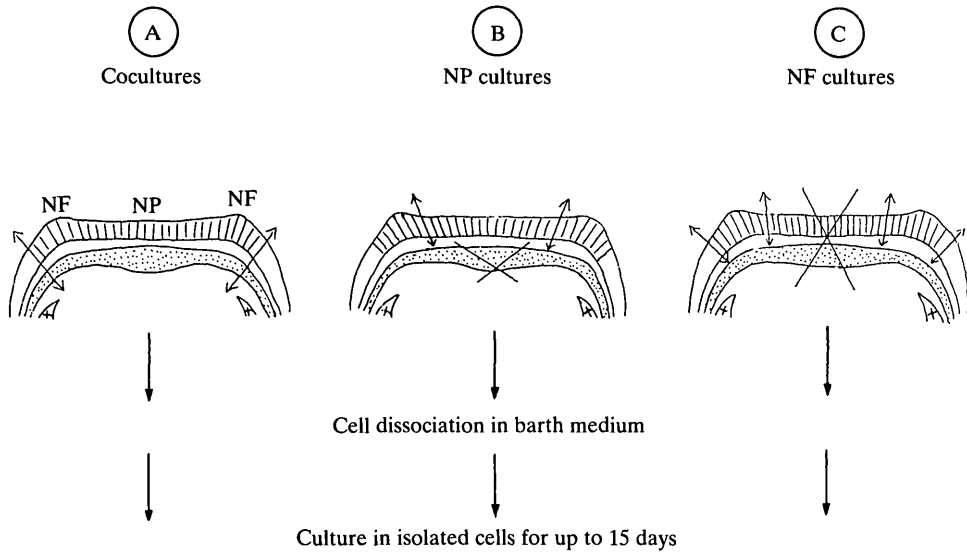


Fig. 1. Experimental procedure

haemocytometer monitored under a phase-contrast microscope. Trypan-blue dye exclusion was used as the criterion for viable cells ( $\leq 5\%$  dead cells).

Three sets of experiments were carried out:

*Cocultures.* – The dissociated cells from neural primordium were cocultured with underlying chordamesodermal cells (Fig. 1A).

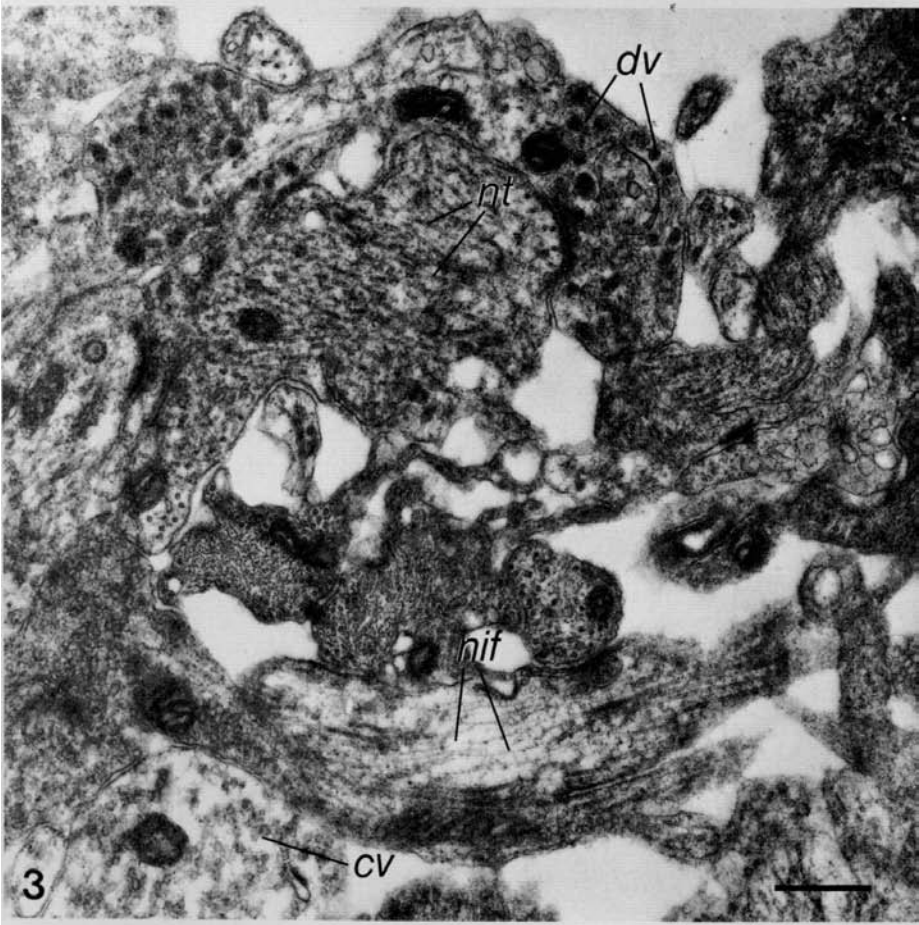
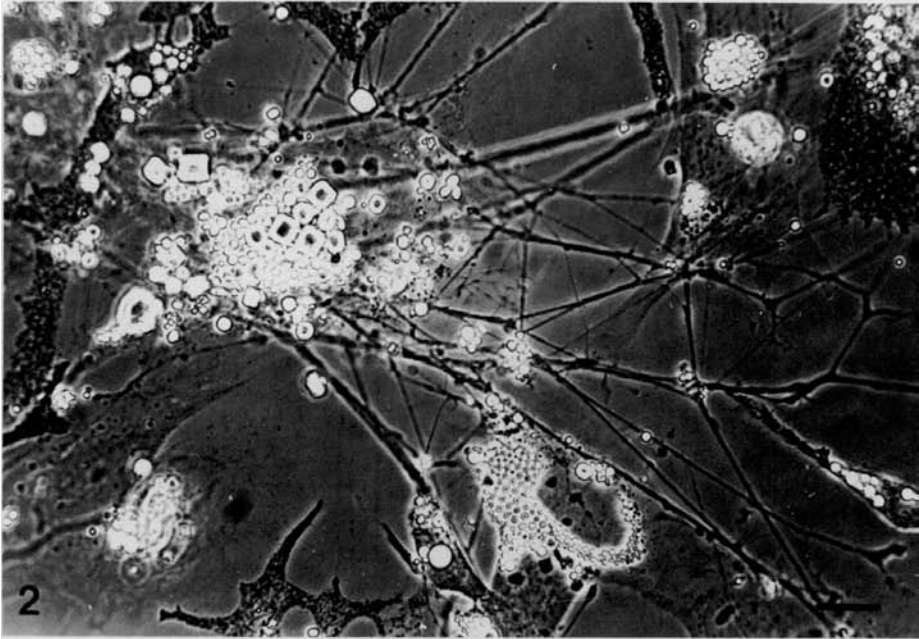
*Neural plate cultures.* – The dissociated cells from isolated neural plate were cultured (Fig. 1B).

*Neural fold cultures.* – The isolated cells from dissociated neural fold were cultured (Fig. 1C).

The cultures were maintained at  $20^\circ\text{C}$  in air atmosphere. After spreading of cells (2- to 3-day-old cultures) the medium was removed and replaced by Barth saline solution without BSA. The cells were then cultured for up to 2–3 weeks without medium change (Fig. 2).

#### *Acetylcholine synthesis and storage*

$[^3\text{H}]$ acetylcholine (ACh) synthesis and accumulation from  $[^3\text{H}]$ choline was measured by the method of Mains & Patterson (1973) adapted for amphibian cell cultures. The cultures were incubated for 6 h at  $20^\circ\text{C}$  in Barth saline solution supplemented with  $69\text{ mM}$ -[methyl- $^3\text{H}$ ]choline ( $5.6\text{--}7.5\text{ Ci mmole}^{-1}$ ) and  $25\ \mu\text{g ml}^{-1}$  ascorbate. Fresh ascorbate was added every second hour as a 100-fold concentrate solution. At the end of the incubation, the cultures were extensively washed and dissolved in  $100\ \mu\text{l}$  formiate–acetate pH2 buffer containing 1% sodium dodecyl sulphate and carrier choline ( $1\text{ mg ml}^{-1}$ ) and acetylcholine



(1 mg ml<sup>-1</sup>). [<sup>3</sup>H]ACh was then purified by high voltage paper electrophoresis at pH2 (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 degrees of freedom = 2 (n-1).

#### *Assay for choline acetyltransferase*

CAT activity was measured in the presence of 37 μM [<sup>3</sup>H]acetyl coenzyme A (936 mCi mmole<sup>-1</sup> from NEN) by the method of Fonnum (1975) with minor modifications (Swerts, Le Van Thai, Vigny & Weber, 1983).

#### *Assay for acetylcholinesterase activity*

The onset of acetylcholinesterase (ACh E) synthesis in cells was studied with the Karnovsky and Roots direct colouring method (1964). Some cultures were preincubated in presence of 10 μM-isoOMPA (tetraisopropyl pyrophosphoramidate) to inhibit non-specific cholinesterase activities. 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. In some cultures the reaction was performed in the presence of 10 μM-BW 284 C 51, a specific inhibitor of acetylcholinesterase, to demonstrate the authenticity of the staining procedure.

#### *Assay for catecholamines*

Cells producing catecholamines were *in situ* identified with a cytofluorescence method (Furness & Costa, 1975). The cultures were 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 supplemented with 10 μM-norepinephrine. This last control 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, dried with an air stream and further 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).

#### *[<sup>3</sup>H]dopamine uptake (radioautography)*

[2, 5, 6-<sup>3</sup>H]dopamine (18 Ci mmole<sup>-1</sup>, Amersham) was purified by HPLC on a μBondapak C<sub>18</sub> column (Waters Associate) equilibrated in 0.25 % acetic acid, 5 % methanol, lyophilized and redissolved in water. For uptake experiments, cultures

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Fig. 2. Phase contrast micrograph. 12-day-old coculture. Differentiated neuronal processes and melanocytes. Bar = 25 μm.

Fig. 3. Electron micrograph. Transverse section in neuronal processes exhibits neurofilament (*nif*), neurotubules (*nt*), clear (*cv*) and dense-core (*dv*) vesicles. Bar = 0.5 μm.

were incubated for 4 h at 20 °C in Barth solution containing 0.1 mM-pargylin and 0.5  $\mu\text{M}$ -purified [ $^3\text{H}$ ]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<sub>10</sub> emulsion and developed 7 days later using Kodak Dektol.

*Immunocyto detection of neuronal markers (neurofilament (NIF) polypeptides and tetanus toxin (Tt) binding molecules)*

(1) Separate polyclonal antibodies against the 200 kD and 70 kD neurofilament polypeptides were kindly provided by Dr D. Paulin (Inst. Pasteur). After fixation (3 % formaldehyde for 30 min at 20 °C, methanol at -10 °C for 6 min and 0.25 % Triton X<sub>100</sub> for 2 min at 20 °C) cells were incubated for 30 min at 20 °C in presence of 200 kD or 70 kD antibodies and then for 30 min in presence of FITC-conjugated goat antirabbit antiserum (Nordic Labs).

(2) Cultures were incubated for 30 min at a time with 10  $\mu\text{g ml}^{-1}$  Tt, a gift from Dr B. Bizzini (Inst. Pasteur), then with rabbit anti-Tt antibodies and then with goat antirabbit antiserum conjugated with FITC. Cells were fixed in 3 % paraformaldehyde for 30 min at 20 °C.

Washings before each incubation were in Barth solution. The coverslips were mounted in Mowiol 4-88 and viewed with epifluorescent Leitz Dialux microscope, incident source HBO<sub>50</sub>, filters I<sub>2</sub> (BP 450-490, LP 515). Controls with non-immune rabbit serum and FITC-GAR were negative.

*Electron microscopy*

Cultures were fixed with 3 % glutaraldehyde, postfixed in 2 % OsO<sub>4</sub> and embedded in Epon. Tangential or transverse sections were contrasted with 7 % uranyl acetate in methanol, lead citrate according to Venable & Goggeshall (1965), (Fig. 3).

## RESULTS AND DISCUSSION

### *I. Expression of neuronal traits acquired during neural induction*

In a previous report (Duprat *et al.* 1985a) we described the early differentiation potential of neuronal precursor cells isolated *in vitro* from amphibian late gastrulae. These experiments indicated that in neural plate (NP), or neural fold (NF) cultures as well as in cocultures, neuroblasts have acquired the capacity to express a high degree of biochemical and morphological differentiation.

#### *(1) Changes in cell adhesive properties*

Changes in cell adhesive properties were the earliest event visualized after induction (approximately after 12 h). Cell adhesive molecules which play a key role in morphogenesis (for review see Edelman, 1984; Thiery, 1984), may early be

differentially expressed on non-induced and induced ectodermal cell surfaces. Similarly, neuroblasts in NP and NF cultures displayed distinctive aggregation properties, apparent after 1–2 days *in vitro*.

### (2) *Expression of neuron-specific markers*

The expression of neuron-specific markers was detected using immunocytochemistry or labelled lectins.

*Tetanus toxin (Tt)*. Tt binds with high affinity to GD<sub>1b</sub>, GT<sub>1b</sub> gangliosides which are components of the toxin receptor molecule in neural plasmalemma. In the three types of cultures (cocultures, NP and NF cultures) immunolabelling of the neurons was very bright and distinctive (collaboration with Dr B. Bizzini).

*Antisera against neurofilament polypeptides*. Experiments performed with antisera raised against 200 kD and 70 kD neurofilament polypeptides showed intense fluorescence on cell bodies as well as neurite processes of differentiating neurons (collaboration with Dr D. Paulin).

The non-neuronal cells did not exhibit any specific labelling with these antisera.

*Changes in cell surface carbohydrates*. Specific changes during the *in vitro* differentiation of cells were studied using FITC- or TRITC-conjugated lectin probes (collaboration with Dr P. Rougé).

At the onset of the culture all the isolated cells from early neurulae, bind soybean agglutinin (SBA; binds  $\alpha$ -D galactose and galactose derivatives), *Pisum sativum* agglutinin (PSA; binds  $\alpha$ -D mannose,  $\alpha$ -D glucose and derivatives) and *Lens culinaris* agglutinin (LCA; binds  $\alpha$ -D mannose,  $\alpha$ -D glucose and derivatives). Later these lectins differentially stain ectodermal (non-induced) and neuronal (induced) cells; SBA did not bind to neurons, while PSA and LCA did not bind to epidermal cells. These data visualized changes or reorganization of the plasma membrane components. It is the transition from an initial phenotypic undifferentiated state into a differentiated state that is correlated with such detectable loss or 'masking' of the plasmalemma glycoconjugates (Duprat *et al.* 1985b). With regard to the events responsible for neural commitment and to our observations on early modifications in the adhesive properties of embryonic cells after neural induction, very early in ontogeny neural commitment did not result in a modification of cell surface polysaccharides detectable with lectin probes. Probably the modifications involved in adhesive property changes are too subtle to be visualized by the technique used.

These molecular markers: Tt binding receptor, NIF polypeptides and surface carbohydrate binding lectins can be considered as early markers of differentiation but not as markers of neuronal commitment because their initial expression is concomitant with the onset of phenotypic differentiation.

### (3) *The development of both catecholamines (CA) and acetylcholine (ACh) metabolism*

In these experiments, we have focused on the metabolism of ACh and CA

although other neurotransmitters as well as neuropeptides may be present in these cultures. No transmitter could be detected at the early neurula stage.

*Catecholamines.* After 10 days in culture, 1–2 % of the neuronal population synthesized and stored CA, as revealed by glyoxylic acid-induced fluorescence. On the other hand, approximately 50 % of the neurons took up [ $^3\text{H}$ ]dopamine by a desmethylimipramine-sensitive high-affinity process. Understanding the reason for the difference between these two figures would necessitate a better knowledge of the specificity of uptake inhibitors for dopaminergic and noradrenergic neurons in amphibians.

Be that as it may, these results emphasized that some neurons isolated from late gastrula stage and differentiated *in vitro* have acquired the potentialities to synthesize, accumulate and take up catecholamines.

*Acetylcholine.* In a previous study, we showed that quantitative measurement of [ $^3\text{H}$ ]ACh synthesis and storage from [ $^3\text{H}$ ]choline were negative at the early neurula stage. They remained negative in NF cultures up to 15 days, low and constant in NP cultures whereas ACh production increased linearly with time in cocultures.

These results suggest that, at the early neurula stage, some neuronal precursors have acquired the capacity to express a high degree of biochemical and morphological differentiation even in the absence of further chordamesodermal cues.

The second part of this report will be focused on studies of cholinergic metabolism and on the chordamesodermal influence on ACh synthesis and accumulation.

## II. Acetylcholine metabolism

The identity of the synthesized product as ACh was confirmed by ascending chromatography in a second dimension following electrophoresis. The chromatography reveals that the majority of the counts in the first dimension co-migrate with ACh (Fig. 4).

Figure 5 shows that the synthesis and accumulation of [ $^3\text{H}$ ]ACh was proportional to the number of explants cultured in the same dish.

### (1) Synthesis and accumulation of ACh (Fig. 6)

No ACh synthesis could be detected in any of the samples examined at the onset of the culture (early neurula stage). The two main enzymes involved in ACh metabolism, choline acetyltransferase (CAT) and acetylcholinesterase (AChE), were not visualized at this early stage.

Significant differences between the three studied sets of cultures were detectable after 5 days. ACh synthesis and accumulation were two to three times higher in cocultures than in NP cultures and remained near background values in NF cultures. This parameter developed linearly with time in culture for cocultures, but remained low in NP cultures. ACh synthesis was always negligible in NF cultures up to 15 days (Fig. 6).



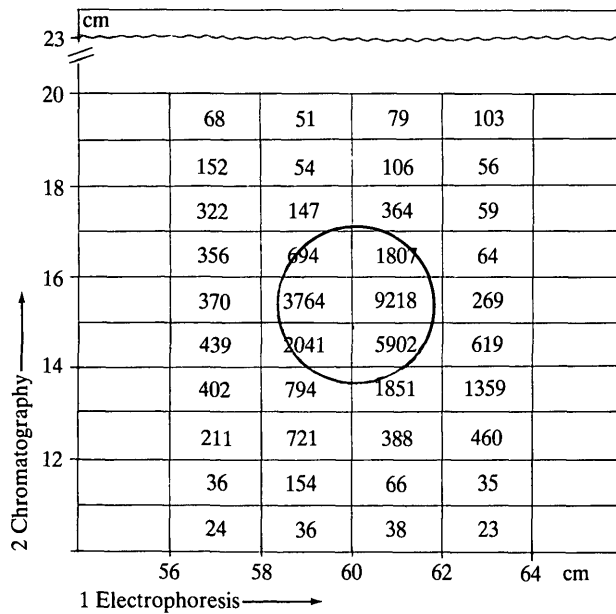


Fig. 4. Identity of ACh produced in culture. This is the profile of the radioactivity obtained when [ $^3\text{H}$ ] choline products were separated by high voltage electrophoresis at pH 2 in direction 1 and by ascending paper chromatography in direction 2 in the 1-butanol : isopropanol : acetic acid : water (20 : 40 : 10 : 20, V : V : V : V) solvent system. The circle indicates the position of the standard ACh, and most of the radioactivity found in the first dimension was recovered in this patch. The numbers in the squares indicate counts per min including a background of 36 c.p.m..

On the other hand, AChE was cytochemically visualized in the three types of experiments; however, the activities present in these cultures have not been measured quantitatively. CAT activity increased linearly with time in cocultures, presented a positive but low and constant pattern in NP cultures and remained negative throughout two to three weeks in NF cultures (unpublished results).

In presence of  $10^{-4}\text{M}$ -NVP (N-hydroxyethyl-4-(1-naphthyl-vinyl) pyridium bromide) a specific inhibitor of CAT activity, the amount of ACh synthesis decreased by 66 % (Fig. 7).

*Control experiments.* In control experiments performed on isolated cultured chordamesodermal cells, no [ $^3\text{H}$ ]ACh synthesis was measured in 15-day-old cultures indicating that the increase of [ $^3\text{H}$ ]ACh synthesis observed in cocultures was not due to synthesis from chordamesodermal cells themselves.

We also have checked that the dorsal ectodermal target cells (presumptive neural ectoderm—early gastrula, st. 8) and the ventral ectoderm (late gastrula, st. 13) did not synthesize detectable amounts of ACh.

## (2) Chordamesodermal influence

To clearly delineate the influence of chordamesoderm on either NP or NF

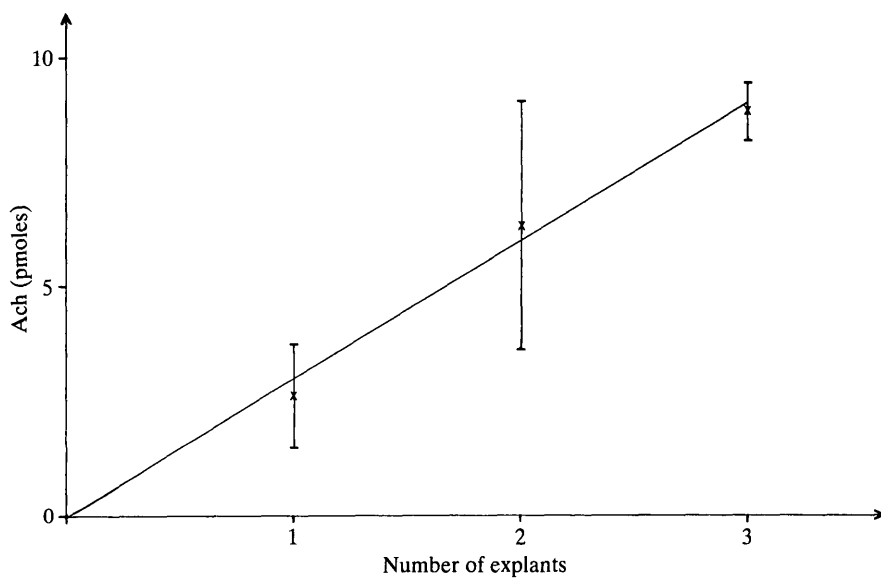


Fig. 5. Linearity of the ACh synthesis measurements when 1, 2, 3 explants were cultured in the same conditions (5-day-old cocultures).

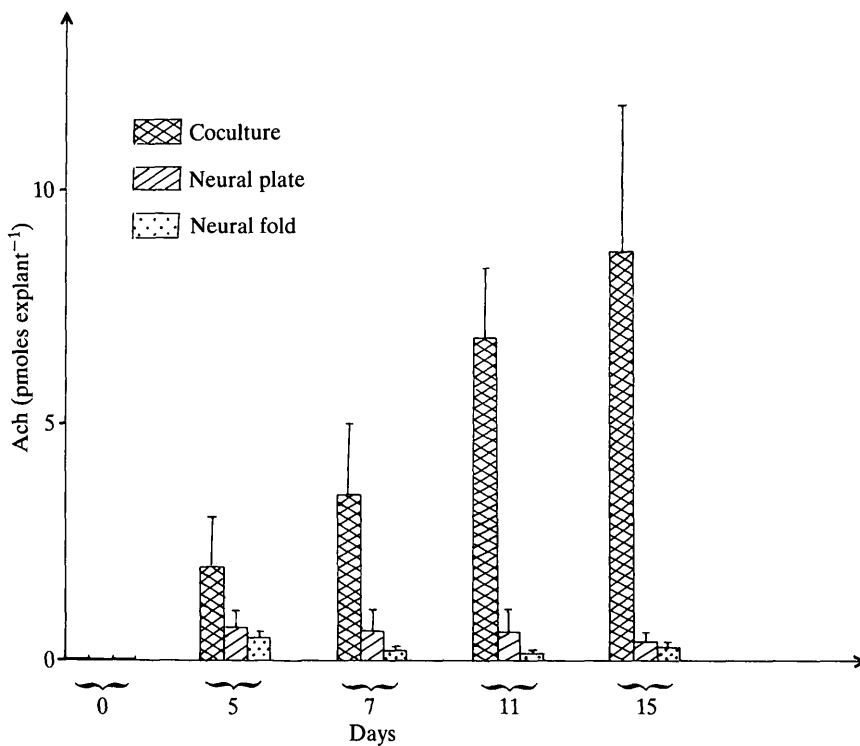


Fig. 6. Ability of cultured cells to synthesize and store ACh up to 15 days.

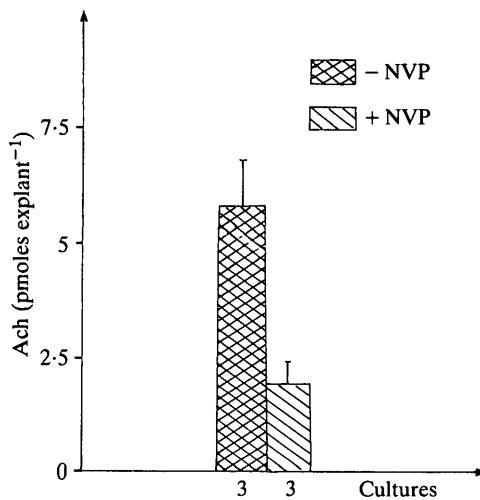


Fig. 7. Depletion of ACh synthesis in presence of  $10^{-4}$ M-NVP, a specific inhibitor of CAT activity. 11-day-old cocultures.

neuroblasts, we have performed cultures with homologous or with heterologous reassociation of these different embryonic areas, as follows:

*Homologous association:*

- culture of NP cells + underlying notochord area cells (NP + CM);
- culture of NF cells + underlying mesodermal cells (NF + M)

*Heterologous reassociation:*

- culture of NP cells + mesodermal cells (NP + M)
- culture of NF cells + notochord area cells (NF + CM)

*Controls* (cf. Materials and Methods)

- Cocultures
- NP cultures
- NF cultures

Figure 8 shows the rate of ACh synthesis detected in each set of cultures after 7 days. Figure 8A shows an important increase of the amount of ACh synthesis in NP + CM cultures and a slight increase in cultures of NF + M. Figure 8B shows heterologous reassociations; cocultured with NF cells, CM cells did not induce any increase in ACh synthesis; and NP cells cultured with mesodermal cells were less stimulated than when cultured with chordal cells (approximately 50%).

*These experiments show for the first time that differences between notochord and mesodermal areas occur in stimulation of ACh synthesis and accumulation in the neural plate or neural fold. Moreover, they also point out differences in the rate of response of NP or NF areas to the stimulating notochord or mesodermal influence.*

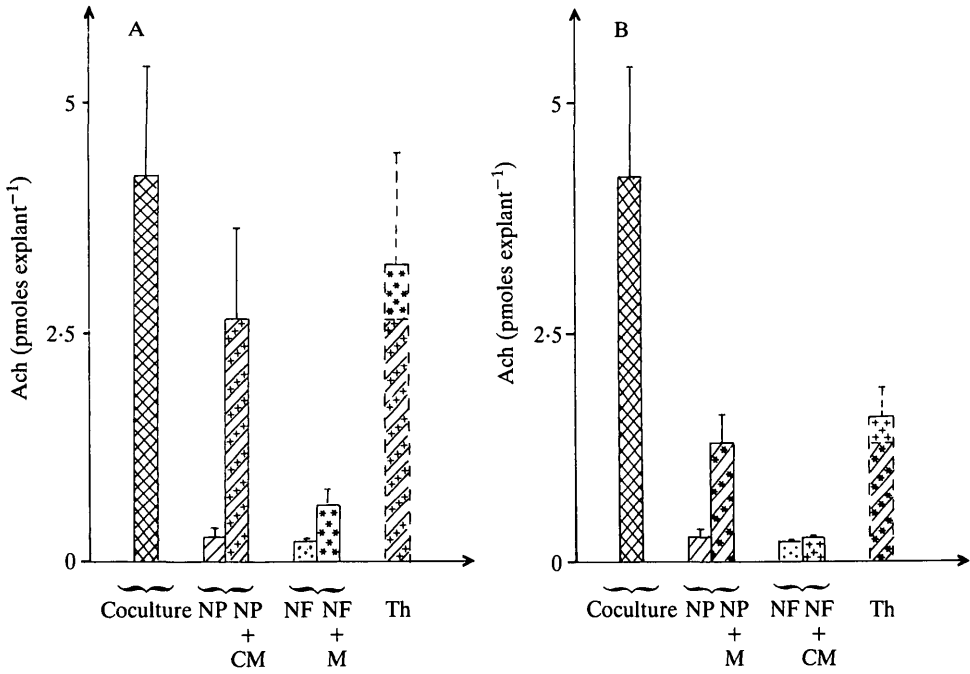


Fig. 8. 7-day-old cultures. Homologous (Fig. 7A) and heterologous (Fig. 7B) reassociations of NP, NF, CM and M. To make results comparable, for each experiment all measurements were performed on five sister-cultures. Eggs were from the same laying.

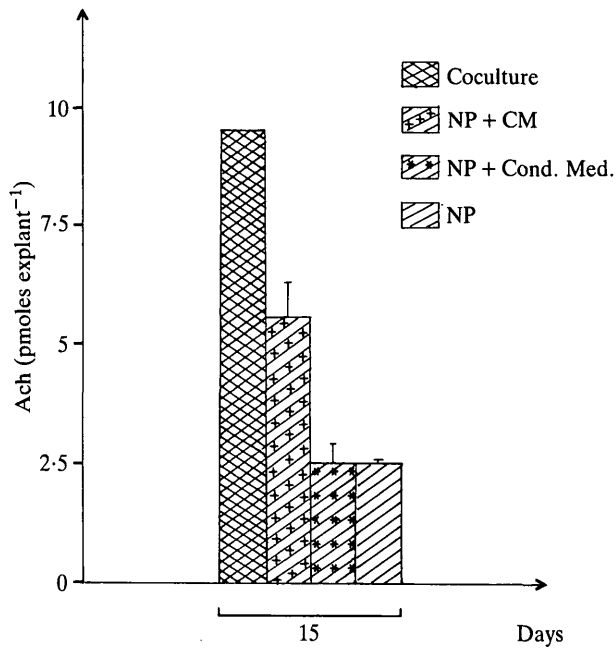


Fig. 9. Absence of ACh synthesis stimulation in isolated NP cells, cultured in a medium 'conditioned' with isolated chordamesodermal cells.

All these homologous and heterologous reassociations were performed on five sister cultures from eggs of the same female (same laying) to make the results comparable.

### (3) Conditioned medium from chordamesodermal cultures

The stimulating influence of chordamesodermal cells could act either by mediation of diffusible factor(s) or by cell contacts. To test the first hypothesis, isolated chordamesodermal cells from early neurulae were cultured for several days; the 'conditioned' medium was then tested with cultures of isolated NP cells.

Figure 9 indicates no stimulating effect of this 'conditioned' medium up to 15 days, even after several 'conditioned'-medium changes. Similarly, the culture of chordamesodermal cells and NP cells in separate parts of the same dish shows an absence of stimulation of ACh synthesis and storage (unpublished results).

These last results suggest that diffusible factor(s) did not seem to be involved in the further stimulation of the neural primordium by chordamesoderm. Moreover it can not be concluded that the chordamesoderm specifically exerts a cholinergic influence on neurectodermal cells, since ACh synthesis is the only neuronal differentiation marker which has been quantitatively studied. New experiments are now carried out to better understand the mechanism of this chordamesodermal influence.

## CONCLUSION

In conclusion, neuronal precursor cells (neural plate and neural fold cells), isolated from late gastrula stage after neural induction and cultured in an unconditioned medium, exhibited a high degree of morphological and biochemical differentiation. Remarkable differences in cell aggregation properties were the first events detected after cell commitment.

In amphibian embryos, as in birds or in mammals, the differentiation of neuroblasts seems to be highly dependent on the microenvironment to which they are submitted during their development. It is of obvious interest to attempt to analyse the mechanisms controlling cell differentiation, in order to obtain a clear picture of the events leading from the committed to the differentiated state.

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## DISCUSSION

*Speaker: M. Weber*

*Question from E. Weston (Oregon):*

Do any of these cells that express the transmitters divide, or are they all post-mitotic? Can you get an increase in the amount of transmitter as a consequence of the increase in the number of cells with a fixed amount of transmitter, or does it increase per cell?

*Answer:*

Cocultures have been incubated with [<sup>3</sup>H]thymidine between days 0–4 and examined by autoradiography at day 12. About 75 % of the neurones were labelled, demonstrating that neurone precursors proliferated during the first days of the culture. It is not known whether precursors for cholinergic neurons proliferated.

*Weston:*

So there is an increase of cell numbers in the cultures?

*Answer:*

Yes, mainly in the cocultures, due to the rapid proliferation of non neuronal cells. Neurone counts in NP and NF cultures have not yet been performed.

*Question from J. Gurdon (Cambridge):*

You say that diffusible factors are not involved in the stimulation of ACh production by chordamesoderm. But when you cultured the two tissues separately in the same dish the arrangement might have reduced the rate at which molecules from one side could reach the tissue on the other. You really need to prove that this does not seriously inhibit the transfer of molecules.

*Answer:*

Due to the low viscosity of the medium and the temperature used, we may expect that a molecule of, let's say, 50 000 daltons would freely diffuse. We cannot exclude, however, the existence of macromolecular factors, which could adsorb at the surface of chordamesodermal cells, or be highly unstable.

*Gurdon:*

What is the minimum time of contact that is needed to get an effect?



*Answer:*

The increase in CAT activity in cocultures over NP cultures was already significant at day 5. It is not known if a permanent contact between neurones and chordamesodermal cells is necessary for the increase in CAT activity between days 5–15, or if a shorter time of contact is enough to trigger this development in CAT activity.

*Gurdon:*

Supposing the minimum time was, say, 4 h and then you do that experiment for that amount of time, surely the molecules could not get across and build up to the same concentration?

*Answer:*

We do not know what is the minimum contact time between neural plate cells and chordamesodermal cells to get this increase in ACh production. To answer this question, one would have to first coculture the NP cells and chordamesodermal cells for 4 h, and then to separate the two cell populations!

*Gurdon:*

My argument is that ideally you would have two to three times longer in that set-up than was the minimum necessary with contact, then one would be convinced that there was not a dilution of components which have to move one side to the other.

*Question from J. Slack (ICRF, London):*

I believe that most of the cells derived from the neural plate are glia and not neurons. Do you get glia in these cultures?

*Answer:*

In the isolated neural plate cultures, the cells form small clumps and one doesn't see many flat cells on the bottom of the dish. Some glia may be present, but they are certainly a minority of the cells. Gliogenesis in the CNS is a late event, and the neural plate may contain a majority of neurone precursors.