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Original Article

Initial GABAergic expression in embryonic amphibian neuroblasts after neural induction

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ABSTRACT At the late gastrula-early neurula stage some embryonic neuroblasts from neural plate and neural fold present apparently as a consequence of neural induction, the capability to develop *in vitro* into different neuronal subpopulations (cholinergic, dopaminergic, noradrenergic, somatostatinergic and some other peptidergic subpopulations) without ongoing influences from the chordamesoderm (Duprat *et al.*, 1987). Using the same *in vitro* model system, the aim of the present work was to delineate the abilities of these neuroblasts to develop GABAergic traits. The initial appearance and development of GABAergic phenotype has been quantitated by assaying the activity of glutamic acid decarboxylase (GAD). GAD activity was undetectable at the early gastrula stage (stage 8a) and was slightly measurable at the early neurula stage (stage 14- onset of the culture). It increased subsequently over the next 14 days *in vitro*. The temporal pattern of appearance and development of GAD activity in culture was in agreement with that observed *in vivo*. Immunocytochemical studies showed that GABA-like immunoreactivity was expressed *in vitro* in a subpopulation of neurons. Thus the developmental program for GAD expression and GABA phenotype maturation is acquired at least in some neuronal precursors. These data together with previously reported results on the expression of cholinergic, catecholaminergic and peptidergic phenotypes demonstrate that different neuronal subpopulations emerge near the end of gastrulation i.e. immediately after neural induction. The embryonic origin of this neuroblast heterogeneity remains to be determined.

KEY WORDS: *neural induction, neurogenesis, GABA, GAD, neural determination*

Introduction

In the gastrulated amphibian embryo, two embryonic territories appear as an immediate consequence of neural induction; these are the neural plate (NP) and the neural fold (NF), which are at the origin of the central and the peripheral nervous systems, respectively.

It is now well established that the definitive development of derivatives of the neurectoderm is greatly influenced by the cellular environment encountered during their ontogenesis. This has been particularly well documented for cells originating from the NF (cf. Le Douarin, 1982, 1986; Landis and Keefe, 1983; Coulombe and Bronner-Fraser, 1986; Bronner-Fraser and Fraser, 1988; Schotzinger and Landis, 1988). However, until recently, it was not clear to what extent the cells that have just undergone neural induction possess the potentialities for differentiation into the various phenotypes that characterize the mature nervous system. It was of interest to study whether NP and NF cells that arise as a direct result of neural induction have the ability to develop mature neuronal phenotypes.

Amphibian late gastrulae constitute a useful embryonic system for this type of experimental approach. In order to analyze their neuronal potentialities, we isolated NP and NF from their embryonic environment immediately after neural induction, dissociated them and cultured the cells in a defined medium. We have already demonstrated that neuron-like cells differentiate *in vitro* under such conditions (Duprat *et al.*, 1984) and that they exhibit characteris-

Abbreviations used in this paper: AET, 2-amino ethylisothiouronium; BSA, bovin serum albumin; CM, chordamesoderm; FITC, fluorescein isothiocyanate; GABA, gamma-amino butyric acid; GAD, glutamic acid decarboxylase; MBN, Na metabisulfite; N-CAM, neural cell adhesion molecule; NF, neural fold; NP, neural plate; SEM, standard error of the mean.

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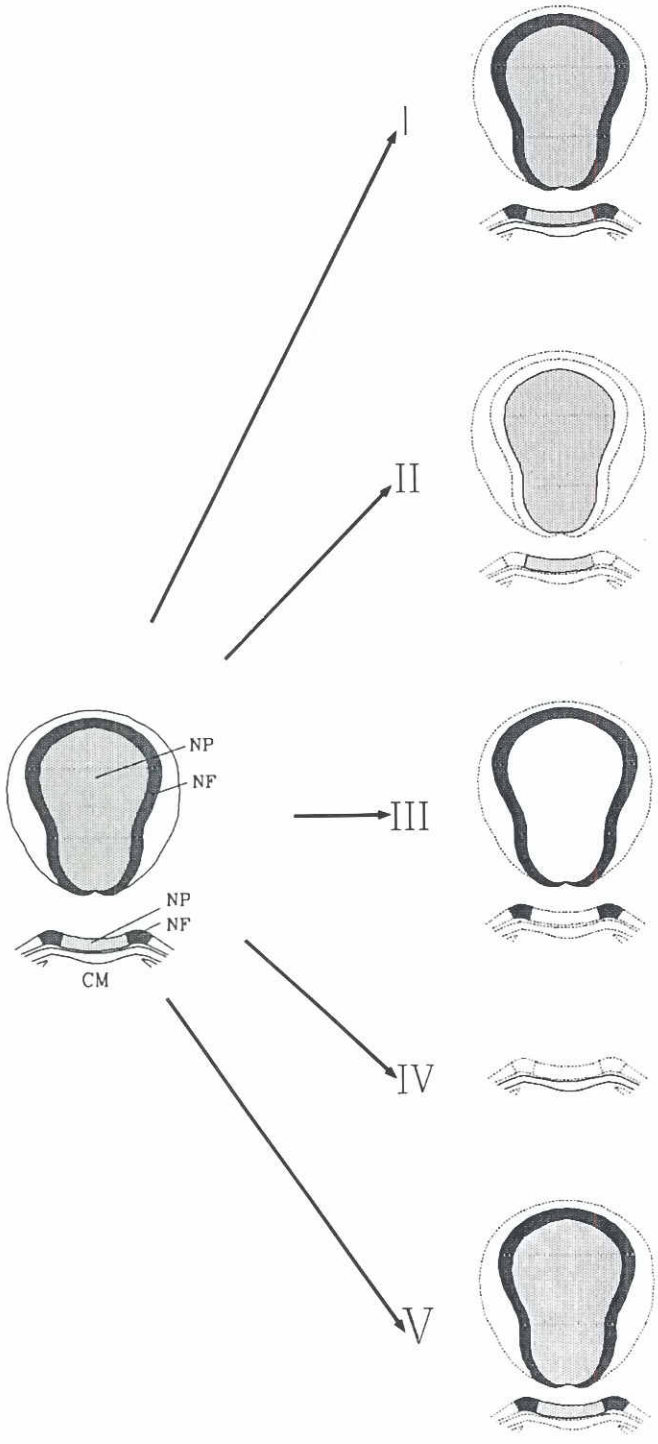

Fig. 1. Experimental procedure for cell cultures. *Five types of cultures were performed from late gastrula embryos (stage 13):* **(I)** *Cocultured neurectodermal cells mixed with underlying chordamesodermal cells, i.e., cocultures (NP + NF + CM).* **(II)** *Isolated neural plate cells (NP).* **(III)** *Isolated neural fold cells (NF).* **(IV)** *Isolated chordamesodermal cells (CM).* **(V)** *Neurectodermal cells (NP + NF) cultured without CM. All the cultures were in Barth's defined saline medium.*

tic traits, such as neurofilament proteins and tetanus toxin binding sites or N-CAM (Duprat *et al.*, 1986; Saint-Jeannet *et al.*, 1989). With regard to expression of neurotransmitters, we have provided evidence that the developmental program for the cholinergic phenotype is determined early (Duprat *et al.*, 1984). Absent at the late gastrula stage, choline acetyltransferase and acetylcholinesterase molecular isoforms appear *in vitro* and differences in the pattern of expression of these enzymes were observed between NP- and NF-derived neurons (Duprat *et al.*, 1985a, 1985b).

Catecholaminergic and peptidergic traits were also demonstrated in culture of NP and NF cells isolated *in vitro* immediately after induction (Pituello *et al.*, 1989).

With respect to the study of early events in neurogenesis, we thought that it would be of interest to determine whether neuronal precursor cells acquire early the ability to express the GABAergic phenotype.

GABA (gamma-amino butyric acid) is considered to be a transmitter as well as a modulator of neuronal activity (Lundberg *et al.*, 1983; Hokfelt *et al.*, 1984; Freund and Antal, 1988). It is assumed to be a major inhibitory transmitter in the central and peripheral nervous systems and GABAergic neurons are believed to be among the main mediators of recurrent inhibition, playing a crucial role in the selection of responses to different stimuli. This regulatory molecule has been implicated in the pathophysiology of a number of neurological and psychiatric disorders.

Differentiation of cells displaying a GABAergic phenotype can be conveniently monitored by measurement of the activity of glutamic acid decarboxylase (GAD), the enzyme catalyzing the synthesis of GABA and/or by immunocytochemical detection of the amino acid itself.

The aims of the present study were to use NP and NF cells isolated *in vitro* straight after neural induction (Duprat *et al.*, 1984, 1985a, 1985b) in order to:

(i) determine the ability of these embryonic cells to develop GABA-related properties (GAD activity, GABA biosynthesis, accumulation and uptake);

(ii) study the temporal pattern with which the GABAergic phenotype emerges and matures in differentiating neurons from NP and from NF at the late gastrula stage, *in vitro* and *in vivo*;

(iii) visualize immunocytochemically and quantify the GABAergic cell population differentiating *in vitro.*

Results

Biochemical studies of initial expression of GAD activity and changes in the kinetics of expression during development in vitro *and* in vivo

Cell cultures

The appearance and the development of GAD, the enzyme responsible for GABA biosynthesis, were analyzed quantitatively in cocultures (NP + NF + CM), and in NP and NF cultures. The data are schematized in Fig. 2. GAD activity, undetectable at the early gastrula stage (stage 8a, data not shown), was very low at the late gastrula-early

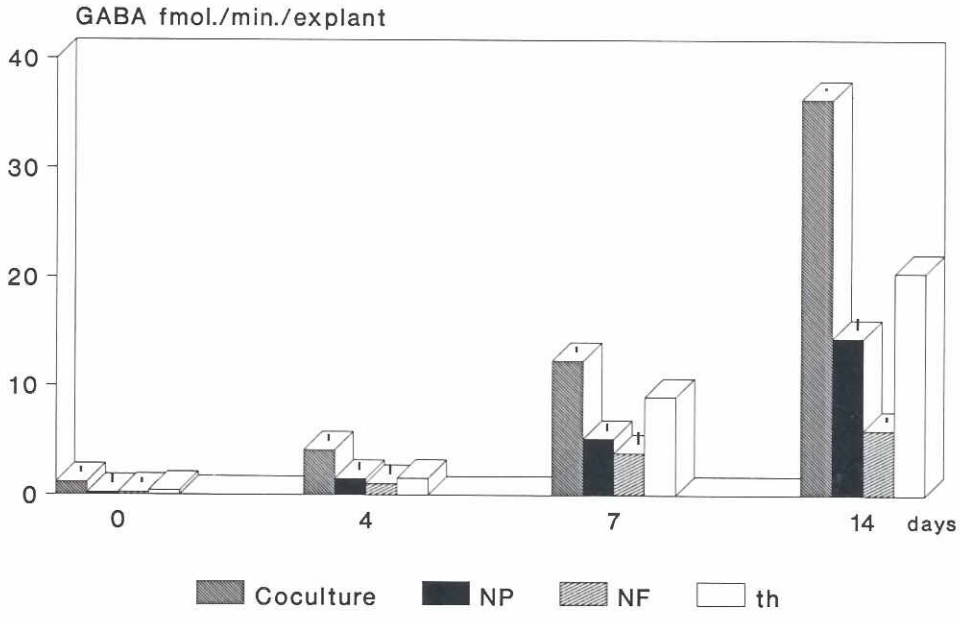

Fig. 2. Appearance and development of GAD activity in cell cultures from late gastrulae. *Analyses were performed at the onset of the culture (t = 0) and after 4-, 7- and 14 days in culture. GAD activity was measured in 4 sibling cocultures (NP + NF + CM), NP cultures and NF cultures in two sets of experiments (cf. Materials and Methods). GAD activity is normalized to "explant" which is one embryo equivalent. The data are means± SEM. Th corresponds to the theoretical value obtained by addition of NP + NF values.*

neurula stage in cocultures assayed at the beginning of the culture period. The level of the enzyme rose regularly from then on and, after 14 days of culture, had increased approximately 35 fold.

In NP cultures, GAD activity was clearly demonstrable at 4 days and subsequently increased. However, the level of enzymatic activity was always lower than in cocultures of identical age.

In NF cultures, GAD activity was also expressed at day 4 and increased throughout the culture period. At all time points, the enzyme levels were lower than in the corresponding NP cultures.

A two-way analysis of variance evidenced a significant difference for GAD activity depending on the culture type ($P<0.01$), and for each one of these types depending on the time course in culture ($P<0.01$). Furthermore, the evolution with time of GAD activity was different for the three types of cultures ($F(4,27)=206$, $P<0.01$).

The theoretical values obtained by adding the activities measured in NP and NF were always less than those measured in corresponding cocultures (Fig. 2). In control experiments conducted by culturing cells of the entire neurectoderm (NP+ NF) in the absence of CM for 14 days, GAD activity (16 fmol/min/explant) was the sum of the individual activities of NP and NF (compare Fig. 2 and Fig. 3).

In an attempt to determine the reason for the enhanced activity observed in cocultures, experiments were carried out on isolated CM cells cultured for up to 14 days without NP and NF. These cultures presented a low but significant GAD activity (7 fmol/min/explant) (Fig. 3). However, the activity detected in cocultures was still higher than the theoretical level obtained by combining the enzymatic activities measured in neurectoderm (NP + NF) cultures and the activity measured in CM cultures. Thus, coculture enhancement could be due either to an increase in the GAD positive neuron differentiation in this culture type or to an increase in the number of positive neurons. Immunocytochemical visualization and quantification of the GABAergic population will provide an answer to the question.

In vivo *emergence and development*

GAD activity was assayed in whole embryos at a number of stages, four of which corresponded to time points at which cultures had been assayed (see legend to Fig. 4).

As mentioned previously, no GAD activity was detected before gastrulation. A barely significant activity (0.65±0.35 fmol/min/embryo) was measured at stage 13-14 (corresponding to zero time of culture). Thereafter the activity rose markedly, roughly paralleling the enzyme levels measured in culture up to the stage 32/7 day time point. However, GAD activity in older embryos was markedly higher than that measured in cultures of equivalent age (14 days).

***Immunocytochemical visualization of the GABAergic cell population* in vitro *and* in vivo**

In vitro *expression of GABA*

To identify the cell population containing GAD and to determine whether GABA was biosynthesized and accumulated, we first performed immunocytochemistry on 14-day-old cocultures, which present the highest level of GAD activity *in vitro*.

A clearly discernible, but faint, GABA immunoreactivity was detected in a few neurons (approximately 1% of the neuronal population). All the non-neuronal cells present in the cultures were negative. Cytochemical experiments were also carried out after preincubation of the cultures with GABA (10^{-5} M) for 5 h. A bright staining was observed in a somewhat greater proportion of cells [188±20 (n=6 cultures) positive neurons, i.e., approximately 6% of the neuronal population]. These observations indicate that

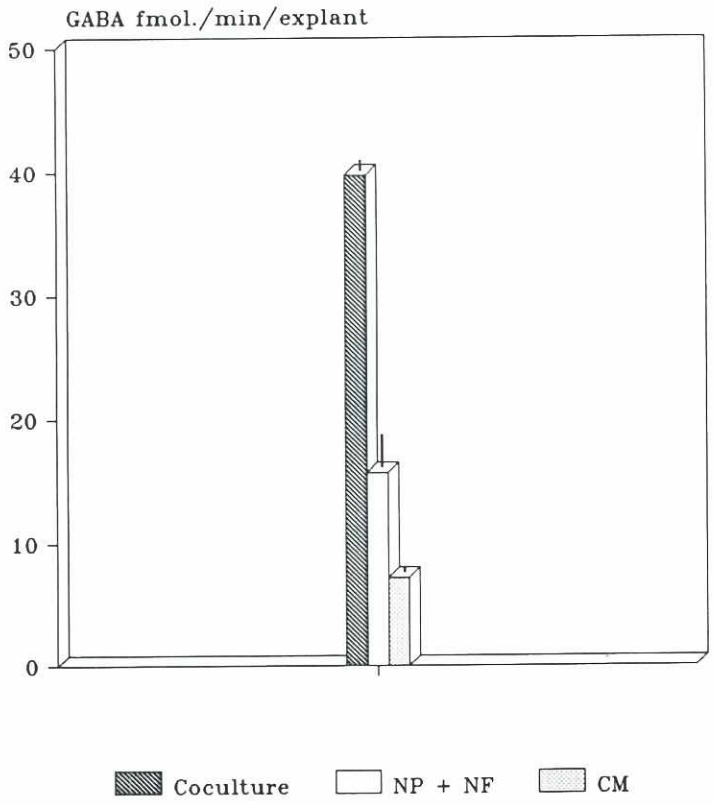

Fig. 3. GAD activity in 14-day-old cell cultures. *Cocultures (NP + NF + CM), cultures from isolated neurectodermal cells (NP + NF) or cultures from isolated chordamesodermal cells (CM) were prepared simultaneously from the same batch of eggs. The data are means ± SEM for respectively four cultures of each type in two sets of experiments (124 gastrulae).*

only a small subset of the differentiated neuronal population is GABAergic and that most of this GABAergic population can be visualized only after loading with GABA.

In culture preincubated with exogenous GABA, some of these GABA-positive neurons occurred in isolation, in which case they either made no apparent contact with other cells or were situated on flattened mesenchymal-like cells (Fig. 5a). Alternatively, they were, together with non-immunoreactive neurons, found in small (Fig. 5b) or much larger (Fig. 5c) neuronal aggregates. The subpopulation of cells expressing GABA-like immunoreactivity exhibited no morphological traits allowing it to be distinguished from the neuronal population as a whole by phase-contrast observation. The positive neurons displayed fluorescence in the cell body as well as in neurites. The fluorescent neuritic network presented a particularly intense immunoreaction at the level of the varicose structures (not shown).

We next carried out a series of experiments in which NP and NF were isolated and cultured separately. The purpose of these studies was two-fold: first, to determine whether neuroblasts were able to express GABA-related properties without further chordamesodermal cues after neural induction and, if so, to quantify GABAergic neurons found in NP and NF cultures respectively, in order to compare them to the GABAergic population found in cocultures.

With regard to the first point, after both 7 and 14 days *in vitro*, NP cultures contained neurons displaying GABA-like immunoreactivity. These cells were most often dispersed in the midst of the large neuronal aggregates characteristic of these NP cultures (cf. Fig. 5c). The number of GABAergic neurons counted in 14-day-old NP-cultures was 71±8 (n=6, cultures) cells per neural plate, i.e. around 3% of the neuronal population.

Similarly, a small subpopulation of neurons also expressed GABA-like immunoreactivity in NF cultures. Positive cells were found alone or in small clusters (cf. Fig. 5a and 5b), and totaled 25±6 (n=6, cultures) per neural fold in 14-day-old NF-cultures, i.e. approximately 1% of the neurons differentiated *in vitro*.

It must be stressed that in cocultures or in CM cultures, myoblasts and chordal cells remained immuno-negative, suggesting that GABA was not biosynthesized or taken up by these cells although the results previously described (Fig. 3) indicated that they contain the biosynthetic enzyme GAD.

The quantitative comparison of GABAergic neurons developing in the three types of cultures (cocultures, NP and NF cultures) revealed that the total number of GABA-positive neurons present in cocultures (188 neurons) was twice that of positive neurons present in NP and NF cultures together (96 neurons).

In vivo *expression of GABA*

Immunohistochemistry was performed on transversal or sagittal frozen sections of embryos at different stages of development, from the early neurula (stage 13-14) to the young-larval stage (stage 38). These analyses showed that during normal development of embryos, expression of the GABAergic phenotype first became faintly discernible at stage 22 (early tailbud stage) and could be clearly visualized at stage 28 (stage corresponding to 4-day-old cultures). At stage 38, a brilliant immunoreaction was observed in cell bodies and in neuritic fibers of neurons whose location within the spinal cord is similar to the ascending neurons described in *Xenopus* by Roberts *et al.*, (1987), (Fig. 6).

Discussion

In vertebrates the phenotypically diverse neuronal cells of the central and peripheral nervous systems are developmentally derived from two embryonic territories, the neural plate (NP) and the neural fold (NF) respectively, which appear immediately after neural induction at the late gastrula-early neurula stage.

The data reported here demonstrate that some of the neuronal precursor cells present in the NP and the NF have acquired, as a direct consequence of this inductive epigenetic process, the potentiality to express the GABAergic phenotype.

Our findings indicate that a faint expression of GAD first emerges at the late gastrula stage both *in vitro* and *in vivo*. No GAD activity was detected in the presumptive ectoderm or in the embryo at the early gastrula stage, before neural

induction had occurred.

In vitro, this expression seems to parallel the morphological differentiation of neurons and develops markedly with time, not only in cocultures but also in cultures of isolated NP and NF. Clearly, then, some embryonic cells have already acquired, shortly after neural induction, the ability to express GAD activity without further influence from chordamesodermal cells.

Nevertheless the enzymatic activity quantified in NP and NF cultures was always, throughout the culture period, lower than in cocultures where NP and NF differentiate in intimate association with chordamesodermal cells. It is particularly interesting to compare this observation with our previous data concerning the development of the cholinergic, catecholaminergic and somatostatinergic phenotypes (Duprat *et al.*, 1985b, 1987; Pituello *et al.*, 1989), where a similar enhancement of the different metabolisms was always observed in cocultures.

It must be pointed out that isolated CM cells differentiated *in vitro* exhibit a low but significant level of GAD activity whereas the neurotransmitter itself is missing. Note that CM cultures contain only myoblasts, typical vacuolated chordal cells, mesenchymal and fibroblast cells but never neurons. A similar result has been obtained for tyrosine hydroxylase which is especially involved in the metabolism of the catecholamines (unpublished data). The role of such enzyme activities in CM is still unsolved.

By staging sibling embryos of ages equivalent to those of cells grown in culture we have provided evidence that differentiation of GABAergic cells in culture follows a time course similar to that observed *in vivo*. However, it is of importance to note that, quantitatively, GAD activity measured at equivalent time points (particularly at late stages) was always lower in cocultures than *in situ*. These observations suggest that although some NP and NF precursor cells have undoubtedly acquired the capability to express a GABAergic phenotype when isolated from further chordamesoderm cues, stimulating influences from the environment are required for a high degree of GAD expression to be attained.

GABAergic phenotypic expression by embryonic neuroblasts cannot be detected immunocytochemically *in situ* until stage 22 (early tailbud stage). This data is consistent with the observations of Roberts *et al.*, (1987), who analyzed the development of GABAergic neurons in *Xenopus* embryos. The time at which a small proportion of cultured neurons contain detectable GABA also corresponds to stage 22. Here again, we observed a good correlation of the time of development of GABA biosynthesis and accumulation *in vivo* and *in vitro*.

Our results provide further evidence that the process of dissociating cells and growing them in culture does not substantially change their early developmental program. Young amphibian neuroblasts are able to complete the development of their mature phenotype while growing in dissociated cell culture in a chemically defined medium. This observation is in agreement with results reported by Spitzer and Lamborghini(1976), Bixby and Spitzer (1982), Blair (1983), O'Dowd (1983), Lamborghini and Iles (1985), Pituello *et al.* (1989).

Our findings obtained in early neuroblasts of *Pleurodeles* in culture are to be compared with those reported by Bixby and Spitzer(1984), Lamborghini and Iles (1985). In *Xenopus* embryo spinal cord the latter authors demonstrated the early development *in vivo* and *in vitro* of a high-affinity GABA uptake system. They showed that these neurons originate near the end of gastrulation, their ability to take up and accumulate GABA first appearing *in vivo* at the tailbud stage. *In vitro*, this transport system develops independently of the ability of neurons to conduct action potentials.

The GABAergic subpopulation we visualized by immunocytochemistry using an anti-GABA antibody in cocultures from *Pleurodeles* late gastrulae could probably correspond to the embryonic neuronal population which develops a GABA-uptake system described in *Xenopus* embryo by these authors.

With respect to the fact that a part of the neuronal GABAergic population seemed to have acquired only the capacity to take up exogenous GABA without being able to biosynthesize this transmitter (at least in amounts detectable by immunocytochemistry), this observation agrees well with the findings of Hollyfield *et al.* (1979). They

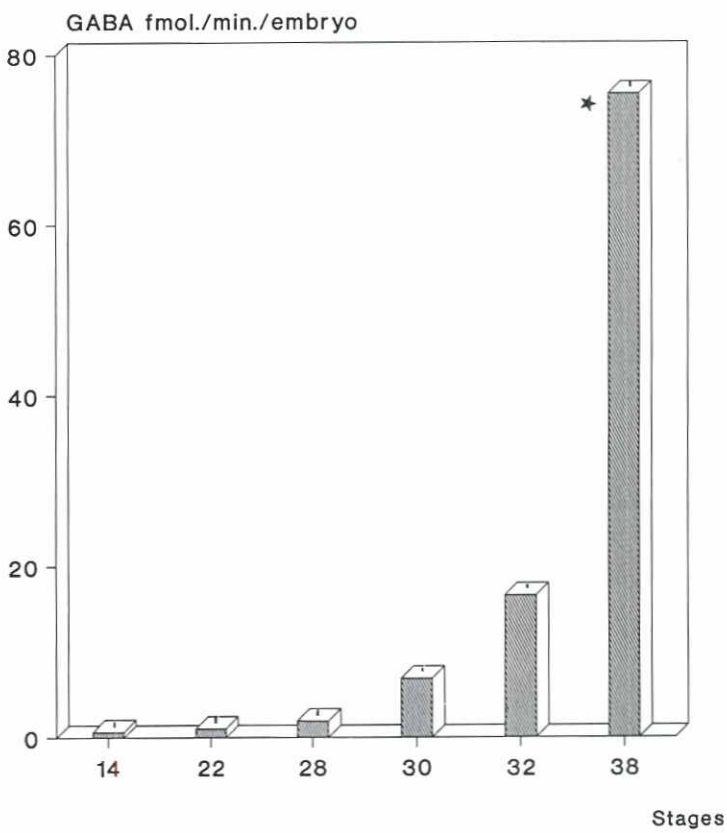

Fig. 4. Appearance and development of GAD activity *in vivo*. *Data are means ± SEM of two sets of experiments carried out on 10 embryos for each point. Stages 14, 28, 32 and 38 correspond respectively to the onset of culture and to 4-, 7-, 14-day-old cultures. *P<0.01, two-tailed, unpaired Student's t test, compared to corresponding cocultures (14-day-old).*

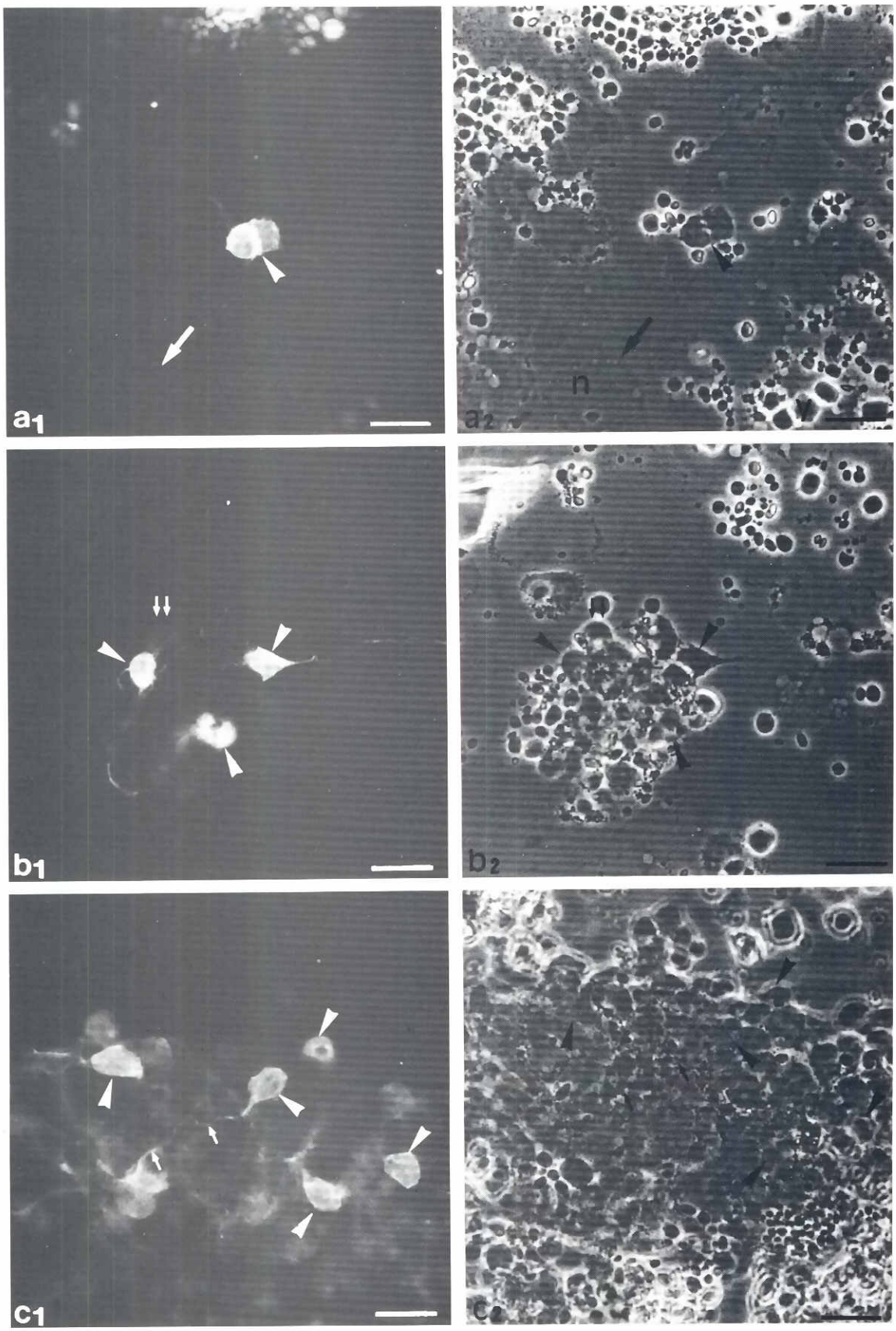

Fig. 5. GABA immunoreactive neurons differentiated *in vitro*. (a1) *Two isolated neuronal cells (➤) display brillant immunostaining throughout their cell bodies and neurites. Non-neuronal cells are negative (➡). (a2) Phase contrast. n : nuclei of non-neuronal cells; y: yolk.* **(b1)** *Three immunopositive neurons visualized in a small cluster (➤). An intense fluorescence is observed both in the cell bodies and in neuritic fibers. Note the presence of negative neurons (→).* **(b2)** *Phase-contrast.* **(c1)** *GABA-like immunoreactive neuronal subpopulation dispersed in a large immunonegative neuronal cluster. Cell bodies (➤) and neuritic processes (→) are fluorescent.* **(c2)** *Phase-contrast. Bar, 20 μm.*

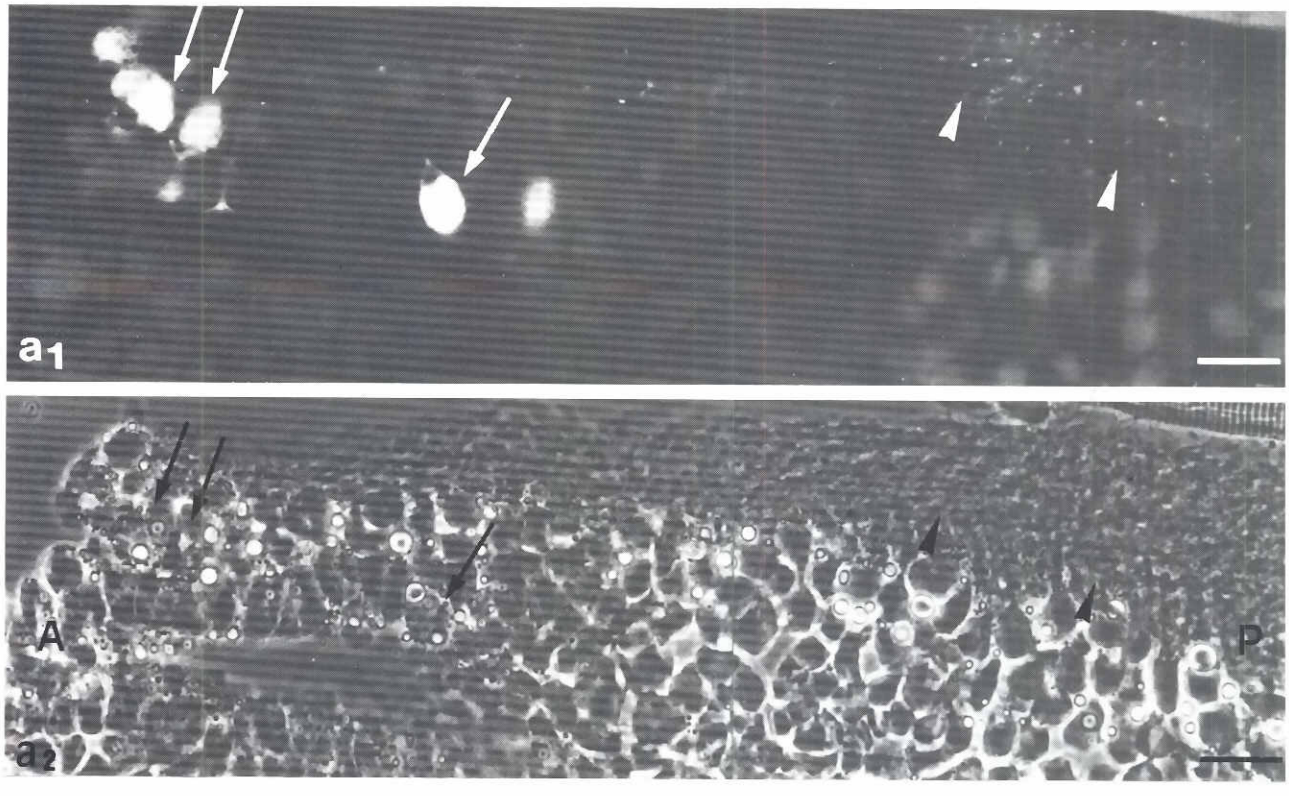

Fig. 6. GABAergic expression in an embryo at stage 38. *Immunohistochemical visualization of GABAergic neurons on oblique sagittal frozen sections in the spinal cord.* **(a1)** *Note the bright fluorescence observed both in some neuronal cell bodies (➡) and in neuritic fibers (➢) dispersed in the white matter.* **(a2)** *Phase-contrast. A: anterior part; P: posterior part. Bar, 20 μm.*

showed that during development of the retina in *Xenopus laevis*, the ability of cells to take up GABA precedes their capacity to synthesize it. Likewise Guerinot and Pessac (1979) demonstrated that the differentiation of the GABA uptake system occurs prior to the development of neurotransmitter synthetic ability in dissociated cell cultures from chick embryo neural retina. Lam *et al.* (1980) have also found that neurons in the rabbit retina possess a mechanism for GABA uptake before they contain appreciable levels of GAD and GABA.

A quantification of GABAergic neurons present in the different types of cultures (cocultures, NP and NF cultures) was carried out to determine whether the enhanced GABAergic metabolism observed in cocultures was due to a numerical increase of the GABAergic neuronal subpopulation or whether it was the consequence of a stimulation of GABAergic expression per neuron. We showed that isolated NP cells plus isolated NF cells developed approximately half as many neurons as did cocultures. This clearly indicated that there is an increase in the number of the GABAergic neuronal subpopulation in the presence of chordamesodermal cells. This observation supports the idea that after neural induction chordamesoderm provides a further environmental stimulating influence for neuronal differentiation.

The nature of the factors involved, and the possible role of cell-cell or cell-substratum contacts in the development of the mature phenotypes remain to be elucidated and to this end, studies are now in progress.

An important aspect of neuronal development that has not been addressed in these studies is the time of cessation of mitotic activity in the neuronal cells. It is generally thought that mitotic activity ceases prior to the development of specific neuronal properties. A precise identification of the stages when the GABAergic neuronal subpopulation becomes post-mitotic will be carried out in further studies.

In conclusion, our experiments carried out on amphibian neuronal precursors from late gastrula neurectoderm demonstrate that neuroblasts have already acquired the capacity to express GAD activity and biosynthesize, accumulate and take up GABA *in vitro*.

By immunovisualization we showed that GABAergic neurons in culture occurred isolated on the substratum or over non-neuronal epithelial or mesenchymal cells, or dispersed among small neuronal clusters or in the midst of much larger neural aggregates.

The results described here, together with previous data already reported on cholinergic, dopaminergic, noradrenergic, peptidergic differentiation, strongly suggest once again that at the late gastrula stage, the neuronal precursor population does not constitute a homogeneous set of cells. The embryonic origin of this neuronal heterogeneity remains to be determined. It will now be of interest to know

whether this neuronal heterogeneity is a direct result of the process of neural induction itself, with stochastic phenomena being involved or not at the cellular level, or whether it reflects a pre-existing heterogeneity in the presumptive ectoderm, target tissue of neural induction.

Materials and Methods

Eggs were obtained by natural matings of *Pleurodeles waltl* and staged according to Gallien and Durocher (1957).

Cell cultures

Cell cultures were performed from embryos at the late gastrula stage (stage 13). As previously described (Pituello *et al.*, 1989), the NP and the NF are well visualized and clearly delimited at this stage, and it is therefore possible to easily separate the two territories.

After removal of the jelly coat and vitelline membrane, the NP and/or the NF were excised with or without the underlying CM (Fig.1), using platinum threads mounted on stalk.

The appropriate tissues were then dissociated in Ca^{++}/Mg^{++}-free Barth's solution (88 mM-NaCl, 1 mM-KCl, 2.4 mM-$NaHCO_3$, 2 mM-Na_2HPO_4, 0.1 mM-KH_2PO_4, 0.5 mM-EDTA, pH 8.7), and the embryonic cells were plated (1.2×10^5 cells/well) on plastic or collagen-coated Nunc multiwell dishes in Barth's saline culture medium (Barth and Barth, 1959) supplemented with 100 I.U. ml^{-1} Penicillin, 100µg ml^{-1} Streptomycin and 0.1% BSA, at 20°C. Following cell attachment and spreading (approximately 48h), the medium was replaced with fresh Barth's medium (without BSA) and the cultures were left to develop for up to 15 days. Over this period, several cell types well identifiable on the basis of specific phenotypic and cytologic features, differentiated (Duprat *et al.*, 1984, 1986):

- Cocultures (neurectodermal cells co-cultured together with chordamesodermal cells, i.e. NP + NF + CM) contained neurons, melanocytes, mesenchymal cells, fibroblasts, ciliated cells, epithelial cells, chordal cells and myoblasts.
- NP cultures showed large neuronal aggregates with thick cables of neurites. Scarce flat cells were also observed.
- NF cultures consisted of neurons isolated or associated in small and loose aggregates among or over flat non neuronal cells. Melanocytes also differentiated. Easily observable is the distinct aggregative behavior of the neurons developing from the NF compared to those from the NP. This observation, analyzed in more detail elsewhere (Duprat *et al.*, 1985a), allows us to easily detect in culture contaminations between the two territories. Thus, when such contaminations were suspected, the culture was systematically eliminated.
- Chordamesodermal cultures contained myoblasts, typical vacuolated chordal cells, mesenchymal and fibroblast cells.

The cell densities that were equal at the initial plating did not remain so throughout the culture period. Indeed, mitosis occurred in the non-neuronal population as well as in an important fraction of the neuronal population (only 25% of the neurons were postmitotic at the beginning of the culture, according to a ^{3}H-thymidine labeling used to define the time of final DNA synthesis).

Assay for GAD activity

GAD activity was assayed on sibling cultures after 0, 4, 7 and 14 days *in vitro*. Cells were scraped off the dishes on ice in cold homogenizing buffer (0.1 M phosphate buffer pH 6.8, 0.2% Triton x100), briefly sonicated and centrifuged for 5 min at 12 000 g at 4°C. The supernatant was immediately processed for the determination of GAD activity by measuring the transformation of the radioactive precursor (^{3}H)-L glutamate into GABA.

Two and 4-µl aliquots were tested in duplicate along with two blanks in which the cell extract was replaced by homogenization buffer. These aliquots were made up to 10µl with 0.15 M phosphate buffer, to which were added 30µl of a cocktail composed of 3µCi L-(3,4-^{3}H)-glutamic acid (NEN) specific activity 41.5 Ci $mmol^{-1}$, 4 mM sodium glutamate, 0.2 mM pyridoxal phosphate, 1mM AET (2-amino ethylisothiouronium bromide), 9µl 0.15 M phosphate buffer and the samples were incubated for 20 min at 37°C. In the latter case, the reaction was linear with time and enzyme concentration. The reaction was then stopped by cooling on ice followed by addition of 10µl of a GABA solution (10 mg ml^{-1}) in electrophoresis buffer (acetic-formic acids, pH 1.9). The samples were stored at -80°C until further processing.

GABA determination by high voltage electrophoresis

Radioactive GABA production was measured after electrophoresis on paper at 4,500 V. until 50% of the labeled substrate was exhausted. After drying the paper, GABA was revealed by spraying with 1% ninhydrin in N butanol and heating for 5 min at 100°C. The appropriate spots were cut out, eluted for 2h in 0.4 ml of 0.1 N HCl and counted in 2 ml Picofluor scintillation liquid, using an Intertechnics-Counter with a 37% counting efficiency for tritium. The results are expressed as fmol GABA/min/explant (lower limit 0.44 fmol) and are means ±SEM of two or more different experiments performed on at least four cultures, i.e. minimum 8 cultures for each time point.

In a parallel series of experiments, GAD assays were performed on whole embryos taken at stage 13-14 for up to 14 days at the same temperature as the dissociated cell cultures (20°C). In this way, it was possible to make quantitative comparisons between the differentiation of GABAergic neurons *in vivo* and *in vitro* over the time period considered. Assay procedure for GAD activity was the same as for *in vitro* experiments.

Immunolocalization

Antibodies

The polyclonal anti-conjugated GABA antibody and its specificity and affinity have been described elsewhere (Seguela *et al.*, 1984; Geffard *et al.*, 1985). This antiserum has been raised in the rabbit. It has already been used to identify GABA-containing neurons in different areas of the nervous system (Gamrani *et al.*, 1986; Buijs *et al.*, 1987; Ontoniente *et al.*, 1987; Eybalin *et al.*, 1988). The corresponding secondary antibody used, goat anti-rabbit-IgG (1/80) conjugated with fluorescein isothiocyanate, was from Nordic, The Netherlands.

Immunocytodetection on cell cultures

Immunocytochemistry was performed using a method adapted from Geffard *et al.* (1985).

All the steps of the immunocytochemical procedure were carried out at room temperature, with the exception of the incubation with the anti-GABA antibodies, which was performed at 4°C. After washing, the cultures were rapidly fixed (1 min) in a freshly prepared mixture of equal volumes of two solutions [solution I: 0.5 M allyl alcohol, 0.1 M Na cacodylate pH 11, solution II: 3.5% formaldehyde, 0.05 M Na metabisulfite (MBN), pH 7.5], pH 10, and then postfixed for 30 min in 3.5% formaldehyde, 0.05 M Na metabisulfite, 0.1 M Na cacodylate pH 7.5. The cultures were carefully washed in 0.05 M/Tris, 0.05 M MBN, pH 7.2 and cells permeabilized with methanol at -10°C for 6 min followed by 0.25% Triton x-100 for 2 min. After a pretreatment with 0.01 M NaBH4, the preparations were incubated with GABA antiserum (1/2000) overnight. They were then washed in 0.14 M NaCl-0.05 M Tris, pH 7.2 and then the goat anti-rabbit-FITC antibody was applied. Finally, the cultures were thoroughly rinsed, mounted in Mowiol 4-88 and examined with a Leitz Dialux microscope equipped with an I_2 filter (BP 355-425, LP 460).

Immunohistochemical detection on embryos

Embryos (stages 14, 22, 28, 30, 32 and 38) were fixed overnight using 3.5% formaldehyde in Barth-Tris solution pH 7.4 at 4°C. After thorough washing for 24h in the same buffer, they were incubated

in a graded series of sucrose solutions (5%, 10%, 15%, 20%, 25%), each for an hour, followed by an incubation overnight at 4°C in a 30% sucrose solution. Frozen sections were prepared and treated for 15 min in 0.25% Triton x-100 and then processed for immunohistochemistry as described above.

Controls

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

Statistics

According to the data, results were examined either with the Student's t test or with a two-way analysis of variance.

Chemicals

Sodium glutamate, pyridoxal-5 phosphate, AET (2-aminoethyl isothiouronium bromide hydrobromide), GABA (gamma-amino butyric acid) were from SIGMA. Acetic and formic acids were from PROLABO. Saline solutions were prepared using MERCK's reagent grade products. Antibiotics were a gift from DIAMANT Lab., France.

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