Int. J. Dev. Biol. 34: 351-364 (1990)

Original Article

Astroglial differentiation from neuroepithelial precursor cells of amphibian embryos: an *in vivo* and *in vitro* analysis

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ABSTRACT Initial development of astroglial phenotype has been studied *in vitro* in an amphibian embryo *(Pleurodeles waltl)*, to document the differentiation potentialities acquired by neural precursor cells isolated at the early neurula stage. In particular, we sought to determine whether interactions between neuroepithelial cells and the inducing tissue, the chordamesoderm, are required beyond this stage to specify precursor cells along glial lineages. Glial cell differentiation was documented by examining the appearance of glial fibrillary acidic protein (GFAp), a specific marker of astroglial lineages. Cells expressing GFAp-immunoreactivity differentiated rapidly, after 48 hours of culture, from cultivated neural plate cells, irrespective of the presence or absence of the inducing tissue. The widespread expression of *Pleurodeles* GFAp protein in neural plate cultures, in which CNS precursor cells develop alone in a simple saline medium, showed that prolonged contact with chordamesodermal cells was not necessary for the emergence of the astroglial phenotype. In addition, the initial development of astroglial phenotype has been defined *in vivo*. The first detectable GFAp-immunoreactivity was visualized in the neural tube of stage-24 embryos, a stage corresponding to 2-3 days in culture, defining radial glial cell end-feet. Thus, dissociation and culture of neural precursor cells did not appear to modify the onset of astroglial differentiation. At stage 32, GFAp-immunoreactivity was observed over the entire length of radial glial fibers and was also evidenced in mitotic cells located in the ventricular zone, suggesting that radial glial cells were not all post-mitotic.

KEY WORDS: *neural development, radial glial cell, gliogenesis, glial fibrillary acidic protein, Pleurodeles waltl*

Introduction

Neurons and glial cells of the vertebrate central nervous system (CNS) are issued from neuroepithelial precursor cells. Within the ventricular zone of the developing CNS, all these precursors proliferate rapidly, as demonstrated by studies of [3H]-thymidine incorporation and have the same morphological characteristics (Schaper, 1897; Fujita and Fujita, 1963). The earliest sign of differentiation in the neural tube is the appearance of neuron-specific markers in a subpopulation of cells withdrawing from the cell cycle, concomitantly with the expression of various markers defining a subpopulation of immature glial cells, the radial glia (Ramon y Cajal, 1909; Rakic, 1971; Levitt *et al.*, 1981, 1983; Tapscott *et al.*, 1981; Hockfield and McKay, 1985). However, mechanisms leading to the segregation of the various neuronal and glial lineages are still largely unknown. In particular, the stage at which cells become specified towards a neuronal or glial fate has not been clearly established. Some neuroepithelial precursor cells could be determined at the early neurula stage, *i.e.* immediately after the process of neural induction. Alternatively, the specification of these cells could occur at later stages, and be dependent upon a prolonged contact of the neural primordium with the underlying inducing tissue, the chordamesoderm.

In our group, several studies have been conducted in amphibian embryos *(Pleurodeles waltl)*, to analyze neuronal potentialities acquired after neural induction by neurectoderm precursor cells and to further define the influence of the chordamesoderm in neuronal differentiation (Duprat *et al.*, 1990a). This model system presents

Abbreviations used in this paper: CC, cocultures of neurectodermal and chordamesodermal cells; CNS, central nervous system; GFAp, glial fibrillary acidic protein; IF, intermediate filament; kDa, kilo-Daltons; NF, neural fold; NP, neural plate; PNS, peripheral nervous system; SDS/PAGE, sodium dodecyl sulfate, polyacrylamide gel electrophoresis.

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0214-6282/90/$02.00

Printed in Spain

the particular advantage of allowing a precise microdissection of neural territories, neural plate (NP), the CNS primitive anlage or neural fold (NF), at the origin of the peripheral nervous system (PNS), at the early neurula stage. Precursor cells can be easily dissociated and cultured in a pure saline medium, totally devoid of hormones or growth factors, to analyze their differentiation potentialities in various environmental conditions, including the presence or absence of chordamesodermal cells. Using this experimental paradigm, we have shown that neuroepithelial precursor cells isolated *in vitro* differentiate into several non-overlapping subpopulations of neurons expressing a variety of neurotransmitter phenotypes, including acetylcholine, catecholamines, GABA (Duprat *et al.*, 1985a, b, 1987, 1990b; Pituello *et al.*, 1989b) and peptides (Pituello *et al.*, 1989a). Thus, chordamesodermal influences that could arise after the early neurula stage do not appear to be instrumental in the emergence of these various neuronal phenotypes. Nevertheless, chordamesodermal cues are not unsignificant since they appear to further stimulate neurotransmitter phenotypes.

In the present study, using glial fibrillary acidic protein (GFAp) as a differentiation marker of astroglial lineages, we have sought to determine whether early precursor cells of the neuroepithelium can also differentiate into glial cells when isolated *in vitro* at the early neurula stage. We demonstrate that cells positive for the glial marker appear rapidly *in vitro*, in both NP and NF cultures, irrespective of the presence or absence of co-cultured chordamesodermal cells. In NP cultures, *Pleurodeles* GFAp was detected in a subpopulation of differentiating neural cells 48 hours after plating, before cells attached to the substratum. In addition, studies *in vivo*, using the same astroglial marker, have allowed us to describe the initial appearance and further development of radial glial cells in the CNS.

Results

Specificity of primary antibodies

Antibody reactivities were analyzed using immunoblotting techniques in cytoskeletal protein-enriched fractions of adult amphibian brains.

The RT97 antibody reacted with a major band at 220 KDa (data not shown) identified as putative neurofilament protein subunit in *Pleurodeles*. Within the limits of molecular weight determination by SDS/PAGE, the size of this protein corresponded well to the 200 kDa heavy neurofilament subunit protein recognized by RT97 in adult *Xenopus* spinal cord (Godsave *et al.*, 1986) and to the 205 kDa protein previously identified as heavy neurofilament subunit protein in *Xenopus* (Szaro and Gainer, 1988b).

The polyclonal antibody directed against mammalian GFAp reacted with a major band at 62 kDa in cytoskeletal protein-enriched extracts of adult brains of *Pleurodeles* (Fig. 1a) and *Xenopus* (Fig. 1b). To ensure that this band corresponded to amphibian GFAp, we performed immunoblotting experiments using XC9D8, a monoclonal antibody directed against an intermediate filament protein antigenically related to GFAp in mammals and specifically expressed in *Xenopus* glial cells (Szaro and Gainer, 1988a). XC9D8 did not react with *Pleurodeles* brain extracts (Fig. 1c). However, in our experimental conditions, this antibody also recognized in *Xenopus* brain extracts a band at 62 kDa (Fig. 1d). Thus, we can conclude that the polyclonal antibody used in this study identifies a GFAp-like protein in *Pleurodeles*.

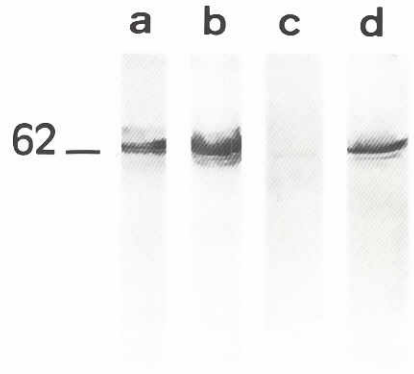

Fig. 1. Immunoblots of cytoskeletal protein-enriched extracts using GFAp-antiserum (lanes a and b) and XC9D8 monoclonal antibody (lanes c and d). *Lanes a and c: adult* Pleurodeles *brain; lanes b and d: adult* Xenopus *brain. Number indicates apparent molecular mass (kDa) of GFAp-like protein. Note that the antiserum stains similar bands on both amphibian species (a and b) whereas XC9D8 stains the glial protein of* Xenopus *(d) but not that of* Pleurodeles *(c).*

Immunohistochemical detection of Pleurodeles-*GFAp* in vivo

Chronological expression of neuronal and glial markers was documented on transverse sections, at various cephalic and truncal levels, of embryos fixed from stage 14 to stage 38 as well as on transverse and longitudinal sections of adult brain.

Stage 14

No labeling was detected with antibodies against GFAp or neurofilaments.

Stage 24

GFAp-like protein was initially identified at this stage in the neural tube. In cephalic and truncal levels, labeling was exclusively localized in small boutons, presumably glial end-feet, regularly spaced along the pial surface, just beneath the external limiting membrane of the lateral part of the neural tube. In the rhombencephalon, some short fine filaments extended from peripheral boutons towards the ventricular surface of the neural tube (Fig. 2A). No labeling was detectable in the retina or in the peripheral structures of the embryos including cells of mesodermal origin.

Stage 28

An increase in both number of stained structures and staining intensity of Pleurodeles-GFAp was observed in the neural tube. Cell processes containing labeled filaments extended radially from the glial end-feet through half to 2/3 of the width of the neuroepithelium (Fig. 2B). As at the previous stage, labeling was localized in the lateral region of the neural tube. Faintly labeled fine filaments, radially oriented, were also observed in the central part of the retina, underneath the vitreal surface. GFAp-immunoreactivity was still undetectable in peripheral structures.

Stage 32

Staining in radial processes extended even further, almost reaching the opposite, ventricular surface of the neural tube (Fig. 3A). Labeled processes were also distributed in dorsal and ventral regions of the neuroepithelium. At this stage, the staining pattern

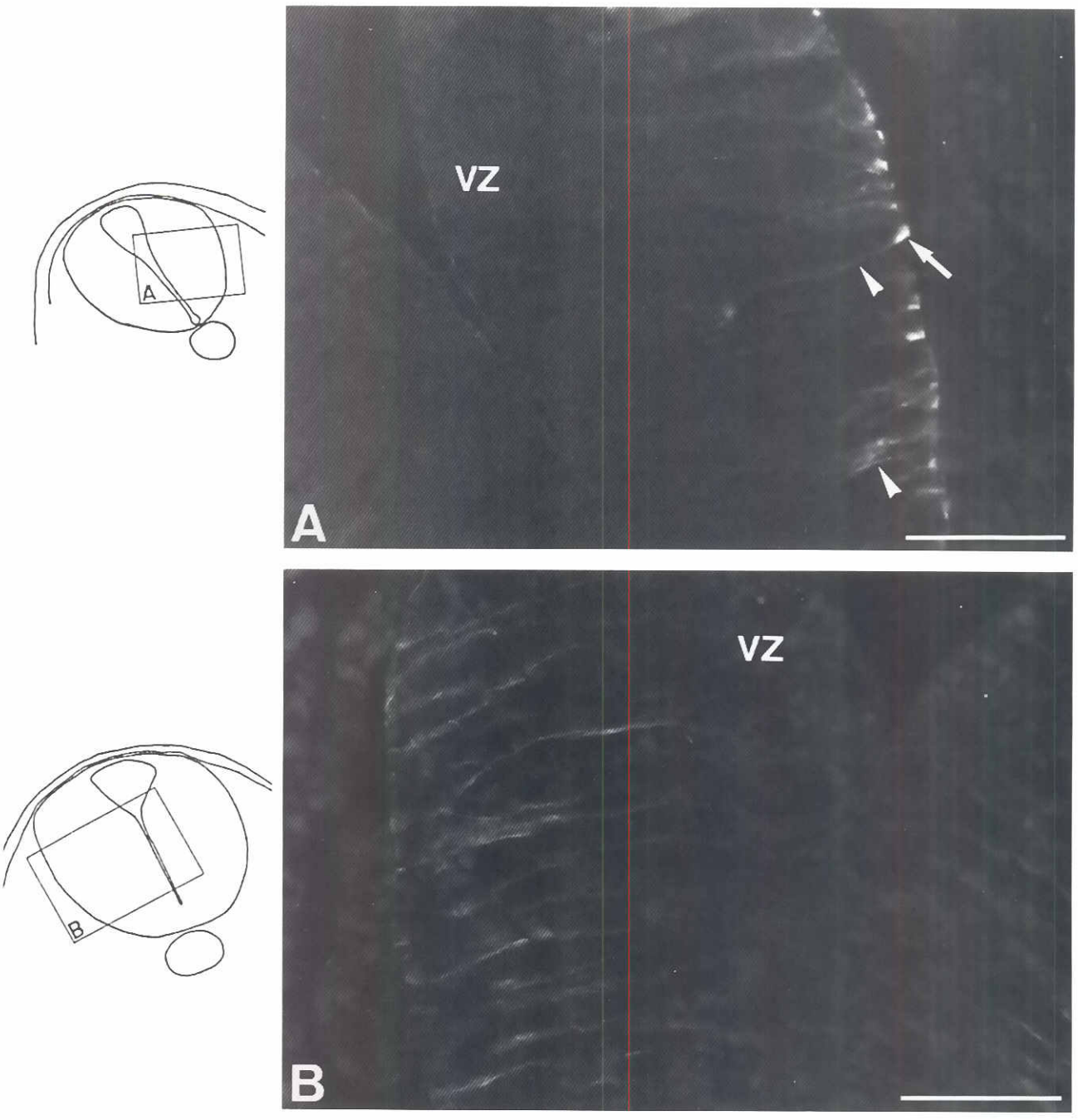

Fig. 2. GFAp-immunoreactivity at early stages of CNS development; transverse sections at the rhombencephalic level of stage-24 and stage-28 embryos; dorsal is at top. *The neural tube areas depicted in micrographs are represented as rectangles in the drawings on the left. GFAp-immunoreactivity is exclusively visualized in neuroepithelium, particularly in end-feet (arrow) of radial glial cells at the pial surface of the neural tube and in the outermost part of some radial processes (arrowheads). Note the absence of staining in the ventricular zone (VZ).* **(A)** *Stage 24, radial processes are very short.* **(B)** *Stage 28, stained processes reach half to 2/3 of the width of the neuroepithelium. Bars represent 50 μm.*

was that of typical radial glial cells, including enlarged glial end-feet beneath the pial surface. Besides, GFAp-immunoreactivity was observed in a small number of spherical cell bodies located in the ventricular zone of the neuroepithelium. In fact, such cells were first observed in the neural tube at stage 30 (data not shown). In some of these cells labeling was faint and diffuse while in others, it was very bright and restricted to a crescent structure at the pole of the cell facing the pial surface (Fig. 3B). A double labeling with a DNA specific fluorescent stain (Hoechst 33258) demonstrated that all of these cells were engaged in mitosis (Fig. 3C). However not all mitotic

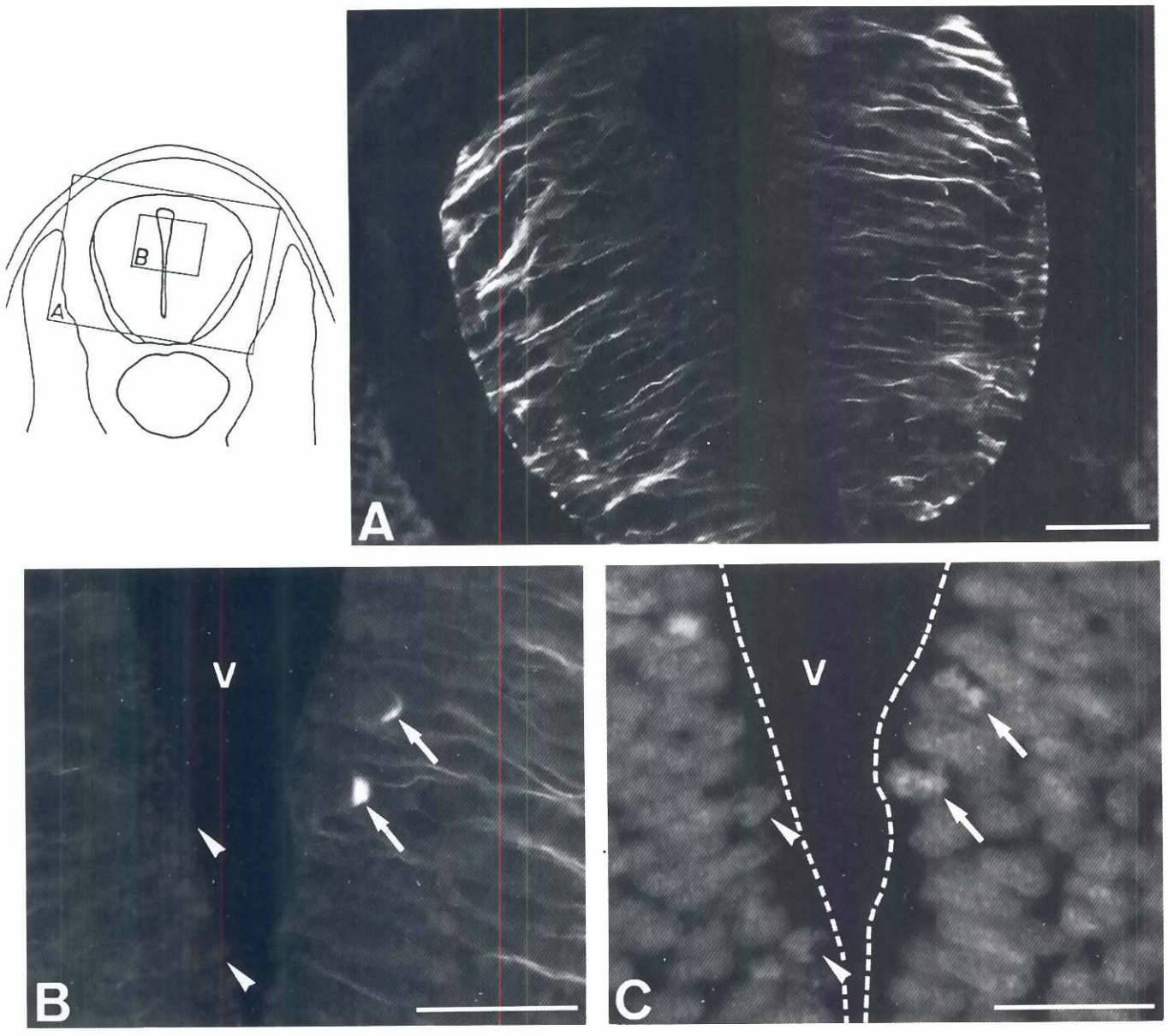

Fig. 3. GFAp-immunoreactivity (A, B) and bisbenzimid staining (C) on transverse sections at posterior rhombencephalic level of stage-32 embryos; dorsal is at top. *Rectangles in inset drawing indicate areas illustrated in A, B and C.* **(A)** *Low power micrograph. Numerous intensely stained radial processes extend now over almost the entire width of the neural tube.* **(B)** *Detail of the ventricular region of the neural tube. In addition to radial processes, the antibody stains two cells (arrows) in the ventricular zone. Fluorescence is restricted to a crescent formation at the pole opposite to the ventricle (V).* **(C)** *The DNA stain demonstrates that the cells labeled in B are dividing (arrows). Note that two other cells also engaged into mitosis on the opposite side of the ventricle (arrowheads) do not exhibit GFAp-immunofluorescence (see B). Bars represent 50 μm.*

cells of the neural tube contained GFAp-immunoreactivity. Cell counts performed on serial sections at various levels of the neural tube showed that GFAp-positive mitotic figures constituted half of the total mitotic cell population in the ventricular zone. This was about 3% of the overall neuroepithelial cell population.

GFAp-staining in the retina was more intense than at stage 28 and extended further internally within radial fibers.

Positive GFAp staining also appeared at this stage in the periphery of the embryo, decorating rounded or ovoid cells. Double immunolabeling experiments of GFAp-antiserum and RT97, a monoclonal antibody directed against the heavy neurofilament protein subunit, indicated the association of these cells with nerve bundles.

Stage 38

The morphological pattern of GFAp-stained structures in the

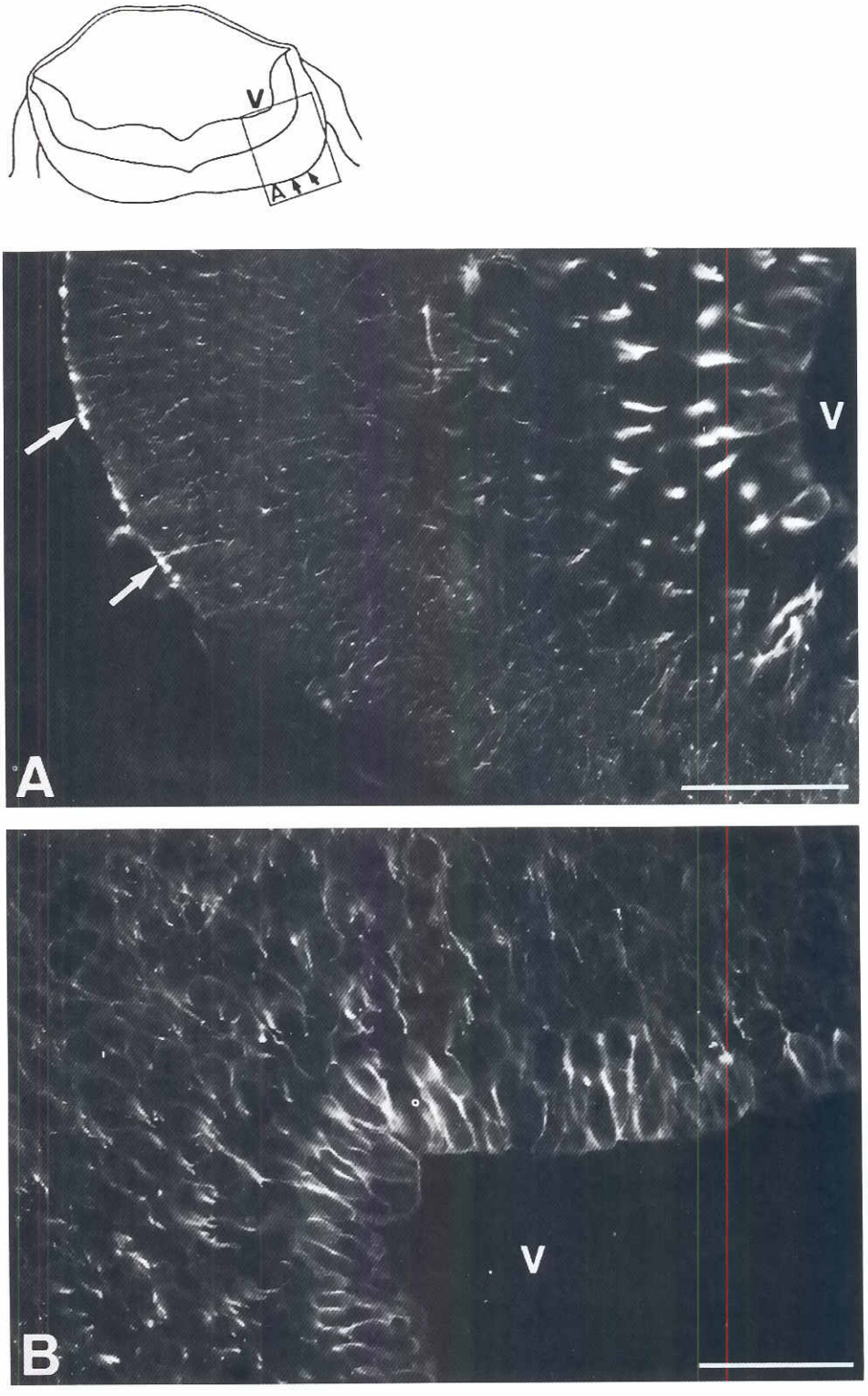

Fig. 4. GFAp-immunoreactivity in transverse sections of rostral cephalic level of stage-38 embryo (A) and in parasagittal section of the adult brain (B). (A) *GFAp-immunoreactive glial cell bodies are now localized underneath the ventricular surface (V). Note the presence of intensely stained endfeet underneath the external limiting membrane (arrows) of brain, dorsal is on the right (see inset drawing for proper orientation).* **(B)** *Somata of radial macrogliocytes lining the lateral ventricle (V) are well visualized. They extend processes towards the pial surface. Bars represent 50 μm.*

neural tube was modified compared to previous stages (Figs. 4A and 5A). In the ventricular zone, GFAp-immunofluorescence was localized at the periphery of rounded cell bodies. Thick and intensely stained filaments emerged from these cells and extended radially within the well individualized intermediate zone, dividing into fine, more faintly stained filaments when entering the marginal zone, easily recognized using RT97 antibody in double-labeling experiments (Fig. 5B). These filaments were less strictly radially oriented

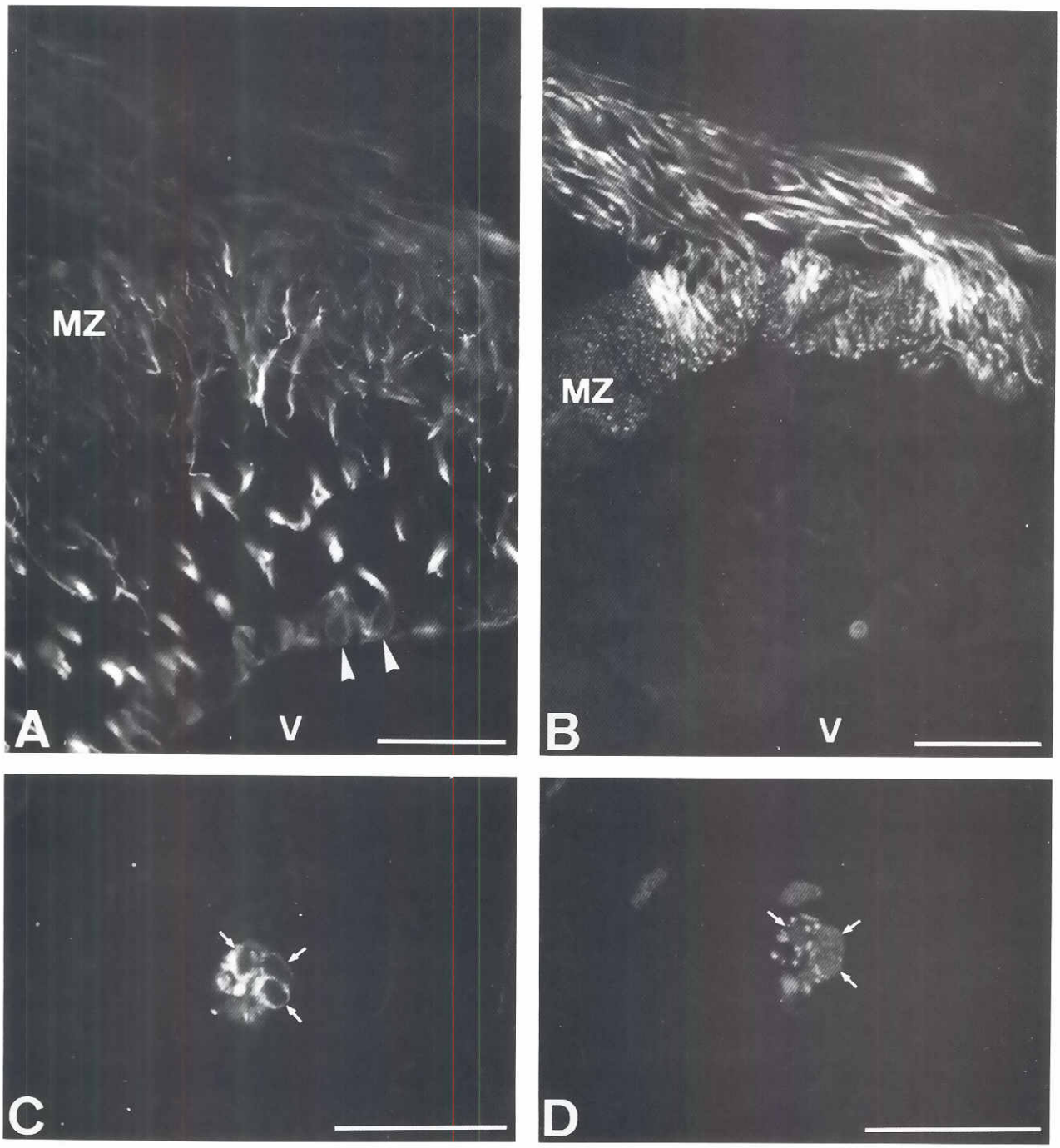

Fig. 5. Simultaneous detection of GFAp (A, C) and neurofilaments (B, D) on transverse sections at rhombencephalic levels of stage 38 embryos. (A-B) *Brain region. Dorsal is on the right of the micrographs. GFAp-antiserum labels macrogliocyte somata (arrowheads) lining the ventricle (V). They extend coarse processes subdividing into fine gliofilaments within the marginal zone (MZ). The marginal zone, containing axon profiles, is clearly identified in B by RT97 labeling. Note in B the emergence of a peripheral nerve with longitudinally arranged axons.* **(C-D)** *Ventral region of the embryo. Three GFAp-labeled cells (arrows in C) are in close apposition to a neurofilament-positive peripheral nerve profile (D). Labeling of cell nuclei by RT97 in D is due to a cross-reactive epitope on histones (Wood* et al., *1985). Bars represent 50 μm.*

and terminated as intensely stained end-feet underneath the external limiting membrane (Fig. 4A). In addition, rounded mitotic cells uniformly labeled with GFAp-antiserum were also found in the ventricular zone of the neural tube. However, they were less numerous than at the previous stage: the mitotic cell population represented only about 0.3% of the total cell population. It is interesting to note that, in contrast to previous stages, all mitotic figures observed expressed GFAp-like protein.

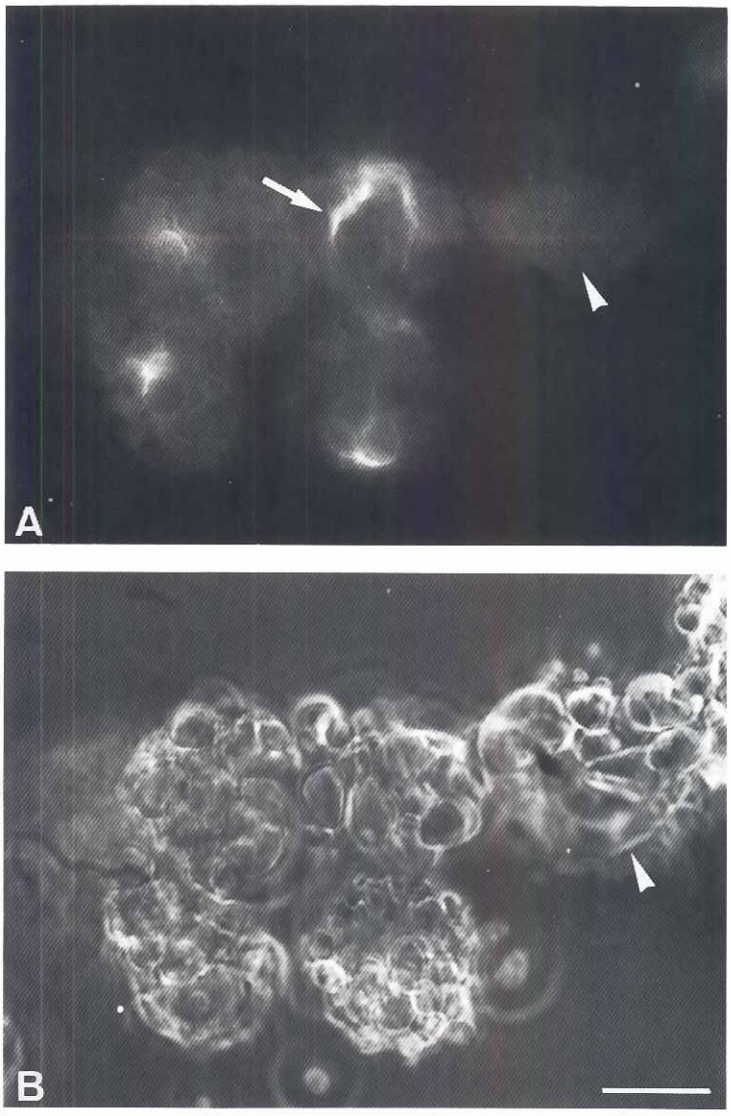

Fig. 6. GFAp-immunoreactivity in a 2-day-old NP culture. (A) *GFAp-antibodies decorate filaments in four neural cells. The stained material is in plane of focus only in one cell (arrow). One cell (arrowheads) appears entirely negative for the glial marker.* **(B)** *Same field viewed in phase-contrast. These early embryonic cells are full of yolk platelets which appear highly refringent. Bar represents 10 μm.*

GFAp-immunoreactivity in the PNS was much more developed than at the previous stage. Intensely stained glial cell bodies and processes were found associated either with peripheral nerves (Fig. 5C and 5D) or with neurons of peripheral ganglia.

Adult brain

GFAp-immunoreactivity defined numerous cells located in the ventricular zone, displaying the typical macrogliocyte morphology previously described in *Pleurodeles* spinal cord (Zamora and Mutin, 1988). Cell bodies and radially oriented proximal processes were intensely colored, whereas ramifying filaments in white matter regions were less stained (Fig. 4B).

Detection of GFAp-immunoreactive cells in vitro

Cultures of isolated neural plate cells

As previously shown, after dissociation and plating, neural plate cells reaggregated rapidly to form large clusters; attachment to the substratum was delayed and took place only after 5 days (Duprat *et al.*, 1985a). Thus, presence of GFAp-like protein was first researched in neural plate cells before they attached to the substratum, *i.e.* from the step of cell dissociation (stage 13) to 4 days after plating. GFAp-immunoreactivity, absent at 24 hours of culture, was detected as soon as 48 hours in the large characteristic neural aggregates. Labeling was restricted to short filaments in cytoplasmic compartment of a subpopulation of cells (Fig. 6). We estimated that GFAp-positive cells represented about 50% of the total neural cell population. After cell attachment to the substratum, GFAp-positive filaments generally adopted a radial repartition and were mostly located in periphery of the neural aggregates; cellular limits of positive cells were not always discernible (Fig. 7A). In addition, fibrillary GFAp-immunoreactivity was also visualized in the cytoplasm of some of the fibroblast-like cells that had migrated out of these aggregates. The morphology of these cells resembled that of protoplasmic astrocytes (Figs. 7B and 7C).

Cultures of isolated neural fold cells

Immunocytochemistry was performed after cell attachment (3

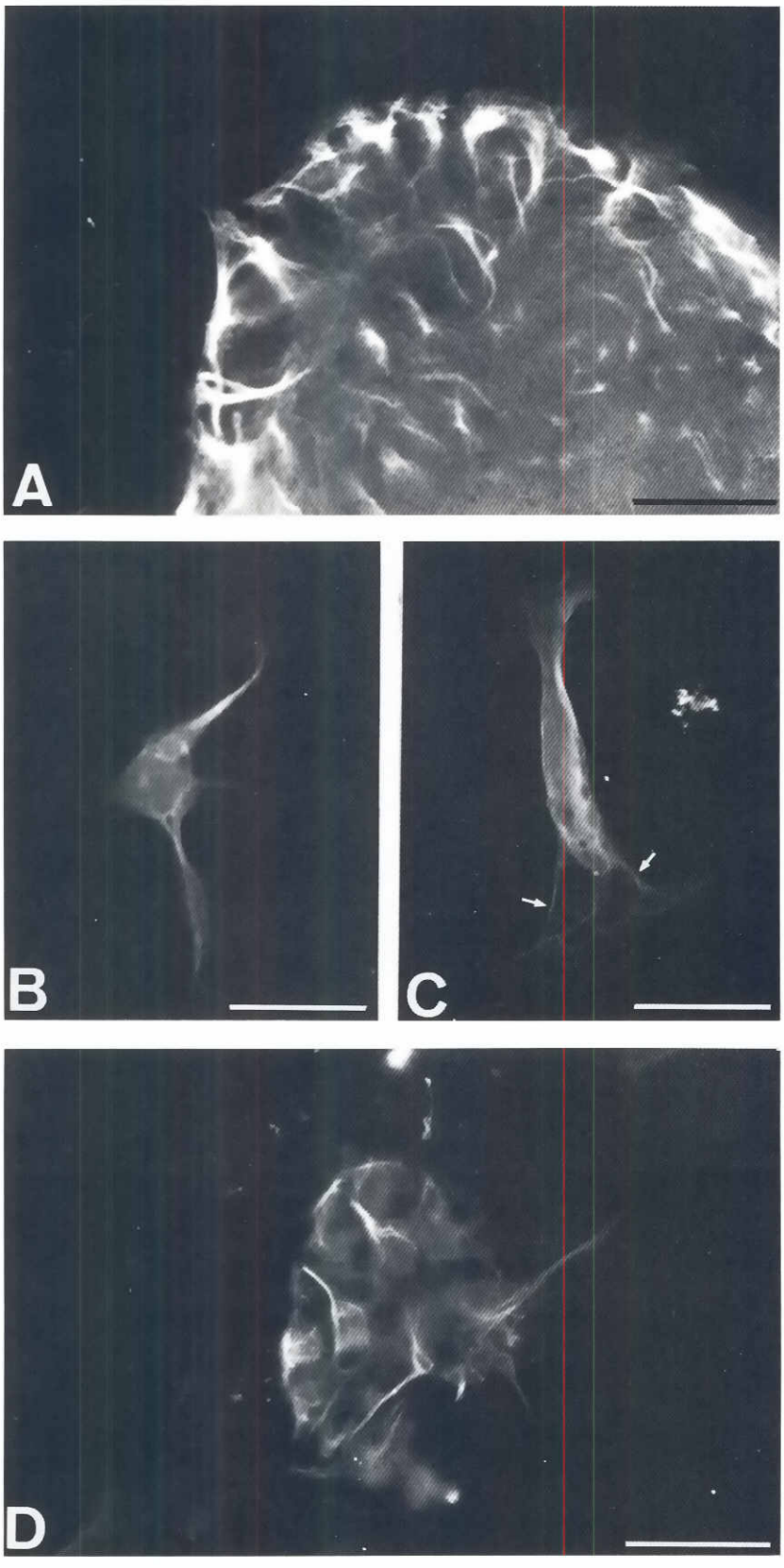

Fig. 7. GFAp-immunoreactivity in NP (A, B, C) and NF (D) cultures. (A) *5-day-old culture. Numerous intensely stained cytoplasmic processes are found at the periphery of a large neural aggregate.* **(B-C)** *7-day-old culture. Two isolated flat cells display GFAp-immunofluorescence. Note in C fine filaments extending in a lamellipodium (arrows).* **(D)** *7-day-old culture. Small cell cluster in which most cells are immunoreactive. Bars represent 50 μm.*

days in culture). GFAp-positive cells were detected in small cellular aggregates, most of which were apparently free of neurons (Fig. 7D). Isolated flat cells, displaying a fibroblastic morphology, were also stained.

Cocultures of neurectodermal and chordamesodermal cells

In these experiments, the entire neural primordium, *i.e.* NP and NF, was cultivated together with chordamesodermal cells. Thus, it was not surprising to find all the different patterns of GFAp-immunoreactivities observed separately in cultures of NP or NF isolated cells, *i.e.* fibrillary staining in small clusters constituted of few cells (Fig. 7A), staining of radial cell processes localized in large neural aggregates (Fig. 7B), and filamentous immunoreactivity in isolated flat cells. We observed no obvious increase in GFAp-like protein expression in cocultures compared to NP and NF cultures.

In all types of cultures, the extent of GFAp-positive structures and the staining intensity increased with time.

***Simultaneous visualization of neuronal and glial phenotypes* in vitro**

Simultaneous detection of *Pleurodeles* neurofilaments and GFAp was performed to document the localization of astroglial cells with regard to neurons in culture. As previously described, initial expression of specific neuronal markers – heavy and light molecular weight neurofilament polypeptides and tetanus toxin binding sites – was correlated with phenotypic differentiation of neurons in culture (Duprat *et al.*, 1986). Likewise, RT97, directed against phosphorylated neurofilament subunits, decorated rounded neuronal cell bodies as well as neuritic processes in the three types of cultures. In NP cultures, neuronal cell bodies were visualized in large aggregates and neurofilament-labeled neuritic processes, often grouped in fascicles, extended from these clusters. In NF cultures, neurons remained isolated or in small aggregates from which a network of fine neurites emerged.

Double immunolabeling experiments showed that, in the large aggregates formed in NP cultures, neuronal cell bodies always intermingled with GFAp-positive cells. In contrast, in NF cultures, we regularly observed small purely neuronal and purely glial cell clusters, in addition to somewhat larger aggregates of mixed phenotype. In cocultures double-stained for neuronal and glial intermediate filaments, the various types of cellular aggregates seen in NP and NF cultures, comprising mixed (Fig. 8) or single phenotypes, were observed.

Discussion

The aim of this work was twofold: i) to determine whether, at the early neurula stage, neuroepithelial precursor cells can differentiate along glial lineages without further cues from the chordamesoderm, and ii) to document *in vivo* the initial appearance of glial cells in the CNS and PNS of the amphibian embryo. Using GFAp, a highly specific marker of cells of astroglial lineages (Bignami *et al.*, 1972; Levitt and Rakic, 1981) as an index of glial cell differentiation, we demonstrate that when explanted *in vitro*, some precursor cells of the neural plate differentiate rapidly into glial cells, in total absence of further chordamesodermal influences.

Specificity of antibodies

The antiserum used in this study was raised against mammalian GFAp. Since class III intermediate filament proteins, GFAp, vimentin and desmin, are closely related, it was necessary to determine whether this antiserum could be used as a reliable glial marker in *Pleurodeles*. In fact, it was especially important to know whether the antibody cross-reacted with amphibian vimentin, since in most vertebrate species, neuroepithelial cells express this intermediate filament at early stages (Tapscott *et al.*, 1981; Cochard and Paulin, 1984; Godsave *et al.*, 1986; Szaro and Gainer, 1988a). Several lines of evidence indicate that this is not the case. On immunoblots of *Pleurodeles* and *Xenopus* brain extracts, the antibody stained bands migrating with similar apparent molecular mass (62 kDa). In *Xenopus*, this band was identified as a GFAp-like protein using monoclonal XC9D8 antibody (Szaro and Gainer, 1988a), whereas vimentin and desmin of lower molecular masses in *Xenopus* (53-57 kDa, Godsave *et al.*, 1986; Szaro and Gainer, 1988a; Dent *et al.*, 1989) were not stained. Furthermore, at all developmental stages studied, intense immunoreactivity was only found in central and peripheral nervous structures. Although myotomes exhibited dull labeling, the antibody did not stain, at any stage, cells of mesodermal or mesectodermal origin, known to contain vimentin (Dent *et al.*, 1989). Therefore, the reagent used in our study demonstrates *Pleurodeles*-GFAp or a closely related molecule.

***Expression of Pleurodeles-GFAp* in vivo**

Our data concerning the adult *Pleurodeles* brain, together with those of Zamora and Mutin (1988) in the spinal cord of the same species, demonstrate the widespread occurrence of GFAp-like protein in radial glial cells throughout the CNS. This also appears to be the case in other amphibian species (Godsave *et al.*, 1986; Miller and Liuzzi, 1986; Szaro and Gainer, 1988a; Messenger and Warner, 1989; Naujoks-Manteuffel and Roth, 1989). In *Pleurodeles* brain, "macrogliocytes" were strongly labeled with anti-GFAp antibodies, and did not appear morphologically different from those described in other species. Most of their cell bodies were located in the ependymal layer, sending a thick process through the gray matter, ramifying extensively into long and fine processes within the white matter which terminated in end-feet at the pial surface. In agreement with the work of Naujoks-Manteuffel and Roth (1989) in *Salamandra salamandra*, displaced gliocytes, with somata situated in the gray matter, were very rarely observed. Thus, radial glia, a transitory embryonic cell type in most brain and spinal cord regions of higher vertebrates (Ramón y Cajal, 1909; Levitt and Rakic, 1981), appears to be the most prominent astroglial cell type in amphibians. It is interesting to note that vimentin, a transitory intermediate filament during glial development in higher vertebrates, is present in adult amphibian gliocytes (Zamora and Mutin, 1988) and thus probably coexists with GFAp-like protein. This further underscores the fact that amphibian astroglial cells remain, in the adult, in an immature state.

Although we did not investigate GFAp-immunoreactivity in the adult PNS, the observation, at stage 38, of intense labeling in cells and processes specifically associated with profiles of peripheral nerves and ganglia indicates that in amphibian as in mammalian species (Jessen and Mirsky, 1980, 1984) *Pleurodeles*-GFAp is also expressed at least in some Schwann and satellite cells. GFAp-immunoreactivity has also been recently reported in putative Schwann cells of *Xenopus* (Szaro and Gainer, 1988a). Thus, GFAp-like protein appears as an excellent marker for various glial cell types in the nervous system of *Pleurodeles*.

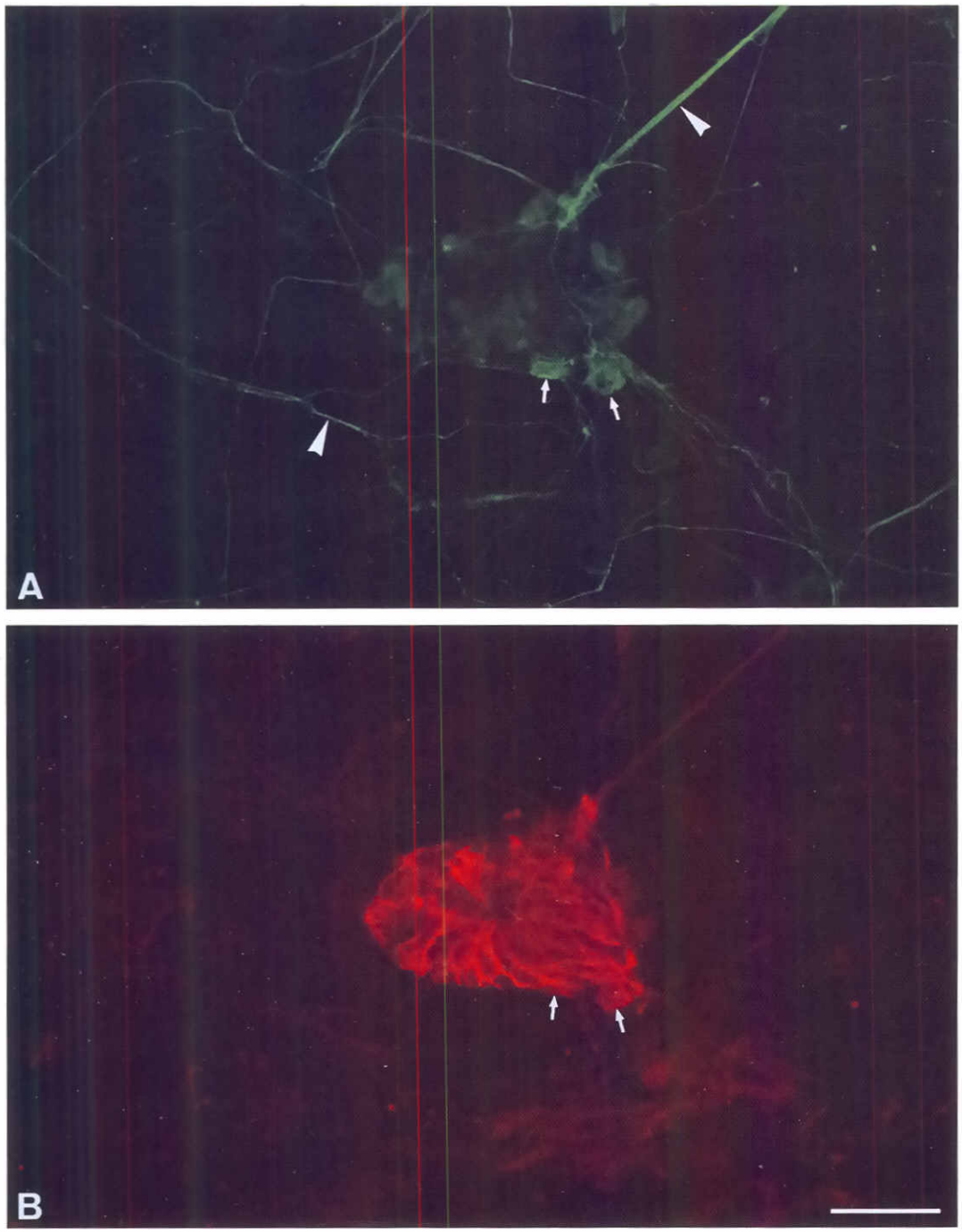

Fig. 8. Double immunolabeling of neurofilaments revealed with a fluorescein conjugate and of GFAp revealed with a rhodamine conjugate in 12 day-old cocultures. Antibodies define a cellular cluster of mixed phenotype. (A) *RT97 stains cell bodies (arrows) and neurites (arrowheads) of neurons localized in the periphery of cellular cluster.* **(B)** *Gliofilaments are radially oriented and intermingled with neuronal cell bodies (arrows). Bar represents 50 μm.*

Our results concerning the initial appearance and development of GFAp-immunoreactivity confirm and extend previous observations. Thus in *Pleurodeles*, as in *Xenopus* and in *Axolotl* (Szaro and Gainer, 1988a; Messenger and Warner, 1989), GFAp-like protein is a relatively precocious marker of radial glial cells although its expression probably follows that of vimentin (Szaro and Gainer, 1988a; Dent *et al.*, 1989).

At stage 38, the morphology of radial glial cells was not much

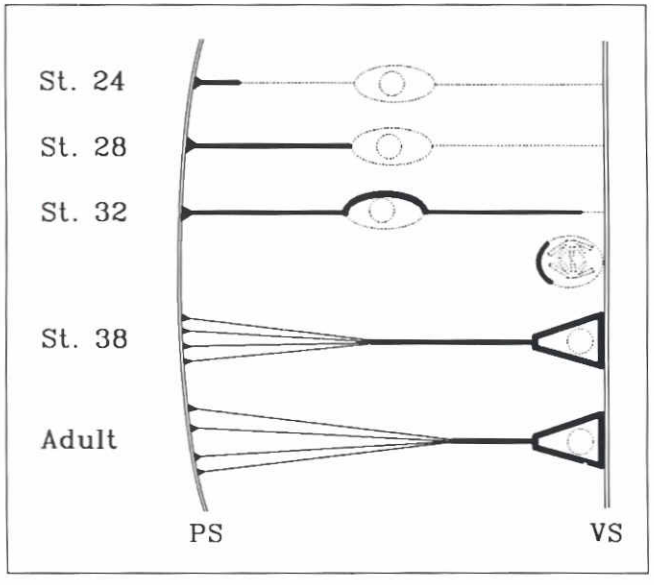

Fig. 9. Schematic drawing recapitulating the localization of GFAp-immunoreactivity in radial glial cells, as evidenced on cross sections of the CNS at the various developmental stages indicated on the left. *GFAp staining is represented as thick and thin solid lines. Dotted lines delineate cell structure. Note that GFAp-immunoreactive mitotic cells, located in the ventricular zone, have been found from stage 30 to 38. PS: Pial Surface. VS: Ventricular Surface.*

different from the morphology we have observed in the adult brain or in the spinal cord (Zamora and Mutin, 1988). Thus the transformation of radial glia from an embryonic morphology to the mature form of the adult radial glia (macrogliocytes) occurs at some time between stages 32 and 38.

It was striking to see that the pattern of expression of *Pleurodeles*-GFAp within radial glial cells is, from the onset, extremely polarized. A recapitulative scheme summarizing our data is presented in Fig. 9. At stage 24, GFAp-immunoreactivity is detectable only at the pial pole of the cell, in typical glial end feet apposed to the external limiting membrane and in the outermost part of the radial fibers. At later stages GFAp-staining extended gradually within radial fibers towards the ventricular surface. Labeling of radial glial cells in their entirety was not seen until stage 32.

Of interest, also, is the observation of GFAp-like protein expression in cells undergoing mitosis, located at the ventricular surface. These cells could either represent newly determined radial glial cells, initiating GFAp expression during a final mitosis as has been described in primates (Levitt *et al.*, 1983), or they could be differentiated radial glial cells still capable of dividing as recently reported in the embryonic murine CNS (Misson *et al.*, 1988). Our observation in some cells of intense GFAp-immunoreactivity exclusively localized in a crescent at the pole of the dividing cell facing the pial surface of the neural tube strongly suggests that these cells are indeed already differentiated radial glial cells having retracted their cytoplasmic process, thus accumulating GFAp-immunoreactive material at this site. On the other hand, in fewer mitotic cells, GFAp-immunoreactivity was faint and uniformly distributed. This could point to an initial synthesis of the intermediate filament protein in newly determined glial cells. At this point of our studies, further experiments are required to draw definitive conclusions about the origin of the GFAp-positive mitotic cells.

Expression of Pleurodeles-*GFAp* in vitro

Glial cell differentiation was also an early event *in vitro*. It occurred in a subpopulation of neural plate cells during the first 48 hours in culture, since these precursor cells, when just plated, do not express the astroglial marker, nor do they show neurofilament (data not shown). After cell attachment, in NP cultures and in cocultures, the radial orientation of some of the immunoreactive cell processes in large neural aggregates may indicate the radial gliocytic nature of the immunoreactive cells. The early appearance of isolated flat cells containing GFAp-immunoreactive fine filamentous arrays suggests that other glial cell types, possibly astrocytes, also differentiate in our cultures. However, astrocyte subtypes (Raff *et al.*, 1983) have not been determined.

It might be of interest to compare the time of onset of GFAp-expression *in vitro* to that observed *in vivo*. This comparison is schematized in Fig. 10. In NP cultures, as pointed out above, GFAp-immunoreactivity was evidenced after 48 hours in culture, a time corresponding to developmental stage 22-23. *In vivo*, GFAp-like protein was already present in radial glial end-feet at stage 24. Thus, initial GFAp-expression in the CNS *in vitro* appears well correlated with the onset of glial cell differentiation *in vivo*.

On the other hand, in NF cultures GFAp-immunoreactive cells were observed 3 days after plating, whereas in the PNS *in situ* the onset of GFAp-expression was found only at stage 32, corresponding to 7 days *in vitro*. Consequently, the appearance of GFAp-

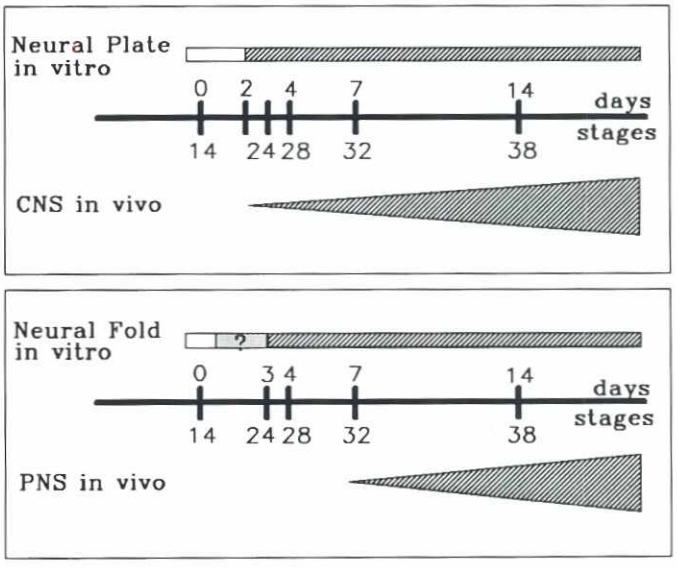

Fig. 10. Comparison of the initial expression of GFAp-immunoreactivity *in vivo* (hatched triangles) and *in vitro* (hatched bars). *The correspondence between* in vivo *stages and days in culture has been established as indicated in the Materials and Methods section. Dotted bar: not determined. Clear bar: absence of GFAp-immunoreactivity. Note that there is a good correspondence in first appearance of the glial marker in neural plate cells* in vitro *and in CNS cells* in vivo *(top panel), whereas the onset of GFAp-immunoreactivity is delayed in the PNS* in vivo *compared to cultivated neural fold cells (bottom panel).*

like protein in NF derivatives differentiating *in vitro* is markedly accelerated, compared to the *in vivo* situation. Several reasons could be invoked to explain this precocious expression in *in vitro* conditions, including changes in the cellular environment of neural fold cells and stimulation of differentiation by the initial dissociation step.

In NP cultures, neuroepithelial precursor cells develop in absence of cells of mesodermal origin, since neural plates were microdissected totally free of contamination by the chordamesoderm. GFAp-expression in these cultures did not qualitatively differ from that observed in cocultures. Furthermore, GFAp-like protein was expressed before cell attachment to the substratum. Therefore, neither further chordamesodermal cues nor cell/substratum interactions are required in our system for astroglial differentiation. In fact, as was predicted by Barth and Barth (1963) in *Rana pipiens*, recent experiments in our group demonstrate that subpopulations of glial cells emerge in cultures of cells dissociated from the amphibian competent presumptive ectoderm, isolated before gastrulation (Saint-Jeannet *et al.*, 1989, 1990). Taken together, these results indicate that the astroglial phenotype can be specified independently of chordamesodermal influences. Nevertheless, it would be of interest to determine whether factors of chordamesodermal origin, which greatly enhance neurotransmitter phenotypic expression (Duprat *et al.*, 1985a, 1987; Pituello *et al.*, 1989b), can also quantitatively stimulate glial differentiation. Emergence of the astroglial phenotype could be the result of an intrinsic developmental program, determined early, possibly before gastrulation (Saint-Jeannet *et al.*, 1989, 1990; Duprat *et al.*, 1990a), in a subpopulation of precursor cells. Such a contention is in agreement with the developmental scheme proposed by Levitt *et al.* (1981), which indicates an early establishment of cellular heterogeneity in the neuroepithelium. Alternatively, astroglial differentiation could be specified through cell interactions between the precursor cells themselves occurring during the first 48 hours of culture, since dissociated neural plate cells reaggregated rapidly after plating.

Experiments devoted to the clonal analysis of the development of cells from both the competent ectoderm and the early neurectoderm are in progress in our laboratory, and may bring new insights in the issue of the state of commitment of CNS precursor cells.

Materials and Methods

Dissociated cell cultures

Neurectoderms were isolated from early neurulae of *Pleurodeles waltl* (stage 14) staged according to Gallien and Durocher (1957). Cell cultures were performed as previously described (Duprat *et al.*, 1985a). Briefly, after removal of the jelly coat and the vitelline membrane, the NP and/or NF were excised with or without the underlying chordamesoderm and dissociated in Ca^{2+}/Mg^{2+} free Barth's medium (Barth and Barth, 1959). Dissociated cells were cultured at 20°C in Barth's solution, on glass coverslips coated with rat tail collagen ($1.2x10^5$ cells per coverslip). Three sets of experiments were carried out:

- Cocultures (CC): neuroepithelial cells cocultured with the underlying chordamesodermal cells.
- Neural plate (NP) cultures: dissociated cells from isolated neural plates.
- Neural fold (NF) cultures: dissociated cells from micro-dissected neural folds. Medium was renewed once, 3 to 5 days after plating.

Correspondence between *in vivo* stages and days in culture was established by raising embryos issued of the same egg laying at 20°C, in parallel to cell cultures. Embryos were staged daily according to the table of development of Gallien and Durocher (1957).

Immunoblotting and immunocytochemical procedures

Primary antibodies

Monoclonal antibody RT97 directed against the phosphorylated 200 kDa neurofilament subunit was provided by Dr. J. Wood (Anderton *et al.*, 1982) and used at 1/400 dilution. XC9D8, a monoclonal antibody directed against *Xenopus* glial fibrillary acidic-like protein (Szaro and Gainer, 1988a) was a gift from Dr. B. Szaro and used undiluted. Rabbit polyclonal antiserum directed against bovine glial fibrillary acidic protein (GFAp) was purchased from Dakopatts and used diluted at 1/100.

Secondary antibodies

- Fluorescein(FITC)-conjugated goat anti-mouse immunoglobulins (GAM-FITC, Nordic, France) used at 1/100 dilution.
- Rhodamine(TRITC)-conjugated goat anti-rabbit immunoglobulins (GAR-TRITC, Immunotech) used at 1/50 dilution.
- Goat anti-rabbit-biotinylated immunoglobulins (Amersham) used at 1/500 dilution.
- Goat anti-mouse-biotinylated immunoglobulins (Amersham) used at 1/500 dilution.
- Streptavidin-horseradish peroxidase conjugated anti-goat antibody (Amersham) used at 1/600 dilution.

Immunoblot analysis

Reactivities of primary antibodies were analyzed on Western Blots (Towbin *et al.*, 1979) of protein extracts separated by sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS/PAGE, Laemmli, 1970). Cytoskeletal-enriched protein extracts were prepared from brains of adult *Pleurodeles* and *Xenopus* according to the method of Szaro and Gainer (1988b). Protein extracts were submitted to SDS/PAGE in reducing conditions and blotted onto nitrocellulose. Individual lanes were treated successively with primary antibodies, appropriate biotinylated secondary antibodies and then with streptavidin-peroxidase conjugated antibody. Reactions were visualized using 4-chloro-1-naphtol and H_2O_2. Control lanes, omitting incubation with primary antibodies, were always included. Background staining of these lanes was negligible.

Immunocytochemical analysis

Immunocytochemistry was first performed on dissociated neural precursor cells of stage 13-14 embryos and on 24- and 48-hour-old cultures, before cell attachment. Cells were suspended in Barth's solution, centrifuged and fixed for 30 min. Cells were subsequently treated as described below except that they were centrifuged between each incubation or washing step. After cell attachment, *i.e.* from 3 to 14 days after plating, cultures were fixed for 30 min. Two kinds of fixations were used:
3.5% formaldehyde in Barth's solution followed by methanol at -20°C for 6 min.

Carnoy's solution (absolute ethanol, chloroform, acetic acid; 60/30/10 v/v). Cells were then permeabilized in Triton-X100 (0.25% in Barth's solution) for 2 min. Next washes were performed in Barth/milk solution (lyophilized skim milk, 1% in Barth's solution) to reduce non-specific labeling.

Double immunolabelings

Cells were incubated with RT97 for 30 min. After 3 x 5 min washes, they were incubated with the secondary antibody: GAM-FITC for 30 min. The GFAp-antiserum was then applied in the same conditions and revealed by 30 min incubation with GAR-TRITC. After washing, coverslips were mounted in Mowiol 4-88 and viewed with an epifluorescence Leitz Dialux microscope equipped with rhodamine (N2) and fluorescein (I2) filter sets. Photographs were taken on Kodak T-Max 400 and on Ektachrome 160 films.

Control coverslips, omitting each primary antibody, were always included in each experiment. None of the secondary antibodies reacted with the inappropriate immunoglobulin.

Immunohistochemistry

Embryos (stages 14, 24, 28, 32 and 38) were fixed with Carnoy's solution for 2h at 4°C. Fixed embryos were embedded in paraffin and cut into

6 μm sections. Immunohistochemistry was then performed on rehydrated sections in the same conditions as those described in the previous paragraph, except that incubations with primary antibodies lasted 1h at room temperature.

Nuclear staining

After immunohistochemistry, sections were incubated for 5 min in an aqueous solution of Hoechst 33258 (bisbenzimid H 33258), (Cesarone *et al.*, 1979) at 0.5 $\mu g.ml^{-1}$ and rinsed in distilled water. The fluorescent dye was visualized using the Leitz D filter set.

Acknowledgments

The authors are indebted to Dr. J. Wood and to Dr. B. Szaro for their generous gift of RT97 and XC9D8 antibodies respectively. We wish to thank Dr. P. Kan for her invaluable help in cell cultures. The excellent technical assistance of F. Foulquier and M.J. Guinaudy and photographic assistance of C. Daguzan are gratefully acknowledged. This work was supported by funds from CNRS, MRES and CNES.

References

ANDERTON, B.H., BREINBURG, D., DOWNES, M.J., GREEN, P.J., TOMLINSON, B.E., ULRICH, J., WOOD, J.N. and KAHN, J. (1982). Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants. *Nature 298:* 84-86.

BARTH, L.G. and BARTH, L.J. (1959). Differentiation of cells of the *Rana pipiens* gastrula in unconditioned medium. *J. Embryol. Exp. Morphol. 7:* 210-222.

BARTH, L.G. and BARTH, L.J. (1963). The relation between intensity of inductor and type of cellular differentiation of *Rana pipiens* presumptive epidermis. *Biol. Bull. 124:* 125-140.

BIGNAMI, A., ENG, L.F., DAHL, D. and UYEDA, C.T. (1972). Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res. 43:* 429-435.

CESARONE, C.F., BOLOGNESI, C. and SANTI, L. (1979). Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal. Biochem. 100:* 188-197.

COCHARD, P. and PAULIN, D. (1984). Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo *in vivo*. *J. Neurosci. 4:* 2080-2094.

DENT, J.A., POLSON, A.G. and KLYMKOWSKY, M.W. (1989). A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus*. *Development 105:* 61-74.

DUPRAT, A.M., GUALANDRIS, L., FOULQUIER, F., PAULIN, D. and BIZZINI, B. (1986). Neural induction and *in vitro* initial expression of neurofilament and tetanus toxin binding site molecules in amphibians. *Cell Differ. 18:* 57-64.

DUPRAT, A.M., GUALANDRIS, L., KAN, P., PITUELLO, F., SAINT-JEANNET, J.P. and BOUDANNAOUI, S. (1987). Review: Neural induction. *Biol. Struct. Morphogen. 75:* 211-227.

DUPRAT, A.M., KAN, P., FOULQUIER, F. and WEBER, M. (1985a). *In vitro* differentiation of neuronal precursor cells from amphibian late gastrulae: morphological, immunocytochemical studies, biosynthesis, accumulation and uptake of neurotransmitters. *J. Embryol. Exp. Morphol. 86:* 71-87.

DUPRAT, A.M., KAN, P., GUALANDRIS, L., FOULQUIER, F., MARTY, J. and WEBER, M. (1985b). Neural induction: embryonic determination elicits full expression of specific neuronal traits. *J. Embryol. Exp. Morphol. 89:* 167-183.

DUPRAT, A.M., KAN, P. and WEBER, M. (1990b). Initial expression of cholinergic traits in embryonic amphibian neuronal cells, choline acetyltransferase and acetylcholinesterase activities. *Ontogenez 5* (In press).

DUPRAT, A.M., SAINT-JEANNET, J.P., PITUELLO, F., HUANG, S., BOUDANNAOUI, S., KAN, P. and GUALANDRIS, L. (1990a). From presumptive ectoderm to neural cells in an amphibian. *Int. J. Dev. Biol. 34:* 149-156.

FUJITA, H. and FUJITA, S. (1963). Electron microscopic studies on neuroblast differentiation in the central nervous system of domestic fowl. *Z. Zellforsch. 60:* 463-478.

GALLIEN, L. and DUROCHER, M. (1957). Table chronologique du développement chez *Pleurodeles waltlii* (Michah.). *Bull. Biol. Fr. Belg. 91:* 97-114.

GODSAVE, S.F., ANDERTON, B.H. and WYLIE, C.C. (1986). The appearance and distribution of intermediate filament proteins during differentiation of the central nervous system, skin and notocord of *Xenopus laevis*. *J. Embryol. Exp. Morphol. 97:* 201-223.

HOCKFIELD, S. and McKAY R.D.G. (1985). Identification of major cell classes in the developing mammalian nervous system. *J. Neurosci. 5:* 3310-3328.

JESSEN, K.R. and MIRSKY, R. (1980). Glial cells in the enteric nervous system contain glial fibrillary acidic protein. *Nature 286:* 736-737.

JESSEN, K.R. and MIRSKY, R. (1984). Non-myelin-forming Schwann cells coexpress surface proteins and intermediate filaments not found in myelin forming cells: a study of Ran-2, A5E3 antigen and glial fibrillary acidic protein. *J. Neurocytol. 13:* 923-934.

LAEMMLI, U.K. (1970). Most commonly used discontinuous buffer systems for SDS electrophoresis. *Nature (London) 227:* 680-685.

LEVITT, P. and RAKIC, P. (1981). Immunoperoxidase localization of glial fibrillary acidic protein in radial glial-cells and astrocytes of the developing rhesus-monkey brain. *J. Comp. Neurol. 193:* 815-840.

LEVITT, P., COOPER, M.L. and RAKIC, P. (1981). Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: an ultrastructural immunoperoxidase analysis. *J. Neurosci. 1:* 27-39.

LEVITT, P., COOPER, M.L. and RAKIC, P. (1983). Early divergence and changing proportions of neuronal and glial precursor cells in the primate cerebral ventricular zone. *Dev. Biol. 96:* 472-484.

MESSENGER, N.J. and WARNER, A.E. (1989). The appearance of neural and glial cell markers during early development of the nervous system in the amphibian embryo. *Development 107:* 43-54.

MILLER, R.H. and LIUZZI, F.J. (1986). Regional specialization of the radial glial cells of the adult frog spinal cord. *J. Neurocytol. 15:* 187-196.

MISSON, J.P., EDWARDS, M.A., YAMAMOTO, M. and CAVINESS, V.S. (1988). Mitotic cycling of radial cells of the foetal murine cerebral wall: a combined autoradiographic and immunocytochemical study. *Dev. Brain Res. 38:* 183-190.

NAUJOKS-MANTEUFFEL, C. and ROTH, G. (1989). Astroglial cells in a salamander brain *(Salamandra salamandra)* as compared to mammals: a glial fibrillary acidic protein immunohistochemistry study. *Brain Res. 487:* 397-401.

PITUELLO, F., DERUNTZ, P., PRADAYROL, L. and DUPRAT, A.M. (1989a). Peptidergic properties expressed *in vitro* by embryonic neuroblasts after neural induction. *Development 105:* 529-540.

PITUELLO, F., KAN, P., GEFFARD, M. and DUPRAT, A.M. (1989b). Initial GABAergic expression in embryonic neuroblasts after neural induction. *Int. J. Dev. Biol. 33:* 445-453.

RAFF, M.C., ABNEY, E.R., COHEN, J., LINDSAY, R. and NOBLE, M. (1983). Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface ganglioside, and growth characteristics. *J. Neurosci. 3:* 1289-1300.

RAKIC, P. (1971). Neuro-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electron microscopic study in Macacus rhesus. *J. Comp. Neurol. 141:* 283-312.

RAMÓN Y CAJAL, S. (1909). *Histologie du système nerveux de l'homme et des vertébrés*, Vol.1 (Ed. A. Maloine). Reprinted by Consejo Superior de Investigaciones Cientificas, Madrid, 1952.

SAINT-JEANNET, J.P., HUANG, S. and DUPRAT, A.M. (1989). Target cell contacts and neural commitment in *Pleurodeles waltl. Cell Differ. Dev. 27 (Supp.):* 52 .

SAINT-JEANNET, J.P., HUANG, S. and DUPRAT A.M. (1990). Modulation of neural commitment by changes in target cell contacts in *Pleurodeles waltl. Dev. Biol. 141* (In press).

SCHAPER, A. (1897). The earliest differentiation in the central nervous system of vertebrates. *Science 5:* 430-431.

SZARO, B.G. and GAINER, H. (1988a). Immunocytochemical identification of non-neuronal intermediate filament proteins in the developing *Xenopus laevis* nervous system. *Dev. Brain Res. 43:* 207-224.

SZARO, B.G. and GAINER, H. (1988b). Identities, antigenic determinants, and topographic distributions of neurofilament proteins in the nervous systems of adult frogs and tadpoles of *Xenopus laevis*. *J. Comp. Neurol. 273:* 344-358.

TAPSCOTT, S.J., BENNETT, G.S., TOYAMA, Y., KLEINBART, F. and HOLTZER, H. (1981). Intermediate filament proteins in the developing chick spinal cord. *Dev. Biol. 86:* 40-54.

TOWBIN, H., STAEHELIN, T. and GORDON, J. (1979). Electrophoretic transfer of

proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA 76:* 4350-4354.

WOOD, J.N., LA THANGUE, N.B., MCLACHLAN, D.R., SMITH, B.J., ANDERTON, B.H. and DOWDING, A.J. (1985). Chromatin proteins share antigenic determinants with neurofilaments. *J. Neurochem. 44:* 149-154.

ZAMORA, A.J. and MUTIN, M. (1988). Vimentin and glial fibrillary acidic protein filaments in radial glia of the adult urodele spinal cord. *Neuroscience 27:* 279-288.

Accepted for publication: June 1990