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# What mechanisms drive neural induction and neural determination in urodeles?

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**ABSTRACT In our laboratory we use Urodeles *(Pleurodeles waltl)* and Anurans *(Xenopus laevis)* to perform comparative studies on neural determination. Urodeles are a good embryological system to study early events in ontogenesis since they present several advantages: slow development, external localization of chordamesoderm at the beginning of gastrulation, large size of cells, diploid genome, etc. I have focused this overview-report on the main findings on *Pleurodeles* neurogenesis. The determination of the two neural lineages (neuronal and astroglial) appears during gastrulation as a consequence of (a) permissive event(s) activated through a $Ca^{++}$-dependent transducing pathway. This signaling-pathway involves L-type $Ca^{++}$ channels. The activation of this $Ca^{++}$ transduction route is sufficient to activate both neuronal and glial structural specific genes, via direct activation of "immediate early genes". The specification of neuronal functional differentiation depends on additional factors of chordamesoderm origin acting during gastrulation and later on. At the early neurula stage, in the neural plate, 20% of progenitor cells present a neuronal fate, 80% are at least bipotential and generate mixed clones (neurons and astrogliocytes). The issue of the state of "commitment" of the precursor cells (competent ectoderm) and the identification of specifying molecules (from Spemann organizer) are underway in *Pleurodeles* and *Xenopus*.**

KEY WORDS: *neural induction, planar signal induction, neuronal determination, astroglial determination, calcium*

## Introduction

In amphibians it is now well known that the induction of neural fate in target ectoderm during gastrulation (Spemann and Mangold, 1924) depends on complex tissue interactions and involves extracellular signal(s) from the dorsal chordamesoderm (Spemann's organizer), the microenvironment, and cell-cell contacts (see Nieuwkoop *et al.*, 1985; Saxén, 1989; Gilbert and Saxén, 1993; Ruiz i Altaba, 1994; and see Holtfreter, 1945; Barth and Barth, 1974; Grunz and Tacke, 1989; Saint-Jeannet *et al.*, 1989, 1990). It is also well understood that the temporally limited competence of the target tissue to be neuralized highlights its importance in neural commitment and suggests that this ectoderm already has some inherent propensity for neuralization (Holtfreter, 1945; Saint-Jeannet *et al.*, 1990, 1993; Pituello *et al.*, 1991; Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.*, 1994; Moreau *et al.*, 1994; Leclerc *et al.*, 1995).

Recent advances have been made in the identification of molecules and in the understanding of mechanisms involved in the patterning of the neural plate of *Xenopus* embryos (for review see Harland, 1994; Kessler and Melton, 1994; Ruiz i Altaba, 1994 and also Otte *et al.*, 1991; Otte and Moon, 1992; Smith and Harland, 1992; Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994). *However, at the present time, the natural inducing molecule(s) remains elusive, and the molecular mechanism(s) of neural induction, from the binding of external signal(s) and transduction pathway(s) to specific neural gene expression, is still poorly understood.*

## What original contributions to this research field do urodeles make?

In our laboratory, we use *Pleurodeles waltl* (Urodele) and *Xenopus laevis* (Anuran) to perform comparative and/or complementary experiments. Gastrulae of *Pleurodeles* are a good, easy and simple experimental biological system in which to study and analyze neural induction mechanisms for a number of reasons:

- at the early gastrula stage, the Spemann Organizer, i.e., chordal and mesodermal area, has an external localization without any underlying contact with ectoderm (see Nieuwkoop this issue; Delarue *et al.*, 1992).

*Abbreviations used in this paper:* NF, neurofilament polypeptides; Tt, tetanous toxin binding sites; GFAp, glial fibrillar acidic protein.

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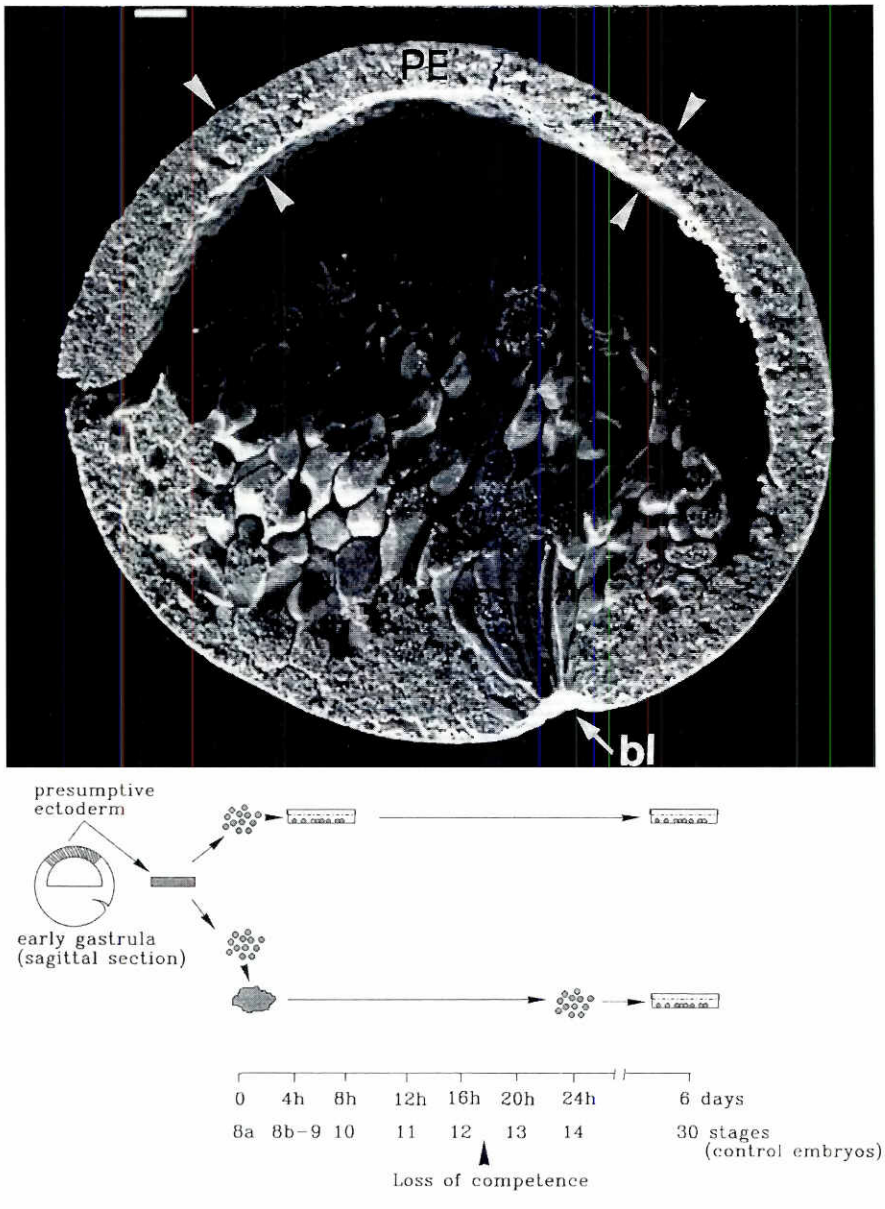


**Fig. 1. Early gastrula of *Pleurodeles waltl* (st. 8a).** *Sagittal section, (above). PE, excised ectoderm; bl, blastoporal lip; bar, 100 µm. Experimental procedure for induction by dissociation and reaggregation of ectodermal cells, below. (Reprinted with permission of Saint-Jeannet et al., 1990).*

- development occurs slowly and the cells are large, easily allowing many aspects of cellular, molecular and ionic processes *in vitro* and *in vivo* to be followed in detail.
- there is no autoneuralization (Duprat *et al.*, 1982).
- the eggs and embryos are diploid, an advantage with regard to *Xenopus* (unfortunately this diploid genome contains about 40 pg DNA with a lot of repetitive sequences. Nevertheless this is not now a problem in the light of modern molecular technology — see Frost-Mason and Frost in this issue).

So, what is known about the mechanisms of neural induction and early neural determination in urodeles?

In this review I will summarize the main findings on *Pleurodeles* neurogenesis.

## Are there neural predispositions in animal cap ectodermal cells before gastrulation?

Some years ago, in agreement with Holtfreter's pioneering observations (1945), we demonstrated with *Pleurodeles*, at the same time as Grunz and Tacke (1989) did so with *Xenopus*, that at the late blastula-early gastrula stages (st. 7-8), dissociation of the competent animal cap ectoderm into isolated cells commits some of them along the neural pathway (Saint-Jeannet *et al.*, 1989, 1990; Duprat *et al.*, 1990). This experimental artifice allowed us to analyze the abilities of these ectodermal cells to express different neural phenotypes in the absence of chordamesodermal cues during gastrulation (Fig. 1).

Using specific neuronal and glial markers (neurofilament polypeptides [NF]), tetanus toxin binding sites (Tt), and glial fibrillar acidic protein (GFAp), we determined whether the two major neural lineages (neuronal and glial lineages) could emerge from the competent ectoderm after induction by dissociation at the late blastula stage. In these experiments all the differentiated cells with neuronal morphology (Fig. 2) stained with one or the other of the neuron-specific immunological markers. Double staining with an anti-GFAp antibody and with a monoclonal neuronal marker (NC1) demonstrated that glial cells and neurons constituted two distinct populations. It was also shown that neural induction observed after dissociation does not reflect a non-specific phenomenon, but is a competence-dependent response.

This direct relationship between induction by dissociation and the competence of the ectoderm was addressed by experiments in which ectoderms were dissociated at various times after their removal at stage 8. The percentage of cultures in which neurons developed decreased with the age of the dissociated explants. From 100% (st. 8) the percentage fell to 15% after 12 h and to 0% after 20 h at 23°C. Also the number of neurons in each culture decreased as a function of the time elapsed between excision and dissociation of the ectoderm (from over 500 neurons per culture to zero after 16-20 h) (Fig. 3).

Reaggregation into a three-dimensional structure immediately after dissociation of cells (15 min) led to a 50-fold reduction in the number of neuronally induced cells. This result is consistent with a role played by cell-cell contacts in the target tissue in eliciting neural induction. Some years ago, Gurdon (1988) reported experiments with regard to mesodermal induction suggesting that a "community effect" was responsible for the expression of the mesodermal phenotype (see Gurdon *et al.*, 1993; Gurdon, 1995). Thus in *Xenopus*, isolated cells of blastula animal caps cultured inside vegetal sandwiches did not express muscle genes, whereas when they were reaggregated in a tridimensional structure, they did. In

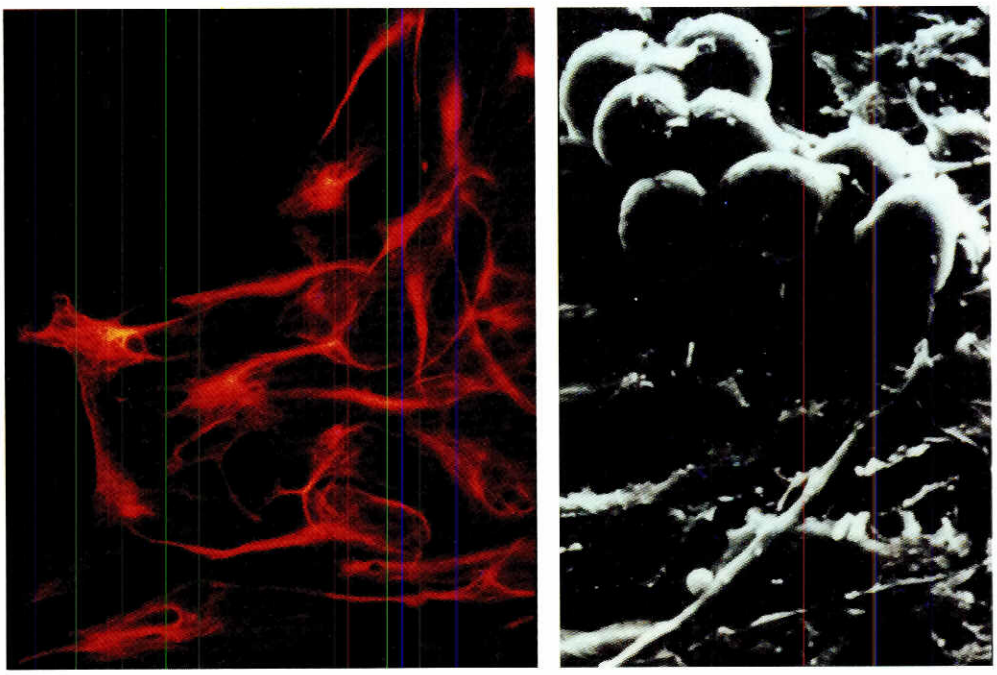

**Fig. 2. The two main neural lineages emerge from competent ectoderm induced by dissociation. (Left)** *Astrogliocytes (GFAp positive).* **(Right)** *Neurons visualized by scanning electron microscopy. These cells are positive for neuronal markers (NC1, NF etc.).*

our experiments, "cell community" appeared to have a negative regulatory effect on the expression of the neural phenotype (Saint-Jeannet *et al.*, 1990).

At that time (1990) we argued that dissociation of competent ectoderm resulted in a commitment of embryonic cells to the neural pathway in a non- definitive and reversible manner, such that most of their epidermal determination was recovered and expressed following the reaggregation step.

With respect to the emergence of the neuronal lineage, an important point concerns the repertoire of neuronal traits expressed after induction by dissociation compared to the repertoire of neuronal phenotypes expressed by neurectodermal cells normally induced during gastrulation and then isolated in culture at the late gastrula stage (without any further influence of chordamesoderm).

Only a limited number of generic neuronal characteristics are expressed after induction by dissociation. Specific morphological neuronal genes (NF, Tt, etc.) are activated without any expression of genes involved in functional traits (genes encoding specific enzymes for biosynthesis of transmitters, transmitters themselves), whereas precursor cells isolated from the neurectoderm immediately after gastrulation are committed to give rise to distinct subpopulations of mature neurons. These neuroblasts differentiate *in vitro* in a simple saline medium (Barth and Barth, 1959) along cholinergic, dopaminergic, noradrenergic, GABAergic and different peptidergic pathways. The neurons that develop acquire in time properties that include the capacity to biosynthesize, store, take up, release or degrade certain of these neurotransmitters.

*These data (natural neural induction versus induction by dissociation) indicate that at the cell and at the gene level, neural induction is not an all-or-nothing event and that the expression of a complete mature neuronal phenotype involves a sequence of inductive events that can be experimentally uncoupled.*

Induction by dissociation could allow the first step of the neural process to occur, with the expression of a subset of specific neuronal genes involved in complete morphological differentiation. Mature functional differentiation needs some other factor(s) or signal(s), probably of chordamesodermal origin, that act during gastrulation (and later during embryogenesis).

Over the last few years, different new dorsalizing factors have been discovered in Spemann's organizer, in *Xenopus* gastrulae: BRACHYURY (Smith *et al.*, 1991), GOOSECOÏD (Cho *et al.*, 1991), X-LIM1 (Taira *et al.*, 1992), PINTALLAVIS (Ruiz i Altaba and Jessell, 1992), NOGGIN (Smith and Harland, 1992), FOLLISTATIN (Hemmati-Brivanlou *et al.*, 1994), CHORDIN (Sasai *et al.*, 1994), Xnr3/Fugacin (Ecochard *et al.*, 1995; Smith *et al.*, 1995).

Some of them (Noggin and Follistatin) can induce embryonic ectoderm to become neural tissue and are expressed *in vivo* in an appropriate temporal and spatial manner. Noggin neuralizes ectoderm in the absence of dorsal mesoderm (Lamb *et al.*, 1993). In the embryo, the normal expression of Noggin in dorsal mesoderm is consistent with its having endogenous neural inducer properties. Follistatin, an activin-binding protein, blocks activin activity. Activin seems to be more than a mesoderm inducer; rather it is a morphogen which distinguishes between epidermal and neural fates and leads also to cell fate determination in all three germ layers (regulated by its concentration) in *Xenopus* (Hemmati-Brivanlou and Melton, 1994). Follistatin could act as a negative feedback signal for activin, blocking the mesoderm (or epidermis)-inducing activity of activin and so allowing neural determination. The appropriate localization of follistatin reinforces its candidacy as an endogenous neural inducer. The neural fate, revealed when the activin signal is abolished, could be in part a "default state."

This concept of a neural default fate can explain the results of cell dissociation experiments and is in agreement with our previous discussion (Duprat *et al.*, 1990): "competent neural ectoderm seems to be a heterogeneous structure with cells presenting distinct neural predispositions (neuronal and glial) that can emerge as a consequence of a permissive (and not instructive) inductive signal."

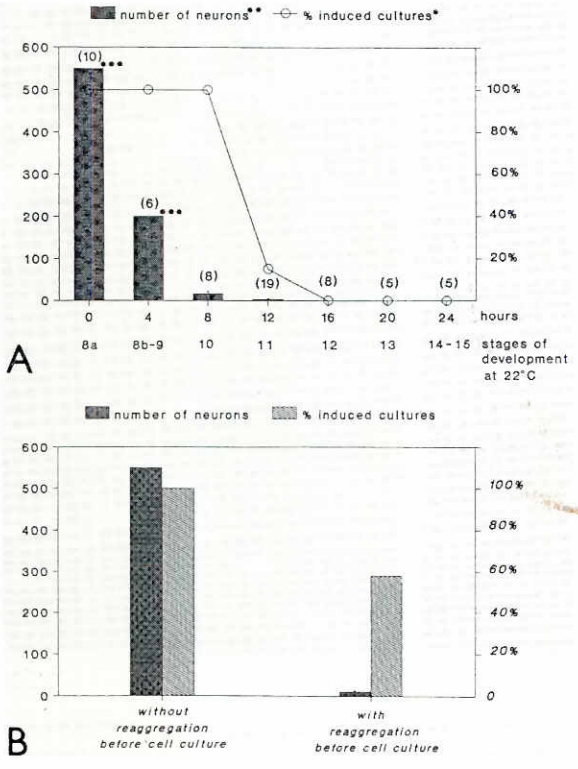


**Fig. 3. Relationship between induction by dissociation and the competence of the ectoderm (A).** *The percentage of induced cultures and the number of neurons per culture were counted. Decrease in the number of induced cultures and of neurons per culture after immediate reaggregation* **(B)**. *(Reprinted with permission of Saint-Jeannet et al., 1990).*

The data obtained after induction by dissociation suggest that early gastrula ectoderm might constitute a heterogeneous cell population presenting distinct predispositions to be neuralized; neural induction without chordamesodermal cues leads to cells of the ectoderm differentiating in culture with the emergence of neuronal and glial lineages.

*What is the molecular basis for the different predispositions of ectodermal cells: a different location of molecular determinants during cleavage stages? A consequence of epigenetic events taking place after the mid-blastula transition, when zygotic genes are activated? A result of planar inductive signals? A stochastic phenomenon? One cannot yet answer. Those questions remains open!*

Neural induction can be brought about by dissociation of ectoderm in many amphibians (Holtfreter, 1945; Godsave and Slack, 1989; Grunz and Tacke, 1989; Saint-Jeannet *et al.*, 1989, 1990; Duprat *et al.*, 1990). Neural commitment can thus occur through a cell autonomous mechanism. The release of ectodermal cells from a repressive effect could be a signal that leads to the development of neural determination as a default state (as in *Drosophila* neurogenesis).

The dissociation-provoked induction procedure provides a suitable model for attempting to screen and identify the factors, probably of chordamesoderm origin, required for the precise and complete determination of neuronal identity.

Neural determination takes place as a result of the activation of specific sets of genes. *What are the molecular mechanisms involved in this inductive process from target plasma membrane receptor(s) or binding site(s) of signal(s) to genes?*

## Mechanism(s) of neural induction in *Pleurodeles*: intracellular signaling transduction via $Ca^{++}$ pathway

It is known from the work of Tiedemann and Born (1978), Born *et al.* (1986), Hemmati-Brivanlou and Melton (1994) that, in *Xenopus*, the neural inducing signal is recognized at the membrane of the target tissue. Identical observations were made on a urodele (Gualandris *et al.*, 1985).

Sater *et al.* (1994) examined ionic signaling during neural induction and demonstrated that dorsal ectoderm of *Xenopus* undergoes an increase in internal pH in response to the natural neural-inducing signal. This result suggests that intracellular alkalinization may participate in gene activation associated with neural induction. Moreover, induction of the target ectodermal tissue toward the neural pathway can also be provoked by modifying the extracellular concentration of divalent cations in *Rana pipiens* gastrulae (Barth and Barth, 1974).

Recently we demonstrated that L-type $Ca^{2+}$ channels are directly involved in the transduction of the neuralizing signal brought on competent animal cap ectoderm (Moreau *et al.*, 1994; Leclerc *et al.*, 1995).

*What could be the role of divalent cations ($Ca^{2+}$) in such an inductive process? What are the mechanisms involved?*

### *Molecular basis of competence*

The results summarized above are consistent with the notion that an extracellular signal, probably acting in a paracrine fashion, is responsible for neural induction. One transducing pathway of this (these) signal(s) appears to involve $Ca^{2+}$ and to be controlled by protein kinase C (PKC; Otte *et al.*, 1989, 1991; Otte and Moon, 1992; Moreau *et al.*, 1994).

In *Pleurodeles*, an *in vitro* stimulation of competent ectoderm by a specific agonist of the L-type $Ca^{2+}$ channel (S-Bay K 8644) triggers an increase in internal cell $Ca^{2+}$ ($[Ca^{2+}]i$), indicating that L-type $Ca^{2+}$ channels are present and functional in the plasma membrane. Using a specific fluorescent probe for these L-type-channels (ST Bodipy-DHP), which enables them to be visualized on living cells, we examined the appearance and the disappearance of these $Ca^{2+}$ channels on the ectoderm surface with respect to acquisition and loss of neural competence. To prevent explants

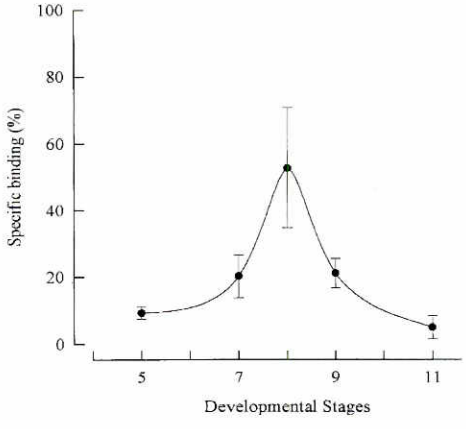


**Fig. 4. Percentage of binding of STBodipy-DHP on animal cap explants of *Pleurodeles waltl* excised at different developmental stages.** *L-type $Ca^{2+}$ channels expression correlates with neural competence. These molecules are at the right place at the right time.*

from rounding up in Holtfreter's medium (Holtfreter, 1933), they were maintained in a gold folding electron-microscopy grid. Fluorescence was detected with a SITS camera, and images were captured at intervals of 5 seconds with 8 bits resolution and processed using the Argus 50 system-Hamamatsu Photonics (for details see Leclerc *et al.*, 1995). We demonstrated that the specific binding was very low at stage 5, i.e., before acquisition of competence. From the midblastula stage onward (stage 6), the percentage of specific binding gradually increased to the late blastula (stage 7), when competence is acquired, and up to the early gastrula (stage 8), corresponding to maximal competence of this target tissue. Subsequently, the decrease and loss of competence correlated well with the decrease in the specific binding of the probe (Fig. 4).

At the early gastrula stage, no significant difference was observed between cells on the dorsal side (from which the nervous system will originate) and cells on the ventral side (which normally produce epidermis, but which can be neuralized at this stage; see Spemann and Mangold, 1924). Cells of the dorsal marginal zone (Spemann organizer), which contribute entirely to chordal and mesodermal derivates, were very weakly labeled with STBodipy-DHP, and endoderm did not bind the probe at all.

These data demonstrate (i) that L-type $Ca^{2+}$ channels are restricted to prospective ectodermal tissue, (ii) that the population of these channels in the plasma membrane increases with time to reach a maximum at stage 8 when neural competence is fully acquired and decreases as competence regresses.

To substantiate the presence and the role of L-type $Ca^{2+}$ channels in early neurogenesis in another amphibian, the kinetics of the expression and the localization of this channel were also described in *Xenopus*. A monoclonal antibody directed against the $\alpha 1$ subunit of this channel, which is a multimeric protein composed of 5 subunits $\alpha 1$, $\alpha 2$, $\beta$, $\gamma$, $\delta$, was used. The $\alpha 1$ subunit carries the ionic pore and binding sites for agonists and antagonists of the channel.

In these experiments, identical results were obtained regarding expression of L-type $Ca^{2+}$ channels and the acquisition and loss of neural competence.

### *Co-localization of L-type $Ca^{2+}$ channel and Go protein*

The results obtained on the pattern of $Ca^{2+}$ channel expression can be compared with earlier results (on *Pleurodeles* and on *Xenopus*) concerning the expression of the transducing molecule Go-protein. This protein is closely associated with neuralization, and the spatial and temporal expression of it correlates suggestively with the state of neural competence (Pituello *et al.*, 1991). Consequently it was of interest to study the respective location of the L-type $Ca^{2+}$ channel ($\alpha 1$ subunit-monoclonal antibody probe) and Go protein ($G\alpha o$-polyclonal antibody probe). Double labeling was carried out on ectodermal cells of *Xenopus* embryos at different developmental stages (st. 8 to 10 1/4). It was found that most of the $\alpha 1$-positive cells were also G-$\alpha$ o-positive and that there was a perfect co-localization of these two molecules (Fig. 5), suggesting the possibility of some interaction between them. The data previously reported show that L $Ca^{2+}$-channels become incorporated into animal cap ectoderm in a progressive manner to reach a maximum at the onset of gastrulation.

With respect to neural competence itself, the molecular basis for this state involves, at least in the cell membrane, some kind of receptor system able to transduce inducing signal(s). L-type $Ca^{2+}$ channels (and maybe Go protein) are good candidates for such a role because of their expression at the right place and time.

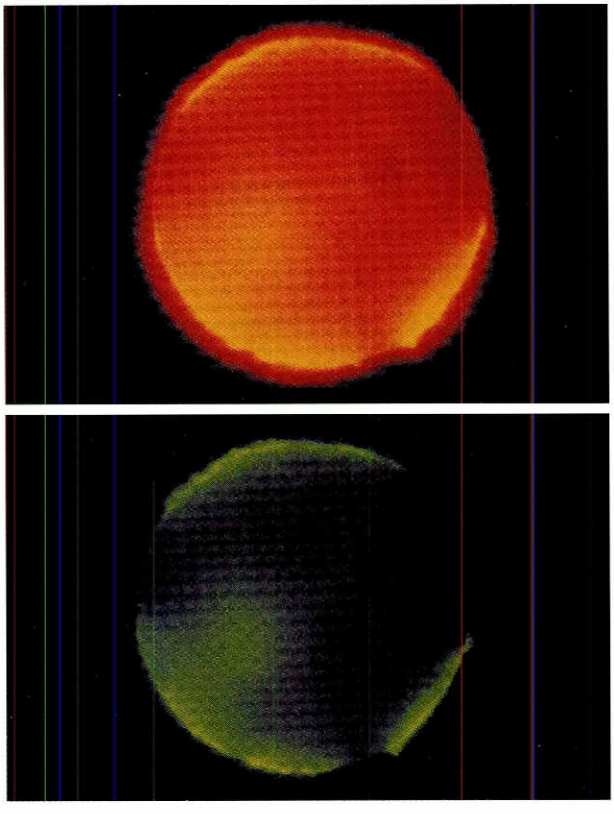

**Fig. 5. Double staining of $G\alpha o$ and L-$Ca^{2+}\alpha_1$ subunits on a competent ectodermal cell *(Xenopus)* shows a perfect co-localization.**

The regulation of the loss of competence is another essential aspect of normal neurogenesis. This loss of competence has been characterized as an autonomous property of cells (Grainger and Gurdon, 1989; Saint-Jeannet *et al.*, 1990; Servetnick and Grainger, 1991). We suggest that in the ectoderm, the loss of neural competence is rather due to the disappearance of the L-type $Ca^{2+}$ channels than to downstream changes in the signaling pathways possibly associated with PKC or Go function. PKC and Go translocations persist in neurectoderm and neural tissue well beyond the loss of competence (Pituello *et al.*, 1991).

Our results further imply that the incorporation of functional L-type $Ca^{2+}$ channels (and Go-protein) in the ectodermal plasma membrane is not a consequence but rather a direct cause of neural competence. This event would apparently be programmed and regulated by a developmentally controlled expression of these channels (transcriptional or/and post-transcriptional regulation?).

### *Increase of internal $Ca^{2+}$ via L-type channels mediates neural induction*

Competent ectoderm of *Pleurodeles* can be induced to differentiate *in vitro* into neural cells by simple activation of the L-type $Ca^{2+}$ channels. Stimulation by the specific L-$Ca^{2+}$ channel agonist S(-)-Bay K 8644 (100 $\mu$M, 30 min, 20°C) is sufficient to induce the differentiation of neuronal and glial lineages. Moreover, *in vitro* neural induction in response to association of the Spemann organizer (blastoporal lip) with dorsal ectoderm (according to Holtfreter's

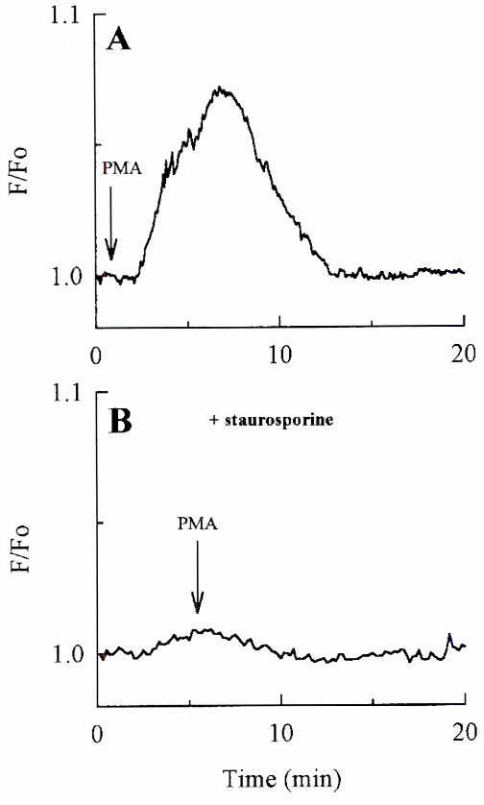


**Fig. 6. Each illustration represents the evolution of $[Ca^{2+}]i$ measured as fluorescence variation of competent ectoderm (st. 8) loaded with fluo-3 AM. (A)** *PMA (500 nm) causes an increase in $[Ca^{2+}]i$.* **(B)** *Inhibition of the effect of PMA by staurosporine (500 nM), an inhibitor of PKC.*

sandwich method) was inhibited when ectodermal cells were loaded with the $Ca^{2+}$ chelator BAPTA (0.4 μM BAPTA-AM), thus blocking $Ca^{2+}$ increase (Moreau *et al.*, 1994).

Previous work on *Xenopus* (Davids *et al.*, 1987; Otte *et al.*, 1988, 1989) has demonstrated that activation of PKC by phorbol esters (TPA) triggers neural induction. Protein phosphorylation by the $Ca^{2+}$/phospholipid dependent protein kinase (PKC) is one of the possible mechanisms of $Ca^{2+}$ channel regulation. PKC has been reported to exert inhibitory or stimulatory effects on L-type $Ca^{2+}$ channels (Shearman *et al.*, 1989; Yang and Tsien, 1993). So the hypothesis was that the inductive effect of phorbol esters might occur via the activation, directly or indirectly, of $Ca^{2+}$ channels.

To test this hypothesis $[Ca^{2+}]i$ was monitored during treatment with PMA, a potent stimulator of different isoforms of PKC. PMA (50 to 500 nm) transiently increased $[Ca^{2+}]i$ in a dose-dependent manner, reaching a maximum in 5 to 10 min and returning to the resting level in 10 to 20 min. In the presence of an inhibitor of PKC (staurosporine, 500 nm) the activation of $Ca^{2+}$ channels by PMA treatment was dramatically decreased. Likewise, 4-PMA, α phorbol ester inactive on PKC, did not trigger significant internal $Ca^{2+}$ increase (Fig. 6). To validate and define the role of PKC on L-type $Ca^{2+}$ channels, we tested the effect of an inhibitor of L-$Ca^{2+}$ channel activity (the antagonist, nimodipine, 10 μM) on the increase of $[Ca^{2+}]i$ triggered by PMA. No increase was detected.

*In conclusion, in Pleurodeles, neural induction involves the activation of L-$Ca^{2+}$ channels which are up-modulated by protein kinase C.* Inductive effects of phorbol esters previously described on *Xenopus* ectoderm (Otte *et al.*, 1988, 1989) may be partly explained by the activation of L-$Ca^{2+}$ channels. Identical results (with $Ca^{2+}$ channel activation regulated by PKC) were also obtained using Concanavalin A as inducer (Con A is an effective inducer for both anurans and urodeles).

The observed time-course of $Ca^{2+}$ increase obtained with Con A was suggestive of the intervention of an internal relay which may be due to a process referred to as *Calcium-Induced Calcium Release* (CICR).

Internal calcium stores can be directly activated by ryanodine or by caffeine, a powerful agonist of intracellular calcium release. In competent ectoderm, caffeine (20 mM) had a pronounced effect on internal $Ca^{2+}$ release, triggering a 30% increase of $[Ca^{2+}]i$, (Fig. 7). Caffeine-treated ectoderm differentiated into neurons and glial cells, confirming the neuralizing effect of raising $[Ca^{2+}]i$. This transitory $[Ca^{2+}]i$ increase (about 20 min) is sufficient to trigger neural induction.

All these data demonstrate that *a rise in internal $Ca^{2+}$, above a threshold value, irrespective of the mechanism by which it is increased within the cell, is sufficient to neuralize competent ectoderm.*

This fact could explain autoneuralization in the axolotl (*Ambystoma mexicanum*). The resting internal $Ca^{2+}$ level in competent ectoderm of the axolotl is higher than that in *Pleurodeles* or in *Xenopus* (there is no autoneuralization in either of these two species). We suggest that a very small change in this $[Ca^{2+}]i$ could

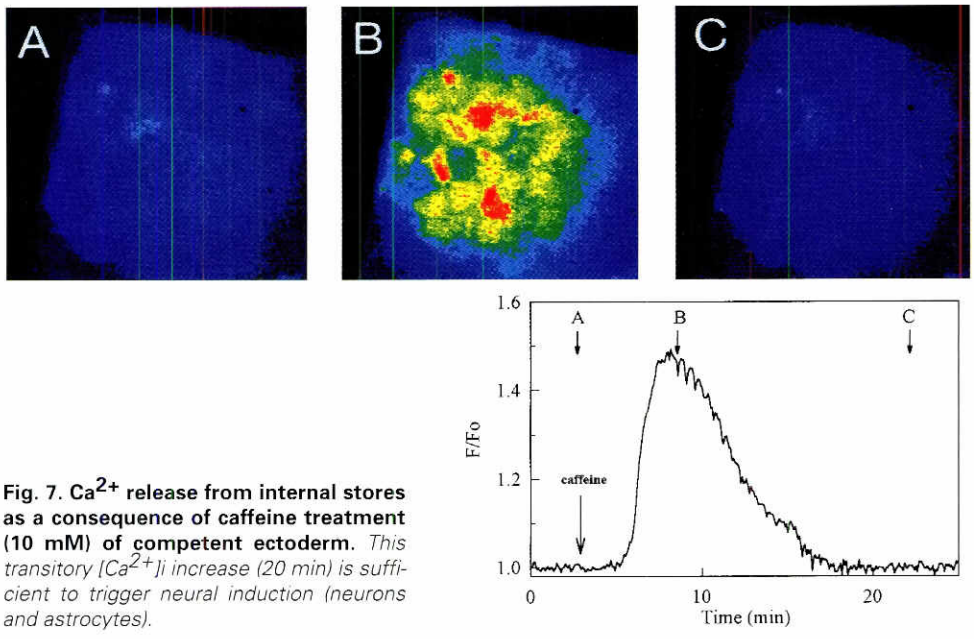


**Fig. 7. $Ca^{2+}$ release from internal stores as a consequence of caffeine treatment (10 mM) of competent ectoderm.** *This transitory $[Ca^{2+}]i$ increase (20 min) is sufficient to trigger neural induction (neurons and astrocytes).*

be enough to exceed the threshold value for neuralization. Thus, a simple removal of animal cap ectoderm from axolotl gastrulae in a saline medium could be sufficient to induce membrane depolarization which might activate voltage-dependent calcium channels and trigger autoneuralization (unpublished results).

In many biological and physiological systems, it is well established that $Ca^{2+}$ changes constitute a common response to external signals (see Berridge, 1993). *Where is the specificity of neural induction which takes place as a result of the activation of specific sets of genes? How might an increase of $Ca^{2+}$ activate specific genes?*

During the differentiation of PC12 cells that results from a stimulation by depolarizing agents, an increase of $[Ca^{2+}]i$ following the activation of L-calcium channels is observed. This $Ca^{2+}$ increase leads rapidly to the activation of IEG (immediate early genes), such as *c-fos* or *Jun-B*. The mechanism by which $Ca^{2+}$ influx activates transcription of *c-fos* or *Jun-B* involves the phosphorylation of transcription factor CREB (a c-AMP response element binding protein) which is directly mediated by calmodulin-dependent protein kinase(s) (Cam-kinase II) (Sheng and Greenberg, 1990; Sheng *et al.*, 1990).

In neural induction, a similar mechanism of CREB phosphorylation and IEG expression following a $Ca^{2+}$ signal is responsible for specific neural gene activation. Preliminary experiments have demonstrated that IEG activation followed an internal $Ca^{2+}$ increase in neural-competent ectoderm (unpublished results).

Sheng and Greenberg (1990) and Sheng *et al.* (1990) have shown that IEG encode regulatory proteins that control expression of the cell genome in response to environmental stimuli. Protein products of IEG might play a crucial role in long-term changes in cell function or determination. Preliminary results indicate that this molecular mechanism is involved in neural induction. It would also be of great interest to ascertain whether Noggin activates L-type $Ca^{2+}$ channels in competent ectoderm (experiments are under way).

## How important is planar induction in urodeles?

Spemann (1938) proposed that neural induction could occur in two ways: "Vertical" induction, due to the involuted dorsal mesoderm, and "planar" induction originating from the organizer region and acting through the plane of the ectoderm layer. This notion of planar induction was abandoned after the experiments of Holtfreter with exogastrulae in urodeles (1933). In these experiments on exogastrulation, the mesoderm undergoes outward movements and does not involute under the ectoderm, which remains empty and has no vertical contact with mesoderm. The Holtfreter's results showed that this ectoderm differentiated into epidermis; there was no neural differentiation after exogastrulation.

Some years ago, the concept of planar induction was reconsidered (using *Xenopus* embryos as a biological model). First, Kintner and Melton (1987) detected N-CAM expression in the ectoderm of exogastrulae. Further, several experiments revealed the expression of various neural markers in exogastrula ectoderm or in cultured explants of dorsal ectoderm associated with dorsal mesoderm such that only planar contact could occur ("Keller sandwiches") (for review see Ruiz i Altaba, 1993; Doniach, 1993). Nevertheless, in such experiments several neural genes failed to be activated (Hemmati-Brivanlou and Harland, 1989; Sharpe and Gurdon, 1990; Dirksen and Jamrich, 1992; Saint-Jeannet and Dawid, 1994; Saint-Jeannet *et al.*, 1994; Taira *et al.*, 1994).

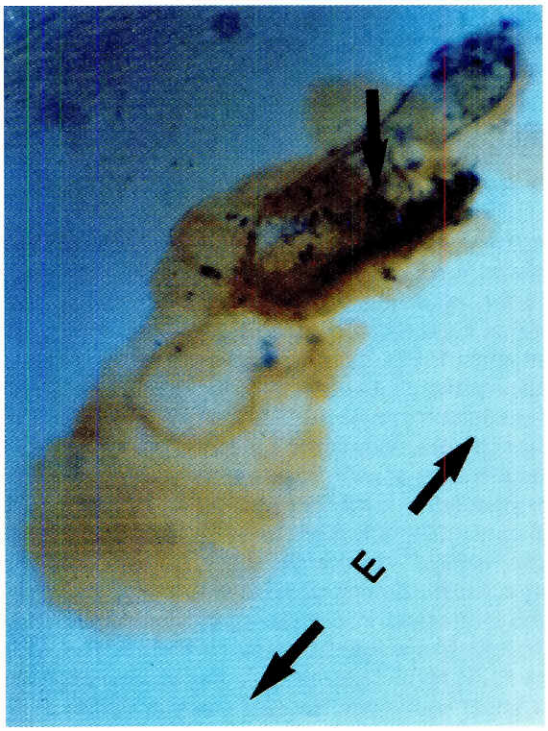


**Fig. 8. Keller sandwich/6 days in culture *(Pleurodeles waltl).*** *Whole-mount immunodetection of neurofilament (brown) and myosin (blue). Only two small neuronal cell clusters (arrow) are differentiated at the contact with chordamesoderm region. No staining is detected in the ectodermal region of the explant (←E→).*

These results obtained in *Xenopus* exogastrulae were discussed with respect to the fact that transient vertical contacts between ectoderm and mesoderm cannot be totally excluded with certainty due to (1) the complex cell movements occurring during exogastrulation, which are incompletely understood, and (2) the internal location of prechordal plate prior to gastrulation in this species (see Nieuwkoop, this issue).

In *Rana pipiens*, Saint-Jeannet and Dawid (1994) demonstrated that dorsal ectoderm differentiated into epidermis and not into neural tissue in blocked gastrulae. Dorsal chordamesoderm could involute laterally to underlie only marginal ectoderm (which was of course induced along the neural pathway).

In *Pleurodeles*, blocked gastrulae or Keller sandwiches did not allow detectable neural differentiation or expression of neural markers such as neurofilaments, specific membrane gangliosides, GFAp, etc. in dorsal ectoderm (Boucaut *et al.*, 1984; Duprat *et al.*, unpublished results). In Keller sandwiches, only neurons and astroglial cells were detected in the ectoderm-chordamesoderm zone, the main ectodermal area remaining of epidermal phenotype (Fig. 8).

*So, it is clear that the relative contribution of planar and vertical signalings vary according to species.* The extent of expression of neural tissue observed when only planar induction is allowed is different in urodele and anuran species *(Triturus torosus, Pleurodeles waltl, Rana pipiens, Xenopus laevis).* This fact highlights once again the importance of comparative studies to analyze and define fundamental and common molecular processes or mechanisms.

An important and difficult question now arises: *What is the relative importance and significance of planar and vertical signalings in normal development in the embryo and in establishment of neural pattern?* Urodeles remain interesting models to address that question.

## Determination of neural lineages

Probably as an immediate consequence of neural induction, two main neural lineages, neuronal and astroglial, differentiate after dissociation of competent ectoderm isolated *in vitro* before gastrulation in the *Pleurodeles* embryo (Duprat *et al.*, 1990; Saint-Jeannet *et al.*, 1990).

In the same way, as a consequence of (natural) neural induction during gastrulation, some neurectodermal cells isolated in culture at the late gastrula-early neurula stage, differentiate into neuronal and into astroglial phenotypes without requiring any subsequent chordamesodermal influences (Duprat *et al.*, 1984, 1985, 1990; Pituello *et al.*, 1990; Soula *et al.*, 1990). Thus, in the differentiated neuronal lineage, cholinergic, dopaminergic, noradrenergic, gabaergic, somatostatinergic, enkephalinergic (leu- and met-enk.) traits are expressed in cultures of isolated neural plate (CNS primitive anlage) and isolated neural fold (origin of neural crest and then the PNS) at the same time as they are *in vivo* . Thus, it was demonstrated that these neurons have acquired the ability to biosynthesize and store, and in some cases to release as well as to take up and degrade the respective neurotransmitters and neuropeptides. These experiments also pointed out that there was no coexistence of different transmitters in neurons, but that distinct neuronal subpopulations were determined. These neurons are completely mature and specified with a status allowing the expression of one transmitter. All the specific genes involved in this functional differentiation are activated and expressed.

For astroglial differentiation, the first detectable expression was at the same time *in vivo* (stage 24, early tailbud) and *in vitro* (2 days in culture) at 20°C (Soula *et al.*, 1990). *In vivo*, in the embryo, most astroglial cells in the CNS appear to develop early (stage 24) and retain throughout life the characteristics of typical radial glia. Different subpopulations of astrogliocytes (radial glial cells, protoplasmic astrocytes) emerge in cultures of cells dissociated from the neural plate or the neural fold, dissected free of contamination by the chordamesoderm, at the late gastrula-early neurula stage.

Emergence of the astroglial lineage could be the result of an intrinsic developmental program, possibly determined before gastrulation (Saint-Jeannet *et al.*, 1989, 1990) and activated during neural induction. Alternatively, we cannot exclude the possibility that astroglial differentiation is specified by cell interactions occurring in culture between the precursor cells themselves.

Clonal analysis *in vivo* and *in vitro* brought new insights to the issue of the state of determination of CNS precursor cells in the neurectoderm.

*What is the fate and the developmental repertoire of the neural progenitors which populate the neural plate immediately after neural induction?*

The possibility that at least some precursor cells of the neurectoderm (and earlier of the competent ectoderm) could already be segregated into separate lineages (neuronal and glial), suggested by experiments and results described above (emergence and rapid differentiation of neurons and astrogliocytes), was examined specifically.

| Site of injection | Mixed clones (●) | Neuronal clones (○) |
|---|---|---|
| Medial | 12/12 | 0/12 |
| Intermediate | 10/17 | 7/17 |
| Lateral | 13/13 | 0/13 |

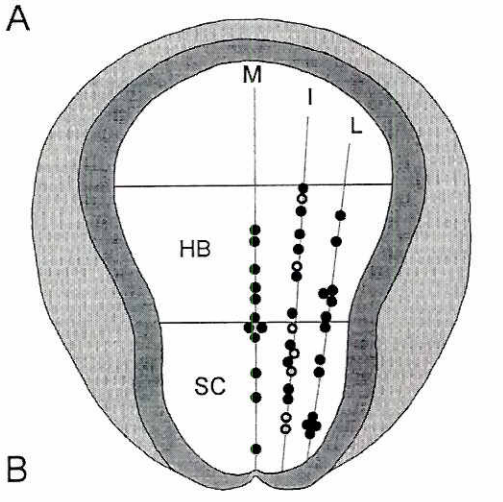


**Fig. 9. The fate map of mixed and neuronal progenitors in the neural plate shows the origin of neuronal cells along the intermediate axis (I).** *Mixed progenitors are found throughout the neural plate area (M, I, L). RB, rhombencephalon; HB, hindbrain; SC, spinal cord. (Reprinted with permission of Soula et al., 1993).*

The *Pleurodeles* embryo was used because (1) neurulation is much slower, allowing manipulations at critical developmental stages over longer periods of time, and (2) *Pleurodeles* neural plate is composed of a single cell layer.

### *Clonal analyses*

Among several cell-cloning strategies, to obtain direct information regarding neural plate cell lineages, iontophoresis (Bronner-Fraser and Fraser, 1988, 1989) was chosen to *in situ* label individual precursor cells in neurulae. Their progeny was then followed *in vivo* (Soula *et al.*, 1993).

At the neurula stage, about 130 cells were labeled in the presumptive territory of the rhombencephalon and of the spinal cord (Fig. 9). Analysis of the clones was performed at different stages of development up to stage 38 (12 days after injection). Phenotypes of labeled cells were identified by positional, morphological and immunocytochemical criteria, using anti-GFAp antibodies as a specific astroglial marker. Clone sizes never exceeded 24 cells, due to a slow rate of division of neural tube cells (maximum 5 generations).

The main result obtained in this study was that about 20% of clones were exclusively composed of neurons, while 80% were mixed clones with both neurons and astroglial cells. Purely astroglial clones were never observed. Of interest is the fact that the range of neuronal clone sizes (3 to 13 cells) was smaller than that of mixed clones (6 to 24 cells).

The position of each labeled cell in the neural plate was precisely recorded at the time of injection and a fate map was

made. It was very striking to observe that the precursors of neuronal clones were exclusively located along the intermediate axes, whereas precursors of mixed clones were distributed homogeneously in the neural plate from the medial to lateral regions (Fig. 9). The significance of such a restriction in space remains unknown at the present time.

In any case, from these observations *the coexistence within the CNS ventricular zone of precursor cells with different fates, already described for later developmental stages, may be traced back to the earliest stage in nervous system ontogeny* (early neurula stage, perhaps earlier for some cells).

A key issue remains: to determine whether such an early heterogeneity in cell fate is the result of a heterogeneity in the neural progenitors or the result of restriction to a neuronal fate by environmental cues. An *in vitro* clonal assay is being developed and will enable the potentialities of individual precursor cells to be studied. Preliminary results seem to indicate that in such a culture system, there is a marked decrease in the percentage of mixed clones (4%) and an increase in the neuronal clones (70%). Few pure astroglial clones are also observed. Thus, in neurectoderm, when precursor cell contacts are prevented by physical isolation, neuronal clones predominate. Furthermore they are always smaller than mixed clones. A relationship between cell division and the emergence of the astroglial lineage can be suggested from these observations.

This culture-assay will be useful to study the regulation and/or the control of the fate of neural precursor cells by defined growth and differentiation factors.

## Conclusions

Experiments described in the first part of this overview-report emphasize that emergence of the two neural lineages (neuronal and astroglial) appears early as a consequence of neural induction. Competent ectoderm could be a heterogeneous cell layer with respect to the different neural potentialities of cells. Neural induction is probably a permissive event activated through different signaling routes. One of them, involving L-type $Ca^{2+}$ channels and then signal transduction via a $Ca^{2+}$ pathway, is sufficient to activate both neuronal-and glial-specific genes in part of the ectodermal cell population. The intracellular machinery includes direct activation of IEG (immediate early genes).

Regarding this early neural determination, the specification of mature functional differentiation of the different neuronal phenotypes probably depends on factors of chordamesodermal origin which remain to be identified.

At the neurula stage, the early segregation of the two main neural lineages concerns only a small number of precursor cells, as indicated in the results of *in vivo* clonal analysis. Approximately 20% of progenitors, located in the neural plate, present a neuronal fate; 80% generate mixed clones with neurons and astroglial cells. The decision as to whether a neural bipotential progenitor becomes a neuron or an astrocyte may be controlled during ontogeny by mitosis, cell interactions, or cell-cell communication mechanisms, including growth and differentiation molecules (notochord and/or floor plate-activated factors).

Experiments devoted to the analysis of the development of cells from both the competent ectoderm before gastrulation and the neurectoderm at the neurula stage should bring new insights to the issue of the state of commitment of the precursor cells and help in the identification of specifying molecules.

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## References

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. (1974). Ionic regulation of embryonic induction and cell differentiation in *Rana pipiens. Dev. Biol. 39:* 1-22.

BERRIDGE, M.J. (1993). Inositol triphosphate and calcium signalling. *Nature 361:* 315-325.

BORN, J., HOPPE, P., JANECZEK, J., TIEDEMANN, H. and TIEDEMANN, H. (1986). Covalent coupling of neuralizing factors from *Xenopus* to sepharose beads: no decrease of inducing activity. *Cell Differ. 19:* 97-101.

BOUCAUT, J.C., DARRIBERE, T., BOULEKBACHE, H. and THIERY, J.P. (1984). Antifibronectin antibodies prevent gastrulation but do not perturb neurulation in gastrulated amphibian embryos. *Nature 307:* 364-367.

BRONNER-FRASER, M. and FRASER, S.E. (1988). Cell lineage analysis reveals multipotency of some avian neural crest cells. *Nature 335:* 161-164.

BRONNER-FRASER, M. and FRASER, S.E. (1989). Developmental potential of avian trunk neural crest cells *in situ. Neuron 3:* 755-766.

CHO, K.W., BLUMBERG, B., STEINBEISSER, H. and DE ROBERTIS, E. (1991). Molecular nature of Spemann's organizer: the role of the *Xenopus* homeo-box gene goosecoid. *Cell 67:* 1111-1120.

DAVIDS, M., LOPPNOW, B. and TIEDEMANN, H. (1987). Neural differentiation of amphibian gastrula ectoderm exposed to phorbol ester. *Roux Arch. Dev. Biol. 196:* 137-140.

DELARUE, M., SANCHEZ, S., JOHNSON, K., DARRIBERE, T. and BOUCAUT, J.C. (1992). A fate map of superficial and deep circumblastoporal cells in the early gastrula of *Pleurodeles waltl. Development 114:* 135-146.

DIRKSEN, M. and JAMRICH, M. (1992). A novel, activin-inducible, blastopore lip-specific gene of *Xenopus laevis* contains a fork head DNA-binding domain. *Genes Dev. 6:* 599-608.

DONIACH, T. (1993). Planar and vertical induction of anteroposterior pattern during the development of the amphibian central nervous system. *J. Neurobiol. 24:* 1250-1275.

DUPRAT, A.M., GUALANDRIS, L. and ROUGE, P. (1982). Neural induction and the structure of the target cell surface. *J. Embryol. Exp. Morphol. 10:* 171-187.

DUPRAT, A.M., GUALANDRIS, L., KAN, P. and FOULQUIER, F. (1984). Early events in the neurogenesis of amphibians. In *The Role of Cell Interactions in Early Neurogenesis.* NATO ASI Series, Life Sci. Vol. 77. Plenum Press, New York, pp. 3-20.

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

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

ECOCHARD, V., CAYROL, C., FOULQUIER, F., ZARAISKY, A. and DUPRAT, A-M. (1995). A novel TGF-ß-like gene, *fugacin*, specifically expressed in the Spemann organizer of *Xenopus laevis Dev. Biol.172:* 699-703

GILBERT, S.F. and SAXÉN, L. (1993). Spemann's organizer: models and molecules. *Mech. Dev. 41:* 73-89.

GODSAVE, S.F. and SLACK, J.M. (1989). Clonal analysis of mesoderm induction in *Xenopus laevis. Dev. Biol. 134:* 486-490.

GRAINGER, R.M. and GURDON, J.B. (1989). Loss of competence in amphibian induction can take place in single nondividing cells. *Proc. Natl. Acad. Sci. USA 86:* 1900-1904.

GRUNZ, H. and TACKE, L. (1989). Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Differ. Dev. 32:* 117-124.

GUALANDRIS, L., ROUGE, P. and DUPRAT, A.M. (1985). Target cell surface glycoconjugates and neural induction in an amphibian. *J. Embryol. Exp. Morphol. 86:* 39-51.

GURDON, J.B. (1988). A community effect in animal development. *Nature 33:* 772-774.

GURDON, J.B. (1995). The formation of mesoderm and muscle in *Xenopus*. In *Organization of the Early Vertebrate Embryo* (Eds. N. Zagris, A.M. Duprat and A.J. Durston). NATO ASI Series, Life Sci. 279. Plenum Press, New York, pp. 51-59.

GURDON, J.B., TILLER, E., ROBERTS, J. and KATO, K. (1993). A community effect in muscle development. *Curr. Biol. 3:* 1-11.

HARLAND, R.M. (1994). Neural induction in *Xenopus*. *Curr. Opin. Genet. Dev. 4:* 543-549.

HEMMATI-BRIVANLOU, A. and HARLAND, R.M. (1989). Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development 106:* 611-617.

HEMMATI-BRIVANLOU, A. and MELTON, D.A. (1994). Inhibition of activin receptor signaling promotes neuralization of *Xenopus*. *Cell 77:* 273-281.

HEMMATI-BRIVANLOU, A., KELLY, O.G. and MELTON, D.A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell 77:* 283-295.

HOLTFRETER, J. (1933). Die totale exogastrulation, eine selbstablosung des ektoderms von entomesoderm. *W. Roux Arch. Entw.Mech. Org. 129:* 669-793.

HOLTFRETER, J. (1945). Neuralization and epidermization of gastrula ectoderm. *J. Exp. Zool. 98:* 161-209.

KESSLER, D.S. and MELTON, D.A. (1994). Vertebrate embryonic induction: Mesodermal and neural patterning. *Science 266:* 596-604.

KINTNER, C. and MELTON, D.A. (1987). Expression of *Xenopus* N-CAM RNA is an early response to neural induction. *Development 99:* 311-325.

LAMB, T.M., KNECHT, A.K., SMITH, W.S., STACHEL, S.E., ECONOMIDES, A.N., STAHL, N., YANCOPOLOUS, G.D. and HARLAND R.M. (1993). Neural induction by a secreted polypeptide noggin. *Science 262:* 713-718.

LECLERC, C., DUPRAT, A.M. and MOREAU, M. (1995). *In vivo* labelling of L-type $Ca^{2+}$ channels by fluorescent dihydropyridine: correlation between ontogenesis of the channels and the acquisition of neural competence in ectoderm cells from *Pleurodeles waltl* embryos. *Cell Calcium 17:* 216-224.

MOREAU, M., LECLERC, C., GUALANDRIS-PARISOT, L. and DUPRAT, A.M. (1994). Increased internal $Ca^{2+}$ mediates neural induction in the amphibian embryo. *Proc. Natl. Acad. Sci. USA 91:* 12639-12643.

NIEUWKOOP, P.D., JOHNEN, A.G. and ALBERTS, B. (1985). *The Epigenetic nature of early chordate development. Inductive interaction and competence.* Developmental and Cell Biology Series 16. Cambridge Univ. Press, Cambridge.

OTTE, A.P. and MOON, R.T. (1992). Protein kinase C isozymes have distinct roles in neural induction and competence in *Xenopus*. *Cell 68:* 1021-1029.

OTTE, A.P., KOSTER, C.H., SNOEK, G.T. and DURSTON A.J. (1988). Protein kinase C mediates neural induction in *Xenopus laevis*. *Nature 334:* 618-620.

OTTE, A.P., KRAMER, I.J. and DURSTON, A.J. (1991). Protein kinase C and regulation of the local competence of *Xenopus* ectoderm. *Science 251:* 570-573.

OTTE, A.P., van RUN, P., HEIDEVELD, M., VAN DRIEL, R. and DURSTON, A.J. (1989). Neural induction is mediated by cross-talk between the protein kinase C and cyclic AMP pathways. *Cell 58:* 641-648.

PITUELLO, F., BOUDANNAOUI, S., FOULQUIER, F. and DUPRAT, A.M. (1990). Are neuronal precursor cells committed to coexpress different neuroactive substances in early amphibian neurulae? *Cell Differ. Dev. 32:* 71-82.

PITUELLO, F., HOMBURGER, V., SAINT-JEANNET, J.P., AUDIGIER, Y., BOCKAERT, J. and DUPRAT, A.M. (1991). Expression of the guanine nucleotide-binding protein Go correlates with the state of neural competence in the amphibian embryo. *Dev. Biol. 145:* 311-322.

RUIZ i ALTABA, A. (1993). Induction and axial patterning of the neural plate: Planar and vertical signals. *J. Neurobiol. 24:* 1276-1304.

RUIZ i ALTABA, A. (1994). Pattern formation in the vertebrate neural plate. *Trends Neurosci. 17:* 233-243.

RUIZ i ALTABA, A. and JESSELL, T.M. (1992). Pintallavis, a gene expressed in the organizer and midline cells of frog embryos: involvement in the development of the neural axis. *Development 116:* 81-93.

SAINT-JEANNET, J.P. and DAWID, I.B. (1994). Vertical versus planar neural induction in *Rana pipiens* embryos. *Proc. Natl Acad. Sci. USA 91:* 3049-3053.

SAINT-JEANNET, J.P., HUANG, S. and DUPRAT, A.M. (1989). Target cell contacts and neural commitment in *Pleurodeles waltl. Cell Differ. 27:* 165.

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:* 93-103.

SAINT-JEANNET, J.P., KARAVANOV, A.A. and DAWID, I.B. (1994). Expression of mesoderm markers in *Xenopus laevis* Keller explants. *Int. J. Dev. Biol. 38:* 605-611.

SAINT-JEANNET, J.P., PITUELLO, F., HUANG, S., FOULQUIER, F. and DUPRAT, A.M. (1993). Experimentally provoked neural induction results in an incomplete expression of neuronal traits. *Exp. Cell Res. 207:* 383-387.

SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L.K. and de ROBERTIS, E. (1994). *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell 79:* 779-790.

SATER, A.K., ALDERTON, J.M. and STEINHARDT, R.A. (1994). An increase in intracellular pH during neural induction in *Xenopus*. *Development 120:* 433-442.

SAXÉN, L. (1989). Neural induction. *Int. J. Dev. Biol. 33:* 21-48.

SERVETNICK, M. and GRAINGER, R.M. (1991). Changes in neural and lens competence in *Xenopus* ectoderm: evidence for an autonomous developmental timer. *Development 112:* 177-188.

SHARPE, C.R. and GURDON, J.B. (1990). The induction of anterior and posterior neural genes in *Xenopus laevis*. *Development 109:* 765-774.

SHEARMAN, M.S., SEKIGUCHI, K. and NISHIZUKA, Y. (1989). Modulation of ion channel activity: a key function of the protein kinase C enzyme family. *Pharmacol. Rev. 41:* 211-237.

SHENG, M. and GREENBERG, M.E. (1990). The regulation and function of C-fos and other immediate early genes in the nervous system. *Neuron 4:* 477-485.

SHENG, M., McFADDEN, G. and GREENBERG, M.E. (1990). Membrane polarization and calcium induce C-fos transcription via phosphorylation of transcription factor CREB. *Neuron 4:* 571-582.

SMITH, J.C., PRICE, B.M., GREEN, J.B., WEIGEL, D. and HERRMANN, B.G. (1991). Expression of a *Xenopus* homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell 67:* 79-87.

SMITH, W.C. and HARLAND, R.M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann Organizer in *Xenopus* embryos. *Cell 70:* 829-840.

SMITH, W.C., MCKENDRY, R., RIBISI, S. and HARLAND, R.M. (1995). A *nodal*-related gene defines a physical and functional domain within the Spemann organizer. *Cell 82:* 37-46.

SOULA, C., FOULQUIER, F., DUPRAT, A.M. and COCHARD, P. (1993). Lineage analysis of early neural plate cells: cells with purely neuronal fate coexist with bipotential neuroglial progenitors. *Dev. Biol. 159:* 196-207.

SOULA, C., SAGOT, Y., COCHARD, P. and DUPRAT, A.M. (1990). Astroglial differentiation from neuroepithelial precursor cells of amphibian embryos: an *in vivo* and *in vitro* analysis. *Int. J. Dev. Biol. 34:* 351-364.

SPEMANN, H. (1938). *Embryonic Development and Induction.* Yale Univ. Press, New Haven (reprinted by Garland Publ., NY, 1988).

SPEMANN, H. and MANGOLD, M. (1924). Über Induktion von Embryonalanlagen durch Implantation art fremder Organizatonen. *W. Roux Arch. Entw.Mech. Org. 100:* 599-638.

TAIRA, M., JAMRICH, M., GOOD, P.J. and DAWID, I.B. (1992). The LIM domain-containing homeobox gene Xlimb 1 is expressed specifically in the organizer region of *Xenopus* gastrula embryos. *Genes Dev. 6:* 356-366.

TAIRA, M., OTANI, H., SAINT-JEANNET, J.P. and DAWID, I. (1994). Role of the LIM class homeodomain protein Xlim-1 in neural and muscle induction by the Spemann organizer. *Nature 372:* 677-679.

TIEDEMANN, H. and BORN, J. (1978). Biological activity of vegetalizing and neuralizing inducing factors after binding to BAC-cellulose and CN Br-sepharose. *Roux Arch. Dev. Biol. 184:* 285-299.

YANG, J. and TSIEN, R.W. (1993). Enhancement of N- and L-type calcium channel currents by protein kinase C in frog sympathetic neurons. *Neuron 10:* 127-136.