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The choice between epidermal and neural fate: a matter of calcium

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ABSTRACT The development of the vertebrate embryo, which includes the induction and the patterning of the three germ layers, requires signaling among cells. In vertebrates, cells of the embryonic ectoderm have a choice during gastrulation between two fates; they give rise to epidermal progenitors on the ventral side and neural progenitors on the dorsal side. It was first shown by Spemann and Mangold in 1924 that the dorsal mesoderm (also called the Spemann-Mangold Organizer) has a potent biological capacity to induce nervous system from the adjacent ectoderm. A similar observation was reported in amniotes, indicating that neural induction by the Organizer is a conserved process controlling the initial steps in vertebrates neurogenesis. Molecular studies of the last decade have led to the identification of signaling molecules which participate in these embryonic decisions. Epidermis induction occurs through a signaling cascade involving Bone Morphogenetic Proteins (BMP 2, 4, 7) and receptor-regulated Smad proteins which translocate into the nucleus to form active transcriptional complexes. These molecules mediate BMPs effect to activate epidermal-specific gene expression and to repress neural-specific gene transcription. Neural fate is revealed by factors secreted by the dorsal mesoderm (Noggin, Chordin, Follistatin, ...) which act by blocking BMP signaling. Consequently, epidermal fate is an induced fate while neural fate is interpreted as a default state of the ectoderm. In this review, we describe the signaling pathways which act to determine the fate of the ectoderm and discuss an alternative model in which neural fate is not a default state. This new model integrates the activation of a calcium-dependent signaling pathway due to an influx of calcium through L-type calcium channels. In this model, calcium plays a central regulatory role. While calcium is required for neural determination, epidermal determination occurs when calcium-dependent signaling pathways are inactive.

KEY WORDS: *neural induction, epidermal determination, transcription factor, amphibian*

Introduction

Neural and epidermal tissues have the same embryonic origin, the ectoderm. In this territory, very early, in the embryo, a sort of binary process determines the cell fate, i.e.; if the cells are not committed in the epidermal fate, they are determined in the neural pathway. For these reasons, we cannot study the epidermal fate of cells without analyzing the neural determination. Major discoveries concerning the choice of neural or epidermal fates were made in amphibians and more particularly in *Xenopus laevis.*

In vertebrates, during gastrulation, the cells of the embryonic ectoderm give rise to epidermal progenitors in the ventral side and neural progenitors in the dorsal side. However, in *Xenopus* dorsal or ventral ectoderm isolated during blastula stage normally develops into epidermis when cultured *in vitro.* Since the experiments performed on the newt by Spemann and Mangold, (1924), we know that the dorsal mesoderm (also called Spemann Organizer) grafted into the ventral side of a recipient gastrula, triggers the formation of a secondary axis with a normal antero-posterior and dorso-ventral axis in the host embryo. The ectopic dorsal mesoderm derived from donor, while the secondary nervous system was recruited from the host ectoderm, which was committed to produce epidermis. These results showed that, during gastrulation, embryonic ectoderm could be induced to form neural tissue in presence of a source of inducing signals secreted by the dorsal mesoderm, this process is called neural induction (Gilbert and Saxen, 1993). At this stage ectodermal cells choose between two fates, neural or epidermal. The role of neural induction is to trigger the respecification of ectoderm to become

Abbreviations used in this paper: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; NCAM, neural cell adhesion molecule.

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neurectoderm leading to the formation of the nervous system of the adult, together with the imposition of anterior-posterior pattern (reviewed by Hamburger, 1988).

There is a conservation of neural induction mechanism in vertebrates. In fact experiments with chick or mouse embryos have shown the existence of organizing regions that could induce a secondary nervous system when transplanted into ectopic regions (Beddington, 1994; Waddington, 1933).

The neural inducers

Several neural inducers have been discovered in *Xenopus,* which respond to precise criteria: i) they have to be expressed at the right place (in dorsal mesoderm) and the right moment (at gastrula stages when neural induction occurs). ii) they should be secreted from the dorsal mesoderm and be able to act on the isolated ectoderm (animal cap) in a non–cell autonomous manner iii) the effect is direct, they should induce neural structures without inducing mesoderm.

Noggin was the first protein to be reported with a direct neural inducing activity (Lamb *et al.*, 1993). Follistatin, an activin antagonist, (Hemmati-Brivanlou *et al.*, 1994) and Chordin, (Sasai *et al.*, 1994) have been also shown to have a direct neuralizing activity. These three factors injected as RNA or *in vitro* in animal cap assay, added in the external medium as a protein, induce the expression of neural-specific genes, such as the neural cell adhesion molecule gene (NCAM). No mesodermal markers are induced. Homologues of these neural inducers have been found in other vertebrates, including zebrafish, chick, mouse and human.

Xnr3, a *Xenopus* homologue of the mouse nodal gene (Hansen *et al.*, 1997; Smith *et al.*, 1995) and Cerberus, are also able to induce neural tissue (Bouwmeester *et al.*, 1996). However, for Cerberus, the induction is accompanied by mesoderm formation and its effect may be indirect.

The fibroblast growth factor (FGF) receptor has also been proposed to mediate neural induction. A dominant negative FGF receptor, when introduced into isolated ectoderms blocks neural induction triggered by Noggin mRNA (Launay *et al.*, 1996). However the same dominant negative FGF receptor, does not prevent neural induction in whole *Xenopus* embryos (Kroll and Amaya, 1996). Basic FGF (bFGF or FGF-2) has been reported to have direct neural-inducing activity when animal caps are incubated in low calcium and magnesium medium (Kengaku and Okamoto, 1995; Lamb and Harland, 1995) but bFGF does not induce neural fate in animal caps when added directly in the medium (Cox and Hemmati-Brivanlou, 1995).

Dissociation inhibits epidermal fate and neuralizes the ectodermal cells

When amphibian animal caps are dissociated in saline divalent ions free medium the cells changed their fates from epidermal to neural. They expressed neural specific markers (e.g., NCAM) and differentiated in neural and glial cells (Godsave and Slack, 1989; Grunz and Tacke, 1989; Saint-Jeannet *et al.*, 1990; Sato and Sargent, 1989). The dissociated ectoderm was not exposed to the inducing effect of the dorsal mesoderm or to any other factors during the process. In amphibians in dissociation context, neural determination can occur autonomously without any inducers. Hemmati-Brivanlou and Melton (1992) obtained similar results when they injected into animal caps a truncated form of the type II activin. Under this treatment, the signaling by activin and BMP was inhibited and the injected animal caps adopted a neural instead of an epidermal fate. The authors proposed a model where epidermal fate is induced and depends of TGF-β signaling while neural fate occurs by disruption of the epidermal-inducing pathway (Fig. 1). According to this model, the neural fate is the default fate of the ectodermal cells (Hemmati-Brivanlou and Melton, 1994).

Bone Morphogenetic Proteins and epidermal induction

The default model of neural induction predicts the existence of inhibitory signals in animal caps to block neural formation. Bone morphogenetic proteins (BMP), members of the TGF-β family have been found to play this role. BMP family members interact through extracellular domains of heteromeric complexes composed of type I and type II of serine threonine kinase receptors. In general type II receptors are constitutive kinases responsible for ligand binding and for transphosphorylation of the type I receptor on a glycine-serine rich domain (GS domain). The phosphorylation of this domain activates the type I kinase which then phosphorylates downstream components of the signaling pathway (members of Smad family). Type I receptors are called activin-receptor-like-kinases (ALKs). ALK2, ALK3, ALK6 are implied in BMP transduction (for review see Itoh *et al.*, 2000). For type II, it can be find BMP receptors type II and receptors for high affinity for activins (ActRII and ActR IIB).

Experiments performed by Wilson and Hemmati-Brivanlou, (1995) revealed that BMP4 was able to suppress expression of neural markers and induce epidermal keratin expression in dissociated ectodermal cells. BMP2 and BMP7, close relatives of BMP4, are also reported to be neural inhibitors and epidermal inducers in the context of dissociated cells (Suzuki *et al.*, 1997b). In ectodermal cells, truncated type I BMP receptors (ALK2 and ALK3) (Sasai *et al.*, 1995; Suzuki *et al.*, 1997b; Suzuki *et al.*, 1995; Xu *et al.*, 1995), dominant negative BMP4 and BMP7 ligands (Hawley *et al.*, 1995), antisense BMP4 RNA (Sasai *et al.*, 1995) produce the same effect i.e. blocked epidermal determination and lead to neuralization. These data suggest that endogenous BMPs are, at least partly, responsible for maintaining epidermal specification in intact animal caps.

To explain the neuralization observed by dissociation of ectoderm cells it has been suggested a dilution of the BMPs. In fact, when BMP4 is added at high concentration during dissociation, the expression of neural markers is totally abolished (Wilson *et al.*, 1997). However, some other explanations can be proposed, as we will see later in this article.

The discovery of BMPs as neural inhibitors and epidermal inducers suggested that neural inducers might work *in vivo* to block BMP signaling. In fact biochemical studies demonstrate that Noggin and Chordin bind directly with BMPs, this binding being specific (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). By binding to BMPs, both Noggin and Chordin prevent BMPs from contacting their receptors, thus inhibiting signal transduction along the BMP pathway. Follistatin has previously been shown to interfere with a number of BMPs by the formation of inactive trimeric complex

(Fainsod *et al.*, 1997; Iemura *et al.*, 1999). Cerberus and Xnr3 may work in a similar way. Cerberus-dependent neural induction is inhibited by BMP4 (Bouwmeester *et al.*, 1996). It is unclear at what level Cerberus and BMP4 interact, but it remains possible that Cerberus binds directly to BMP4. Moreover, BMP4 or an activated BMP receptor (Hansen *et al.*, 1997) also blocks xnr3-induced neural formation. A new ligand of the TGF-β family, Growth and Differentiation Factor-6 (GDF6), is an other antagonist of neural inducers. In dissociated cells, GDF6 induces epidermis and inhibits neural tissues. Its activity is blocked by Noggin and it has been demonstrated that GDF6 binds directly to Noggin. GDF6 can form heterodimers with BMP2, which can play an important role to regulate cell fate determination (Chang and Hemmati-Brivanlou, 1999).

BMP antagonists secreted by dorsal mesoderm seem sufficient to neuralize the ectoderm. However, it appears that BMP activity must be regulated at additional levels. A level of regulation probably exists during neural induction to reduce the expression of BMP genes in the dorsal ectoderm. BMP4 transcripts are excluded from the dorsal side at gastrula stage (a time when neural induction occurs), including the dorsal ectoderm whose fate is to become the future neural plate (Hemmati-Brivanlou and Thomsen, 1995). However, BMP2 and BMP7 RNAs are expressed in the entire presumptive ectoderm at gastrula stage. In 1998, Kroll *et al.* have identified Geminin, a new neuralizing molecule. The maternal geminin mRNA is found throughout the animal hemisphere from oocyte through late blastula stage. At early gastrula, its expression is restricted to a dorsal territory corresponding to the future neural plate. In this territory, at this time, the expression of BMP4 is absent. Misexpression of Geminin in gastrula both suppresses the expression of BMP4 and neuralizes the prospective epidermis. It can upregulates the expression of proneural genes such as X-ngr-1. The dominant negative Geminin suppress neural differentiation and when misexpressed in the dorsal ectoderm, it produces islets of epidermal genes expression within the neurectoderm. These effects are rescued by the coexpression of the full-length molecule. Therefore, the role of Geminin in early development is to define the future territory of the neural plate and to downregulate the expression of epidermal inducers (BMPs).

Smad proteins as BMP signal transducers are involved in epidermal determination

It has been reported that BMPs can activate a MAP kinase pathway, leading to apoptosis in *Xenopus* embryo (Shibuya *et al.*, 1998). However, some evidence shows that the prominent role in BMP signaling is performed by Smads proteins (Dale and Jones, 1999; Heldin *et al.*, 1997), (see Fig. 1). Smads are the substrates for type I receptor kinase. They were identified as the products of the *Drosophila* Mad (mothers against dpp) and *Coenorahabditis* Sma genes, which act downstream of the BMP homologous ligand receptor in these organisms (Newfeld *et al.*, 1996; Savage *et al.*, 1996; Sekelsky *et al.*, 1995). The vertebrate homologues of Mad and Sma have been cloned and they were designated as Smad for Sma and Mad. At least nine vertebrate homologues have been identified (Smad1-9) (Heldin *et al.*, 1997; Itoh *et al.*, 2000).

The Smad family can be divided into three distinct groups: receptor-regulated Smads (R-Smads which involved Smad 1, 2, 3, 5, 8; common partner Smads (Co-Smads), with Smad 4 and XSmad 4β and inhibitory Smads (I-Smads) with Smads 6,7 (for review see Itoh *et al.*, 2000). Activated type I receptor kinase interacts transiently with the R-Smads and phosphorylates them. The activated R-Smad recruits Co-Smad and the heteromeric complex accumulates in the nucleus. Nuclear Smad complex can bind to DNA directly or indirectly through other DNA binding proteins and regulates the transcription of target genes (Derynck *et al.*, 1998; Massagué and Wotton, 2000). Smad1, Smad5, (and maybe Smad9) mediate BMP signaling during ectodermal patterning. Smad1 has been shown to inhibit neural induction induced by a truncated BMP receptor in intact animal caps and both Smad1 and Smad5 can mimic BMP4 in inhibiting neural formation and inducing epidermal-type keratin in the cell dissociation assay. Smad1, Smad5 and possibly Smad9 are specific signals in the transduction of the BMP pathway (Chen *et al.*, 1997b; Graff *et al.*,

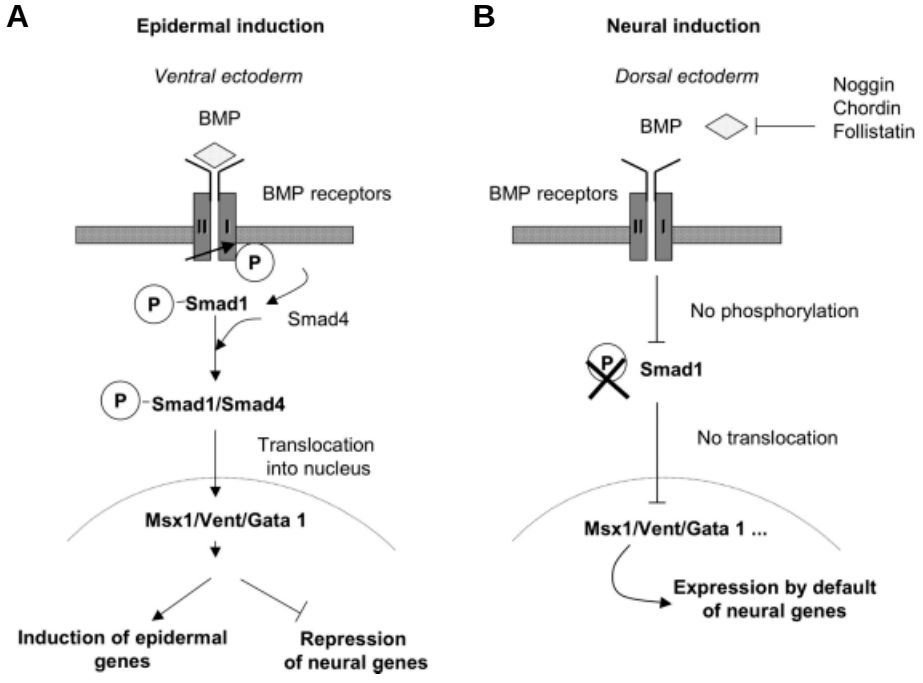

Fig. 1. Model for epidermal and neural inductions in *Xenopus laevis* embryo. *In the case of epidermal induction* **(Left)**, *the transduction of BMP pathways leads to the formation of the complex between phosphorylated Smad1 and Smad4, which is translocated into the nucleus. This complex activates DNA binding proteins such as Msx1, which activates epidermal gene expression and represses neural gene transcription. In case of neural induction,* **(Right)** *neural inducers such as Noggin prevent the binding of BMPs to its receptors, leading to a blocking of BMP signal transduction. This inhibition of BMP signaling results in a de-repression of neural gene expression. This model known as neuralization by default does not take into account the necessary and sufficient role played by calcium ions, as discussed in this article.*

1996; Suzuki *et al.*, 1997a; Thomsen, 1996; Wilson *et al.*, 1997), The Co-Smad, Smad4, is a partner for the R-Smads. In mammalian cell culture, Smad4 forms a heterodimer with Smad1 upon BMP4 stimulation. In amphibian ectoderm, both Smad1 and Smad5-dependent neural inhibition is blocked by the mutant Smad4 (Suzuki *et al.*, 1997a). Smad6, Smad7, Smad8, (Hata *et al.*, 1998; Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997; Nakayama *et al.*, 1998; Topper *et al.*, 1997; Tsuneizumi *et al.*, 1997) act as inhibitors of TGF-β signal transduction. Overexpression of Smad6 or Smad7 is sufficient to induce neural markers in the absence of mesoderm in *Xenopus* ectodermal cells and the corresponding RNA can induce a partial secondary axis when injected in the ventral side of embryos (Casellas and Hemmati-Brivanlou, 1998; Hata *et al.*, 1998; Nakayama *et al.*, 1998).

Smad7 seems to inhibit all TGF-β ligands with no discrimination, such as TGF-β1, activin, Vg1 and BMP4 by a non selective binding to the TGF-β type I receptor (Hata *et al.*, 1998; Hayashi *et al.*, 1997). Smad8 preferentially blocks BMP signaling, although it may also be involved in other pathways. In ectodermal determination, inhibitory Smads may play an important role in balancing epidermal and neural induction by blocking activities of Smad1/ Smad5/ Smad9 (Fig. 1).

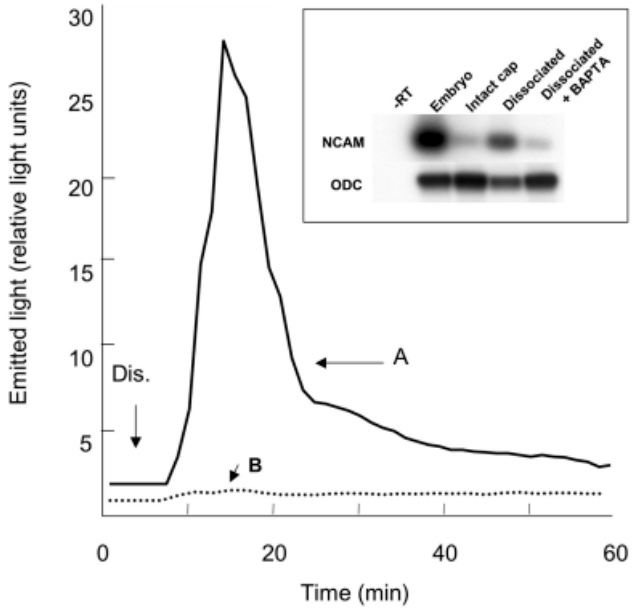

Fig. 2. Recording of internal calcium concentration during dissociation of *Xenopus* ectodermal cells. *Trace A represents concentration in medium free of divalent ions. Trace B is from an experiment performed after loading ectodermal cells with BAPTA-AM (0.4 mM in the external medium). To measure calcium, cells were previously loaded with aequorin as previously described (Leclerc* et al.*, 2001). Inset: expression of the pan-neural marker NCAM in the animal caps was measured by RT-PCR. Dissociated caps differentiated into neural cells expressing NCAM. BAPTA-AM loaded caps before dissociation (Dissociated+BAPTA) show a dramatic reduction in NCAM expression. Intact cap: animal caps not dissociated; sibling control embryos served as positive control (embryo) and PCR on the same RNA without reverse transcription was done to check the absence of genomic DNA (-RT). ODC is the control with ornithine decarboxylase.*

Some evidence shows that the Smad complex can bind directly with DNA (Dennler *et al.*, 1998). However in *Xenopus* it has been shown that the complex Smad 2/Smad 4 binds to Fast1 a winged helix DNA binding protein. The complex Smad2/Smad4/Fast1 interact with specific sequence on the DNA within the mix2 promoter (Chen *et al.*, 1996; Chen *et al.*, 1997a). For the moment, no such a sequence has been described for the Smad1/Smad4 complex.

Transcriptional regulation of epidermal and neural fates by BMP signaling

In early *Xenopus* embryos, a number of genes have been identified that are transcriptionaly regulated in response to BMP signaling. These include homeobox proteins Xmsx1, mix1, Xhox3, Xfd1 and Xmyf5, the intracellular signaling molecule XSmad8 and the intercellular signaling molecules Xwnt8 and XBMP4 (Dale and Jones, 1999) and a new family of homeobox transcription factors such as Xvent-1, Xvent-2 (also known as Xom) (Gawantka *et al.*, 1995; Ladher *et al.*, 1996; Onichtchouk *et al.*, 1996). All these genes can be upregulated by BMP4 and the overexpression of these factors in *Xenopus* results in ventralized phenotypes (i.e. promote epidermal differentiation at the expense of neural structures), as does BMP4 (Chang and Hemmati-Brivanlou, 1998 for a review). The expression is blocked by the inhibitors of BMP 4 signaling such as dominant negative type I BMP receptors. Expression of dominant negative constructs of Xvent-1, Xvent-2 and Xmsx1, inhibit epidermal determination and neuralize the isolated ectoderms. Xmsx1 and Xvent2 are activated in absence of protein synthesis and certainly represent immediate early response to BMP4 signaling (Ladher *et al.*, 1996; Suzuki *et al.*, 1997c).

Neural plate-specific markers (expressed both in neurons and glia), such as NCAM, can be activated in the animal caps by zinc finger transcription factors of the Zic family (Zic3; Zic-related-1) (Mizuseki *et al.*, 1998; Nakata *et al.*, 1997) or by a POU domain-containing DNA binding factor, XlPou2 (Witta *et al.*, 1995). Zic3, Zic-r1 and XlPou2 are expressed in the neurectoderm at gastrula stages, suggesting that they may participate in neural induction *in vivo*. In addition, these genes are induced by BMP antagonists (Noggin and Chordin) and suppressed by BMP4. However, the mechanism which regulates their expression in the ectoderm remains to be found. Xvents genes have been reported to behave as repressors of transcription (Friedle *et al.*, 1998; Trindade *et al.*, 1999). A consensus Xvent-2 DNA-binding site has been identified. Furthermore, the goosecoid promoter contains a sequence which is required for the Xvent-2 mediated repression of this homeobox gene (Trindade *et al.*, 1999).

This suggests that in ectodermal cells, BMP-regulated DNA-binding proteins, such as Xvents or Xmsx1, may repress expression of neural-specific transcription activators to block neural commitment. Alternatively, BMP-dependent transcription factors may suppress neural gene expression directly by blocking access of neural-specific activators to DNA.

Altogether, these results suggest that the determination of the epidermal and neural cell fates may rely on the balanced interaction of the opposing transcription factors. In the absence of BMP signals, neural inhibitory transcription factors are ren-

dered inactive, whereas neural genes are activated by DNA-binding proteins i.e. Zic3, Zic-r1 or XlPOU2.

Calcium is involved in the choice between neural and epidermal fate

Data obtained in the last ten years suggest that the choice of fate of the ectoderm results from an antagonism, at extracellular level, between ventralizing signals (Bone Morphogenetic proteins, BMP 2,4,7) and dorsalizing signals secreted by the dorsal mesoderm (Noggin, Chordin, Follistatin, XnR3, Cerberus…). Mechanisms involved in specifying the fate of the ectoderm suggest that the epidermal induction results from the activation of a transduction cascade and have been therefore defined as an instructive process involving the binding of BMPs on their receptors (Fig. 1). This cascade has two effects: it induces epidermis and inhibits neural fate. On the contrary, neuralization would simply results from the inhibition of the instructive process and is therefore regarded as a permissive process (for review see Weinstein and Hemmati-Brivanlou, 1999).

If the by default model has allowed to understand a part of the processes of early neurogenesis and epidermal determination at the molecular level, a number of important questions remain to be resolved. In particular, what is the transduction cascade involved between plasma membrane and nucleus (downstream of Noggin and BMP) which leads to the neural fate. Indeed, the default model hardly explains the inhibition of the neuralization triggered by Noggin on isolated ectoderms which express truncated forms of FGF receptors (Launay et al., 1996). We have identified several elements belonging to the transduction of the neuralizing signal, which allows us to ask the question: is neural determination really a by default process?

Several old works have shown that calcium might play an important role in the choice of determination (neural vs epidermal). Barth and Barth, (1964) have suggested that calcium is important to trigger neuralization in Rana pipiens ectoderm. Patch clamp experiments performed in our laboratory have shown that calcium permeability intervenes during gastrulation (unpublished results). Moreover, it is well known that dissociation of animal caps in divalent ions free medium orientated the cells toward a neural fate (Godsave and Slack, 1989; Grunz and Tacke, 1989; Saint-Jeannet et al., 1990; Sato and Sargent, 1989).

Neuralization by dissociation of ectodermal cells is associated with a calcium signal

Neuralization by dissociation can be explained by the dilution of epidermal inductors (BMPs) from the receptors. However we have shown that the dissociation of animal caps in calcium free medium triggers an increase in intracellular calcium concentration $[Ca^{2+}]_i$ (Fig. 2). This increase is probably due to an efflux of calcium from internal stores resulting from the inversion of the calcium gradient of concentration between intra and extracellular compartments (Leclerc et al., 2001). When ectodermal cells are loaded with the calcium chelator BAPTA, neuralization by dissociation was inhibited and the neural marker NCAM is not expressed (Leclerc *et al.*, 2001). This suggests that a dilution of epidermal inducers is not sufficient to explain the results. A calcium dependent signal appears to be required to trigger the neuralization of the ectoderm and consequently, to inhibit epidermal fate.

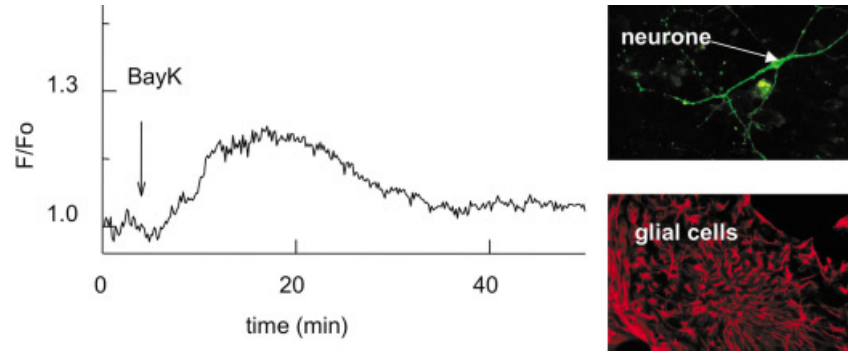

Fig. 3. Effect of direct stimulation of L-type calcium channels on isolated animal caps by a specific agonist S(-)Bay K8644 (5 μM). *This treatment triggers an intracellular calcium increase and, after 36 hours of culture, the differentiation of neurons and glial cells. Calcium was recorded by loading the animal cap with the fluorescent probe Fluo-3. Neurons and glial cells were respectively evidenced with NC1 and GFAP antibodies.*

Noggin triggers an increase in intracellular calcium concentration

The isolated ectoderm follows epidermis fate in absence of inductive signals. In the presence of an inductive signal such as Noggin, it will adopt a neural fate. Addition of Noggin on isolated ectoderms triggers an increase in $[Ca^{2+}]_i$. This increase has 10-20 minutes duration and represents about 15 % of the resting level of $[Ca^{2+}]_i$ (Moreau *et al.*, 1994). This increase is totally inhibited by L-type channel antagonists, such as nifedipine or nimodipine. In addition, the blockade of L-type calcium channels inhibits neural induction (Leclerc *et al.*, 1997). It is not excluded that other sources of calcium may be involved, at least partially. In fact methylxanthines such as caffeine or theophyline, known to release calcium from internal stores, are potent neural inducers (Moreau *et al.*, 1994).

Direct activation of L-type calcium channels triggers neural induction on isolated ectoderms

The isolated ectoderm directly stimulated by specific agonists of L-type calcium channels (S(-)Bay K 8644) presents a transient increase in $[Ca^{2+}]_i$ of 20 minutes duration. This transient increase is sufficient, even in an active BMP context, to trigger neural induction. The treated ectoderm cells differentiate in neural and glial cells, which can be characterized by immunocytochemistry (Moreau *et al.*, 1994) (Fig. 3).

All the previous results have been obtained on animal caps, which is an *in vitro* system. However, we have confirmed that during gastrulation, calcium dependent phenomenon also occurs in a whole embryo. We have visualized calcium movements in each cell of the dorsal ectoderm in a whole *Xenopus* embryo during gastrulation using the photoprotein aequorine and a new

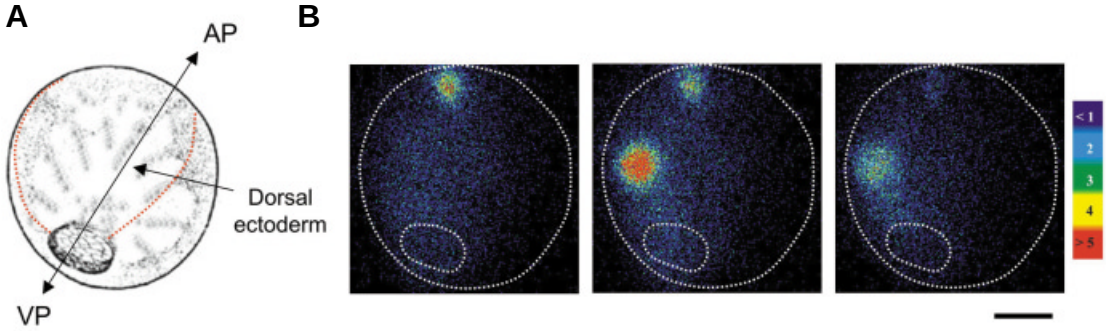

Fig. 4. An example of a sequence of Ca^{2+} transients which occur in the dorsal ectoderm from a representative embryo at stage 11.5 (12 hours post fertilization). *Each aequorin-generated photon image represents 120 seconds of accumulated light and is superimposed on its corresponding image of the embryo. These images show the different intensities of transients occurring simultaneously but at independent positions of the dorsal ectoderm.* **(A)** *Schematic of the embryo shows the dorsal ectoderm and the antero-posterior axis. AP, animal pole; VP, vegetal pole. Color scale indicates luminescent flux in photon per pixel. Scale bar, 0.3 mm.*

technique of Ca^{2+} imaging system which has a high sensitivity (Miller *et al.*, 1994). This technique allows visualizing calcium movements in the whole embryo (Fig. 4). The onset of calcium signaling activity occurs at late blastula stage (stage 8-9), at a time when zygotic transcription starts. As gastrulation proceeds, calcium flashes increase both in number and intensity, to reach a peak activity by mid-gastrulation (stage 11-11.5), a stage where neural determination is thought to have occurred. This activity was found strictly restricted to dorsal ectoderm (the tissue where neural induction takes place) and never occurs in ventral ectoderm cells, which are at the origin of epidermis. Ventral cells never receive neural inductive signals.

The increase in $[Ca^{2+}]_i$ is determinant since neural induction was totally blocked in ectoderm cells preloaded with the potent calcium chelator BAPTA or if specific antagonists of L-type calcium channels blocked Ca^{2+} channels. In these last conditions the embryos lack anterior brain structures (Leclerc *et al.*, 1997; Leclerc *et al.*, 2001; Moreau *et al.*, 1994), (Fig. 5).

Our results suggest that the increase in $[Ca^{2+}]_i$ occurring during neural induction in dorsal ectoderm creates calcium compartments of high level. These compartments can activate transcription factors that have a proneural activity.

Calcium targets

To understand how the $[Ca^{2+}]_i$ can control neural determination, we have to determine the elements, which belong to the excitation-transcription coupling (reception of Noggin signal/neural genes expression). We have shown that calcium controls the expression of c-fos (Leclerc *et al.*, 1999) and at least two other transcription factors: XlPou2 and Zic3. If Fos is a ubiquitous transcription factor, XlPou2 and Zic3 are specific of neural determination. They belong respectively to the POU domain family and odd-paired domain family of transcription factors (Nakata *et al.*, 1997; Witta *et al.*, 1995). They are primary neural regulators and we have found that their expression is calcium dependent. Indeed, our experiments performed on isolated *Xenopus* ectoderms show that XlPou2 is induced very early after an increase in $[Ca^{2+}]_i$. The mRNA for XlPou2 is detectable as early as 30 minutes after $[Ca^{2+}]_i$ increase. In addition, specific antagonists of L-type calcium channels block the expression of XlPou2 in response to Noggin. The expression of Zic3 is dramatically reduced when the embryos are incubated in presence of antagonists of L-type calcium channels (Leclerc *et al.*, 2000).

Neural competence of the ectoderm is linked to the expression of L-type calcium channels

The ability of the ectodermal cells to be induced and to differentiate toward neural tissue in response to signals coming from the mesoderm is called neural competence. This competence is acquired shortly before gastrulation and lost during late gastrula stages in amphibian embryos. Both protein kinase C (PKC) and G-protein pathways have been proposed to affect the ectoderm competence (Otte *et al.*, 1991; Otte *et al.*, 1992; Otte and Moon, 1992; Pituello *et al.*, 1991). However, PKC or G protein alone are unable to induce neural tissue. We have demonstrated that the appearance of dihydropyridine sensitive calcium channels (L-type Ca^{2+} channels) is correlated with the acquisition of neural competence by the ectodermal cells. The highest density of these channels is reached when competence of the ectoderm is optimal. Conversely, the decrease of L-type Ca^{2+} channel density occurs simultaneously with the normal loss of competence. In addition, we show that these channels are functional. We propose that the molecular basis of the gain and loss of neural competence is linked to the presence of L-type Ca^{2+} channels in ectodermal cell membranes of amphibian embryos (Dréan *et al.*, 1995; Leclerc *et al.*, 1995).

Control of the choice of determination: a new model

Our results show that an increase in $[Ca^{2+}]_i$ is a necessary and sufficient event to turn the fate of ectoderm cells from epidermis to neural tissue. However, one of the important unresolved questions raised by our data, concerns the mechanism by which the L-type Ca^{2+} channels are activated during gastrulation in the dorsal ectoderm. This question is linked to an other one: how Noggin may produce an influx of Ca^{2+} through L-type Ca^{2+} channels? In this respect, it is interesting to further consider the relation between Noggin and the FGF receptor. On animal caps, it has been shown that truncated FGF receptor prevents neuralization triggered by the BMP antagonist, Noggin (Launay *et al.*, 1996). We think that FGF receptor might be implied in the transduction of this signal via the activation of calcium dependent pathways. Indeed, during mesoderm induction FGF produces the phosphorylation of a phospholipase (PLCγ), which in turn activates a protein kinase C (PKC) (Gillespie *et al.*, 1992). Activation of PKC by phorbolesters has been shown to cause neural differ-

entiation in *Xenopus* dorsal ectoderm (Otte *et al.*, 1991) and to triggers an increase in internal calcium concentration associated with neural induction on isolated ectoderms of the amphibian *Pleurodeles*. This increase in internal concentration is due to the activation of L-type calcium channels (Moreau *et al.*, 1994). Furthermore, on chick embryo neurons, activation of the FGF receptor leads to membrane potential depolarization, which causes Ca^{2+} influx through voltage, operated Ca^{2+} channels (Distasi *et al.*, 1995). It will be interesting to test whether the suppression of the BMP pathway by Noggin is associated with a change in membrane potential.

It is important to emphasize that a Ca^{2+} increase is sufficient to induce neural tissue even in an active BMP context. This suggests that calcium participates in the inhibition of the BMP pathway and leads us to propose a new model (Fig. 6) to explain what prompts a cell to select an epidermal versus a neuronal pathway. This model involved the activation by calcium of specific kinases and phosphatases. The action of BMP antagonists (i.e. Noggin, Chordin) is one way to inhibit BMP signaling by preventing BMP4 to interact with its receptor; it is so far the most studied way. However, an other way is to act downstream and to block either BMP receptor activation or Smad phosphorylation. It is reported that the spatial distribution of activated Smad1 (phosphorylated Smad1) changes at the onset of gastrulation. Prior to gastrulation, phosphorylated Smad1 is equally distributed between dorsal and ventral side of the embryo, reflecting activation of the BMP4 signaling pathway, whereas at stage 9.5, Smad1 phosphorylation is enriched in the ventral side and by stage 10.25 (early gastrulation) most of the activated Smad1 is localized to the ventral side (Faure *et al.*, 2000). This result correlates with the pattern of calcium increase which starts in dorsal ectoderm at stage 9 and is maximum by stage 11.5 (mid-gastrulation) (Leclerc *et al.*, 2000). *Xenopus* calcineurin, a Ca^{2+}/calmodulin dependent phosphatase 2B, has been cloned. It is 94% identical at the amino acid level with the murin and human calcineurin. It is expressed through out early development (Saneyoshi *et al.*, 2000). One can hypothesized that dephosphorylation of Smad1 in the dorsal ectoderm during gastrulation is controlled by calcineurin.

FKBP, a cytosolic protein that binds the calcium dependent protein phosphatase calcineurin, appears to inhibit BMP4 signaling in *Xenopus* embryos. Injection of FKBP mRNA into 8-cell stage embryos generates the formation of ectopic axis. No secondary axis formation occurs when FKBP was coinjected with Smad1, a downstream signaling molecule of BMP4 receptor. This effect is probably mediated by calcineurin since injection of constitutively active murin calcineurin produces a similar phenotype. Furthermore, a FKBP mutant unable to bind calcineurin did not induced ectopic axis (Nishinakamura *et al.*, 1997).

Taken together these lines of evidence suggest that the increase in intracellular calcium recorded during gastrulation may activates the calcium dependent protein phosphatase calcineurin, which in turn will block BMP4 signaling. As a consequence of this inhibition, ectoderm will adopt a neural instead of an epidermal fate.

The activation of calcium calmodulin kinase II (CaMkinase II) has been reported to occurs during the transcriptional activation of immediate early gene such as c-fos (Greenberg *et al.*, 1992). In isolated ectoderm from the newt *Pleurodeles walt*, a Fos-related protein is upregulated during neural induction. This is triggered by Noggin and involves an increase in intracellular calcium mediated by the activation of L-type Ca^{2+} channels. The expression of this Fos-related protein is dependent on the activation of a CaMkinase, blocked by KN62, which in turn phosphorylates the Ca^{2+}/cAMP response element binding protein (CREB) (Leclerc *et al.*, 1999). Future considerations will include identifying downstream targets of calcium and Ca^{2+} response element on promoters of early neural-specific transcription factors.

The model (Fig. 6) for the choice of determination (epidermal vs neural) involves two instructive mechanisms; an instructive mechanism (epidermal determination) and a permissive mechanism (neu-

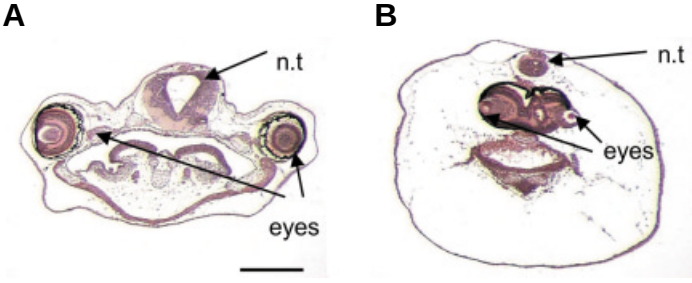

Fig. 5 (Left). Transverse section of the head of a *Xenopus* embryo (stage 42, 3 days) stained with hematoxylin-eosin. (A) *Control embryo.* **(B)** *Embryo incubated in the presence of nifedipine 10 μM (L-type calcium channels antagonist). n.t, neural tube; bar, 100 μm.*

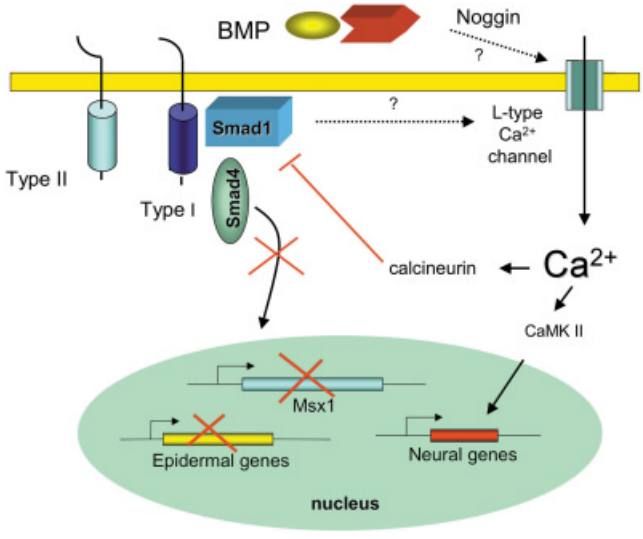

Fig. 6 (Right). New model for neural induction in *Xenopus laevis* embryo. *In this model, calcium plays the central role, by activating a calcineurin which prevent the phosphorylation of the Smads and by the control of neural gene transcription, either directly or via the activation of a calcium calmodulin kinase type II (CaMK II) (see text for more details). L-type calcium channels are activated by unidentified mechanisms at the level of the binding of BMP by neural inducers. Under these conditions, neuralization is not a permissive process, but an instructive mechanism as epidermal induction.*

ral determination). Neural determination requires numerous BMP4 signaling inhibitors both outside (Noggin, Chordin…) and inside the cell (calcium). In this model, calcium is a central regulator that inhibits BMP signaling pathways (epidermal determination) and activates neural pathway. The instructive mechanism involved in epidermal induction is well known, but the model we propose for neural induction remains to be analyzed in details.

Summary

The development of the vertebrate embryo, which includes the induction and the patterning of the three germ layers requires signaling among cells. In vertebrates, during gastrulation, the cells of the embryonic ectoderm have the choice between to fates; they give rise to epidermal progenitors on the ventral side and neural progenitors in the dorsal side. It was first shown by Spemann and Mangold in 1924 that the dorsal mesoderm (also called the Spemann-Mangold Organizer; see Sander and Faessler, 2001) has the potent biological activity to induce nervous system from the adjacent ectoderm. A similar observation was reported in amniotes, indicating that the neural induction by the organizer is a conserved process controlling the initial step in vertebrates neurogenesis. Molecular studies of the last decade have led to the identification of signaling molecules that participate in these embryonic decisions. Epidermis induction occurs through a signaling cascade involving Bone Morphogenetic Proteins (BMP 2, 4, 7) and receptor-regulated Smad proteins which translocate into the nucleus to form active transcriptional complexes. These molecules mediate BMPs effect to activate epidermal-specific gene expression and to repress neural-specific gene transcription. The neural fate is revealed by factors secreted by the dorsal mesoderm (Noggin, Chordin, Follistatin, …) which act by blocking BMP signaling. Consequently, epidermal fate is an induced fate while neural fate is interpreted as a default state of the ectoderm. Here, we discuss an alternative where neural fate is not a default state; this new model integrates the activation of a calcium-dependent signaling pathway due to an influx of calcium through L-type calcium channel. In this model, calcium plays a central regulatory role. While calcium is required for neural determination, epidermal determination occurs when calcium-dependent signaling pathways are inactive.

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