Calcium signalling during neural induction in *Xenopus laevis* embryos

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In *Xenopus*, experiments performed with isolated ectoderm suggest that neural determination is a 'by default' mechanism, which occurs when bone morphogenetic proteins (BMPs) are antagonized by extracellular antagonists, BMP being responsible for the determination of epidermis. However, Ca^{2+} imaging of intact *Xenopus* embryos reveals patterns of Ca^{2+} transients which are generated via the activation of dihydropyridine-sensitive Ca^{2+} channels in the dorsal ectoderm but not in the ventral ectoderm. These increases in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) appear to be necessary and sufficient to orient the ectodermal cells towards a neural fate as increasing the $[Ca^{2+}]_i$ artificially results in neuralization of the ectoderm. We constructed a subtractive cDNA library between untreated and caffeine-treated ectoderms (to increase $[Ca^{2+}]_i$) and then identified early Ca^{2+}-sensitive target genes expressed in the neural territories. One of these genes, an arginine methyltransferase, controls the expression of the early proneural gene, *Zic3*. Here, we discuss the evidence for the existence of an alternative model to the 'by default' mechanism, where Ca^{2+} plays a central regulatory role in the expression of *Zic3*, an early proneural gene, and in epidermal determination which only occurs when the Ca^{2+}-dependent signalling pathways are inactive.

Keywords: calcium; dihydropyridine-channels; neural determination; *Xenopus laevis*; gene expression

1. INTRODUCTION

In amphibians, the formation of the nervous system occurs during gastrulation, with a process called neural induction. In the past 15 years, it has been suggested that neural induction results from the opposing action of ventralizing signals such as bone morphogenetic proteins (BMPs) from the ectoderm, which are responsible for the determination of the epidermis, and dorsalizing signals, such as noggin, chordin, follistatin, XnR3 and Cerberus, from the dorsal mesoderm (reviewed by Sasai & De Robertis (1997)). However, mounting evidence suggest that antagonizing BMP signalling is not sufficient to explain neural induction and that other signalling components such as fibroblast growth factor (FGF) are also required (Delaune *et al.* 2005; Stern 2005).

We suggest that neural induction is controlled by a different signalling pathway, in which transient rises in the concentration of intracellular calcium ($[Ca^{2+}]_i$) play a role in controlling the binary determination decision (i.e. epidermis versus neural tissue). We present a new model to explain the role of Ca^{2+} in neural induction and to revaluate the concept of 'by default' neural induction.

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2. CALCIUM IS INVOLVED IN THE CHOICE BETWEEN NEURAL AND EPIDERMAL FATE

Ectoderm (i.e. the animal cap), dissected at the blastula stage, exhibits a high level of plasticity. Without inducing factors it develops into atypical epidermis and with appropriate neural inducers such as noggin, the animal cap cells express a variety of neural markers.

Over 40 years ago, Barth & Barth (1964) were the first to suggest that Ca^{2+} is required to trigger neuralization in *Rana pipiens* embryos. In addition, the dissociation of animal caps in Ca^{2+}- and Mg^{2+}-free medium propelled the cells towards a neural fate (Grunz & Tacke 1989; Saint-Jeannet *et al.* 1989, 1990, 1993). Furthermore, we have shown that the dissociation of animal caps in Ca^{2+}-free medium triggers an increase in $[Ca^{2+}]_i$. This increase is due to an efflux of Ca^{2+} from internal stores, resulting from the inversion of the gradient of concentration in Ca^{2+} between intra- and extracellular compartments (Leclerc *et al.* 2001). In addition, neuralization by dissociation is blocked when animal cap cells are loaded with the Ca^{2+} chelator N,N'-[1,2-ethanediylbis(oxy-2, 1-phenylene)]bis[N-[2-[(acetyloxy)methoxy]-2-oxo-ethyl]]bis[(acetyloxy)methyl]ester (BAPTA); the neural marker neural cell adhesion molecule (NCAM) is not expressed (Leclerc *et al.* 2001). This shows that in animal caps a Ca^{2+}-dependent signal is necessary both
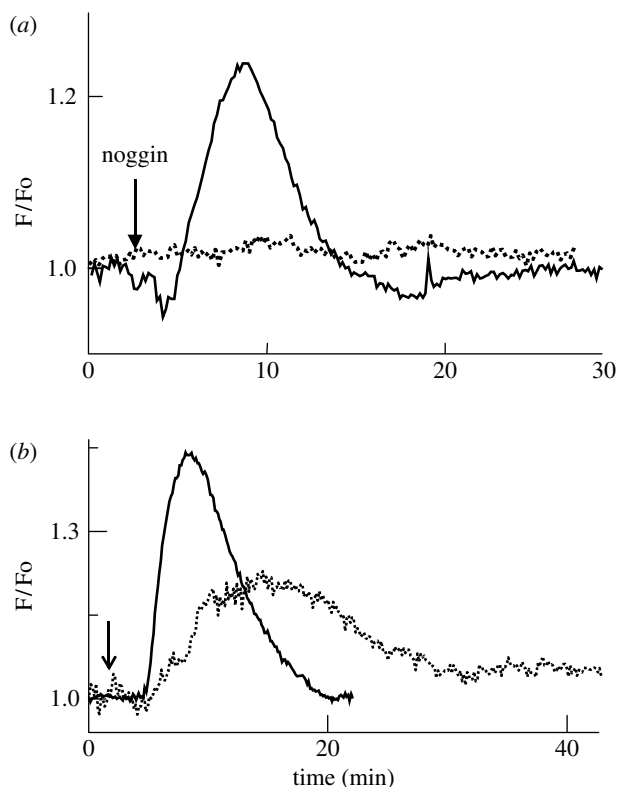
Figure 1. An increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is necessary and sufficient to trigger neural induction on isolated animal caps. (*a*) Noggin protein triggers an increase in $[Ca^{2+}]_i$ via DHP-sensitive Ca^{2+} channels (solid line) since its effect is blocked by nimodipine, a specific agonist of DHP-sensitive Ca^{2+} channels (dotted line). Nimodipine blocks neural induction. (*b*) An artificial increase in $[Ca^{2+}]_i$ either by a release from internal stores with caffeine (solid line) or by direct stimulation of DHP-sensitive Ca^{2+} channels with a specific agonist S(−)BayK 8644 (dotted line), triggers neural induction. These treatments lead to the differentiation of neurons and glial cells. Ca^{2+} was recorded by loading the animal caps with the fluorescent probe fluo-3.

to trigger neuralization of the ectoderm and to inhibit epidermal determination.

3. DHP-SENSITIVE Ca^{2+} CHANNELS AND NEURAL INDUCTION

Addition of noggin to animal caps triggers an increase in $[Ca^{2+}]_i$ (figure 1*a*). This increase has a duration of approximately 10–20 min and represents approximately 15% of the resting level of $[Ca^{2+}]_i$ (Moreau *et al.* 1994; Batut *et al.* 2005). This $[Ca^{2+}]_i$ increase is completely inhibited by antagonists of dihydropyridine (DHP)-sensitive Ca^{2+} channels, such as nifedipine or nimodipine (figure 1*a*). On the other hand, animal caps that are treated with specific agonists of DHP-sensitive Ca^{2+} channels, such as S(−)Bay K 8644, generate a transient increase in $[Ca^{2+}]_i$ with a duration of approximately 20 min (figure 1*b*). This increase is sufficient, even in an active BMP context, to trigger not only the expression of neural markers but also the formation of neurons and glial cells (Moreau *et al.* 1994). Conversely, the inhibition of DHP-sensitive Ca^{2+} channels inhibits neural induction (Leclerc *et al.* 1997). In addition, methylxanthines, such as caffeine

or theophylline, which are known to stimulate the release of Ca^{2+} from internal stores, are also potent neural inducers (figure 1*b*; Moreau *et al.* 1994; Leclerc *et al.* 1995). These latter experiments suggest that Ca^{2+} plays a crucial role, since whatever its provenance is, it triggers neuralization of the ectoderm.

4. IMAGING Ca^{2+} TRANSIENTS DURING NEURAL INDUCTION IN INTACT AMPHIBIAN EMBRYOS

Using intact embryos during gastrulation, we have confirmed all the results obtained with the *ex vivo* animal cap system. Using the Ca^{2+}-sensitive photoprotein, aequorin in conjunction with a custom-designed photon imaging microscope (Webb *et al.* 1997), we have directly visualized the Ca^{2+} dynamics that occur in *Xenopus* ectodermal cells. The onset of Ca^{2+} signalling activity occurs at the blastula stage (i.e. stage 8), long before the start of gastrulation (i.e. before mesoderm invagination) and the Ca^{2+} transients are localized in the most anterior part of the dorsal ectoderm. These observations indicate that neural induction might be initiated earlier than was previously thought, but this possibility still needs further investigation. Recent studies have demonstrated that neural induction requires the combined activity of the Nieuwkoop centre and the blastula chordin and noggin-expressing (BCNE) centre located in dorsal animal cells (Kuroda *et al.* 2004). The BCNE centre contains the prospective neuroectoderm and Spemann organizer precursor cells, and is required for brain formation. We suggest that the Ca^{2+} transients observed in the dorsal ectoderm during the blastula stage might well be localized in the BCNE centre. Thus, these transients appear so far to be the first visualized events linked to neural induction. As gastrulation proceeds, the number and intensity of the Ca^{2+} transients increasingly reach a peak of activity by mid-gastrulation (i.e. stage 11–11.5). This activity was found to be restricted to the dorsal ectoderm (i.e. the tissue where neural induction takes place) and never occurred in the ventral ectoderm cells (i.e. those which do not receive neural inductive signals).

Intact embryos also yielded results (i.e. neural induction was blocked) similar to the animal caps on treatment with either the calcium chelator BAPTA or specific antagonists of the DHP-sensitive Ca^{2+} channels. In addition, when treated with DHP-sensitive Ca^{2+} channel blockers, the embryos lacked anterior brain structures (Moreau *et al.* 1994; Leclerc *et al.* 1997, 2001). This phenotype is similar to the one obtained when the BCNE centre is removed (Kuroda *et al.* 2004).

In an attempt to simplify the experimental model, Keller open-face explants (Keller & Danilchik 1988) were used as a two-dimensional system to study neural induction. This model is sufficient to reproduce many aspects of neural induction observed *in vivo*, such as the expression of neural marker genes, neuronal differentiation and the induction of a regionalized neural plate along the antero-posterior axis. In this model, we observed that Ca^{2+} transients start from the most anterior part of the open-face explant as was observed in the intact embryo. (Leclerc *et al.* 2003).
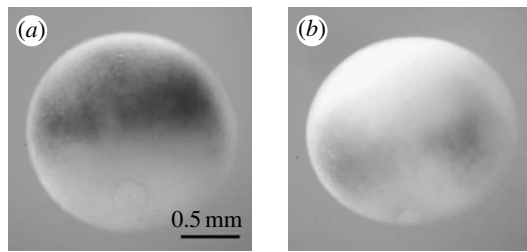
Figure 2. Loss of function of *xPRMT1b* decreases *Zic3* expression. Late gastrula embryos (stages 12–12.5) were injected at the 2-cell stage with a morpholino (Mo1b) against *xPRMT1b* and then probed for the expression of *Zic3*. (*a*) Control. (*b*) Mo1b strongly reduces *Zic3* expression when compared to the control embryo.

5. WHAT ARE THE Ca^{2+} TARGET GENES?

We have previously shown, with animal caps, that Ca^{2+} controls the expression of the immediate early gene *c-fos* (Leclerc *et al.* 1999) and of two other transcription factors: *XlPou2* and *Zic3*. While Fos is a ubiquitous transcription factor, XlPou2 and Zic3 are involved in neural determination and are primary neural regulators (Witta *et al.* 1995; Nakata *et al.* 1997). We demonstrated that specific antagonists of DHP-sensitive Ca^{2+} channels blocked the expression of *XlPou2* in response to noggin in animal caps, and dramatically reduced the expression of *Zic3* in the whole embryo (Leclerc *et al.* 2000). In addition, in planar explants, the accumulated pattern of Ca^{2+} correlated with the expression of *Zic3*, and treatment with nifedipine (a DHP-sensitive Ca^{2+} channel antagonist) blocked the Ca^{2+} transients and reduced the level of *Zic3* expression (Leclerc *et al.* 2003). These results suggest that the increase in $[Ca^{2+}]_i$ occurring during neural induction in the dorsal ectoderm can create compartments of high Ca^{2+} level, which might activate genes with proneural activity.

To identify new Ca^{2+} target genes involved in neural induction, we constructed a subtractive cDNA library between untreated (i.e. ectodermal) and short duration (i.e. 15–45 min) caffeine-treated (i.e. neuralized) animal caps. This treatment that triggers neural induction via an increase in $[Ca^{2+}]_i$ (Moreau *et al.* 1994) allows the differential isolation of the earliest Ca^{2+}-dependent genes involved in neural determination (Batut *et al.* 2003). We selected one gene, *xPRMT1b*, from the 30 early genes identified that were found to be controlled by Ca^{2+} and expressed in the presumptive neural territories. *xPRMT1b* is the *Xenopus* homologue of the mammalian arginine methyltransferase *PRMT1* gene (Batut *et al.* 2005). On animal caps, the expression of *xPRMT1b* is an early response to a Ca^{2+} increase that does not require de novo protein synthesis. Its expression is triggered following the application of noggin or by the inhibition of BMP signalling with tBR (a non-functional form of the BMP4 receptor). These effects are specifically blocked by BAPTA, a calcium chelator. In the whole embryo, *xPRMT1b* is expressed in neural territories. The early expression of *xPRMT1b* at the gastrula stage also occurs through a Ca^{2+}-dependent mechanism mediated by the activation of DHP-sensitive Ca^{2+} channels. Overexpression of *xPRMT1b* in the neural territories activates the expression of the neural precursor gene *Zic3*. A morpholino approach, with an oligonucleotide against *xPRMT1b*, blocks the expression of the neural markers

induced by an increase in Ca^{2+} such as *Zic3* in animal caps, and in the whole embryo it impairs anterior neural development (figure 2; Batut *et al.* 2005). Identical phenotypes are obtained with antagonists of DHP-sensitive Ca^{2+} channels (Leclerc *et al.* 2000), or when the BCNE centre is deleted (Kuroda *et al.* 2004). These results suggest that the *xPRMT1b* is a direct link between the $[Ca^{2+}]_i$ increase and downstream events involved in neural induction.

6. DISCUSSION

Acquisition of a neural fate has been, until recently, considered as a permissive event, only requiring the inhibition of BMP signalling. While this 'by default' model has allowed us to understand part of the process of early neurogenesis and epidermal determination at the molecular level, a number of important questions still remain to be addressed. The 'by default' model conflicts in particular with data from chick and ascidian embryos, which indicate that neural induction is initiated by FGF signalling in a partly BMP-independent manner (Bertrand *et al.* 2003; Stern 2005). The 'by default' model also cannot fully explain the inhibition of neuralization triggered by noggin on isolated ectoderm that expresses truncated forms of FGF receptors (Launay *et al.* 1996). In addition, in intact *Xenopus* embryos, it has been recently shown that BMP inhibition is required but is not sufficient to trigger neural induction, and that pre-gastrula FGF signalling is required in the ectoderm for the emergence of neural fates (Delaune *et al.* 2005).

Finally, our results indicate that an increase in $[Ca^{2+}]_i$ is a necessary and sufficient event to neuralize the ectoderm. These results suggest a permissive role played by Ca^{2+}. The identification and functional characterization of new Ca^{2+} target genes, such as *xPRMT1b*, will help us to make the link between Ca^{2+} influx and neural determination.

However, several important and as yet unsolved questions have been raised by our data; for example, the mechanism by which the DHP-sensitive Ca^{2+} channels are activated during gastrulation in the dorsal ectoderm is still unknown, as is how noggin can stimulate an influx of Ca^{2+} through DHP-sensitive Ca^{2+} channels. In this respect, it is important for us to further consider the relationship between noggin and the FGF receptor. It has been demonstrated, for example, in chick embryo neurons and endothelial cells that the activation of the FGF receptor stimulates the release of arachidonic acid and its metabolites, which in turn activate a Ca^{2+} influx probably via transient receptor potential channels (TRP; Distasi *et al.* 1995; Antoniotti *et al.* 2003).

Another important question is why the Ca^{2+} signals are initially generated in the anterior part of the ectoderm, since the inducing signal is supposed to be via the diffusion of molecules secreted by the dorsal mesoderm. The anterior region of the Ca^{2+} transients in the blastula stage might correspond to the BCNE centre. The presence of neural inducers, such as noggin, in the blastula ectodermal precursor cells (Kuroda *et al.* 2004) and the evidence that noggin activates DHP-sensitive Ca^{2+} channels on animal caps (Leclerc *et al.* 1999; Batut *et al.* 2005) support this hypothesis.
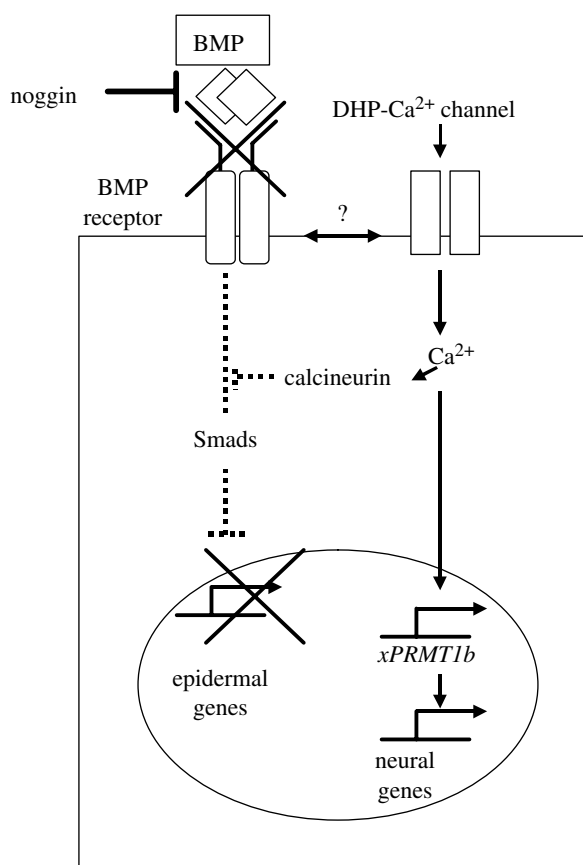
Figure 3. A new model for neural induction in *Xenopus laevis* embryos. In this model Ca^{2+} plays a central role, by directly activating Ca^{2+} target genes such as *xPRMT1b*, which in turn control neural gene transcription either directly or via the activation of a Ca^{2+}/calmodulin kinase type II. The Ca^{2+} signals may also inhibit the BMP signalling pathway by activating a calcineurin, which prevents the phosphorylation of Smads (see text for more details). The activation of the DHP-Ca^{2+} channels is performed by an as yet unidentified mechanism (indicated by the '?') at the level of the binding of BMP by neural inducers.

7. HOW GENE EXPRESSION DURING NEURAL INDUCTION MIGHT BE CONTROLLED BY Ca^{2+}

Control of gene expression by Ca^{2+} very often involves changes in the transactivating properties of transcription factors after the induction of Ca^{2+}-dependent kinases and phosphatases. However, direct effectors of Ca^{2+}-induced gene expression have also been suggested to exist in the nucleus. An important *cis*-regulatory element, downstream regulatory element (DRE), has been implicated in what can be called excitation– or stimulation–transcription coupling mechanisms (Carrion *et al.* 1999). The DRE sequence, which is located downstream from the TATA box, is the target of the DRE antagonist modulator (DREAM), an EF-hand Ca^{2+}-binding protein of the recovering subfamily, which in the absence of Ca^{2+} binds to the DRE site and represses transcription. To date, DREAM is the only Ca^{2+} sensor known to bind specifically to DNA and directly regulate transcription in a Ca^{2+}-dependent manner. Some evidence shows that promoters of some neural-specific genes contain putative Ca^{2+}-dependent *cis*-regulatory elements.

Recent findings suggest an alternative mechanism by which Ca^{2+} might control gene expression directly. In our model, the DHP-sensitive Ca^{2+} channel plays a crucial role in triggering neural induction and neural gene expression. The mechanism that links the activity of this Ca^{2+} channel to the nucleus is not well understood. The DHP channel may be related to an L-type Ca^{2+} channel. Recently it was shown that the C-terminal fragment of an L-type Ca^{2+} channel translocates to the nucleus, binds to a nuclear protein, associates with an endogenous promoter and regulates transcription of a wide variety of endogenous genes important for neuronal signalling (Gomez-Ospina *et al.* 2006). This work suggests that during neural induction, the activity of DHP-sensitive Ca^{2+} channels might be involved in a similar way to control the expression of neural genes.

Another way for Ca^{2+} to control neural gene expression is through the inhibition of BMP signalling, by acting downstream of Smad phosphorylation. The spatial distribution of activated Smad1 (i.e. phosphorylated Smad1) has been reported to change at the onset of gastrulation. Prior to gastrulation, phosphorylated Smad1 (which reflects the activation of the BMP4 signalling pathway) is equally distributed on the dorsal and ventral sides of the embryo. In contrast, at late blastula, Smad1 phosphorylation is enriched on the ventral side, and by early gastrulation most of the activated Smad1 is localized to the ventral side (Faure *et al.* 2000). This correlates with the pattern of Ca^{2+} increase, which starts in the dorsal ectoderm at the blastula stage and is maximal at mid gastrulation (Leclerc *et al.* 2000). One can hypothesize that the dephosphorylation of Smad1 in the dorsal ectoderm during gastrulation is controlled by calcineurin, a Ca^{2+}-/calmodulin-dependent phosphatase 2B. *Xenopus* calcineurin is expressed throughout early development (Saneyoshi *et al.* 2000). Furthermore, injection of constitutively active mouse calcineurin into a ventral position produces a double axis (Nishinakamura *et al.* 1997).

To conclude, we propose a new model of neural induction to modulate the concept of the 'by default' mechanism. Our new model integrates the activation of a Ca^{2+}-dependent signalling pathway due to an influx of Ca^{2+} through DHP-sensitive Ca^{2+} channels. While Ca^{2+} is required for the activation of neural-specific genes, epidermal determination occurs when the Ca^{2+}-dependent signalling pathway is inactive (figure 3).

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