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Cell communication with the neural plate is required for induction of neural markers by BMP inhibition: evidence for homeogenetic induction and implications for Xenopus animal cap and chick explant assays

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Abstract

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In *Xenopus*, the animal cap is very sensitive to BMP antagonists, which result in neuralization. In chick, however, only cells at the border of the neural plate can be neuralized by BMP inhibition. Here we compare the two systems. BMP antagonists can induce neural plate border markers in both ventral *Xenopus* epidermis and non-neural chick epiblast. However, BMP antagonism can only neuralize ectodermal cells when the BMP-inhibited cells form a continuous trail connecting them to the neural plate or its border, suggesting that homeogenetic neuralizing factors can only travel between BMP-inhibited cells. *Xenopus* animal cap explants contain cells fated to contribute to the neural plate border and even to the anterior neural plate, explaining why they are so easily neuralized by BMP-inhibition. Furthermore, chick explants isolated from embryonic epiblast behave like *Xenopus* animal caps and express border markers. We propose that the animal cap assay in *Xenopus* and explant assays in the chick are unsuitable for studying instructive signals in neural induction.

Keywords

Xenopus; chick; neural induction; default model; neural plate border; homeogenetic induction; neural crest; pre-placodal region; animal cap assay; BMP signaling; GATA

Introduction

Since the discovery of neural induction by Spemann and Mangold in 1924 (Spemann and Mangold, 1924), there has been considerable interest in identifying the signals responsible. Relatively little progress was made until about a decade ago, when the “default model” was proposed (Hemmati-Brivanlou and Melton, 1997a, 1997b; Harland, 2000; Muñoz-Sanjuán and Brivanlou, 2002). This model states that Bone Morphogenetic Proteins (BMP) are initially active throughout the entire ectoderm. As gastrulation starts, the organizer and dorsal mesoderm secrete BMP antagonists generating a dorso-ventral gradient of BMP activity. Consequently neural tissue, neural crest and epidermis arise in the ectoderm at progressively higher levels of BMP activity as they are situated further away from the dorsal mesoderm. Since the default model was first proposed there has been considerable controversy concerning whether or not it provides an adequate explanation for neural induction. Recent experiments in chicken and *Xenopus* embryos indicate more complexity to the establishment of a functional neural plate (Streit et al., 1998; Streit and Stern, 1999c, 1999b; Streit et al., 2000; Linker and Stern, 2004; De Almeida et al., 2008). In particular, one set of experiments in the chick raised the possibility that not all of the ectoderm, as the default model predicts, but only cells close to the neural/epidermal border are sensitive to BMP and its antagonists (Streit et al., 1998; Streit and Stern, 1999b). We therefore re-examined this issue in *Xenopus* and chick to determine whether the two systems behave in a comparable way. In both, we find that non-neural ectoderm can be neuralized by BMP inhibition only when the BMP-inhibited cells form a continuous trail from the neural plate or its border. This suggests that homeogenetic (induction of like by like – in this case induction by the neural plate; (Mangold and Spemann, 1927; Mangold, 1929, 1933; Nieuwkoop et al., 1952; Servetnick and Grainger, 1991) inducing signals from the neural plate can only travel between BMP inhibited cells. We wondered whether the animal cap, which is easily neuralized by BMP inhibitors, might be equivalent to the neural-epidermal border. Detailed fate maps reveal that even the smallest caps contain cells fated to contribute to this border. Finally we show that chick epiblast explants express markers consistent with a border-like identity and behave like Xenopus animal caps.

Materials and methods

Xenopus embryology

Fertilization, staging, injections, lineage tracing, animal cap assays and in situ hybridisation were performed as described (Linker and Stern, 2004). mRNA was transcribed from *Smad6-pCS2+* (Linker and Stern, 2004). *CerberusShort-pCS2+* was kindly provided by E. de Robertis (Piccolo et al., 1999), Δ*Smad7-pCS2+*, *TEV2GR-pCS2+* by M. Whitman (Wawersik et al., 2005), *FGF8a-pCS2+* by R. Harland (Fletcher et al., 2006) and *eFGF-pCS2+* (*Xenopus* FGF4) by J. Slack (Isaacs et al., 1994). Nuclear-*LacZ* mRNA or 5-10ng lysine-fixable-fluorescein (FDX, 40,000 M_r; Molecular Probes) were used as lineage tracers. Where noted, dexamethasone (DEX) was added (final: 10μM).

Animal caps of different sizes were transplanted from FDX-injected embryos into uninjected hosts (stages 8.5-9; (Nieuwkoop and Faber, 1967). Embryos were allowed to heal in ¾ Normal Amphibian Medium (NAM) for 1 hour and grown overnight (to stage 19) in 1/10 NAM at 14° C. After healing, fluorescent and bright-field pictures of animal views of the embryos were taken. From these, the projected surface area of the transplanted tissue was calculated using ImageJ. Transplants were categorized as smaller or larger than a “typical” animal cap (Sive et al., 2000) and fate maps generated for each of these. Standardized outlines of embryos at stages 9 and 19 were created by averaging the outlines of 10 embryos at each stage. Fluorescence and bright-field photographs were taken after transplantation, just before fixation and after processing for *Sox3* expression. Images of the embryos were then morphed to the standard outline and the overlap between transplanted areas in different embryos calculated.

Chick experiments

Fertilized hens' eggs (Brown Bovan Gold; Henry Stewart) were incubated at 38°C. Factors were delivered at stage $3^+/4$ (Hamburger and Hamilton, 1951) by electroporation, by grafting transfected COS cells or as proteins adsorbed to heparin-coated acrylic beads. Electroporation was performed (Sheng et al., 2003) using the following cloned into *pCAβ: XSmad7* (Casellas and Brivanlou, 1998; De Almeida et al., 2008), *cSmad6* (Yamada et al., 1999; Linker and Stern, 2004), *cChordin* (Streit et al., 1998), *Xenopus* truncated BMP receptor (*tBR*; (Suzuki et al., 1994) and *cCerberus* (Zhu et al., 1999; Bertocchini et al., 2004). Expression plasmids (*pCDNAII*) encoding *Noggin* (Streit and Stern, 1999b), *Dkk1* (gift of E. Laufer; (Foley et al., 2000), *Crescent* (gift of P. Pfeffer and J.C. Izpisua-Belmonte; (Pfeffer et al., 1997) or soluble *NFz8* (Deardorff et al., 1998) were used to transfect COS cells (Streit et al., 1998; Linker and Stern, 2004; De Almeida et al., 2008). FGF8 (R&D systems, 50μg/ml) was delivered on heparin beads (Streit et al., 2000). Movies of cultured embryos (New, 1955) were made as described (Foley et al., 2000).

In situ hybridisation and whole mount immunocytochemistry were performed as described (Stern, 1998). *Sox2* produces background staining in grafted cell pellets; expression of the markers in the host was therefore assessed in histological sections.

A fluorescein-labelled morpholino (MO) against GATA2 was designed to target the first splicing site: GGGATGCTCATTTACCGTGTGCCTG. Fluorescent GATA3-MO targeted the initial ATG: AGACCTCCATCTTCCGCG. They were co-electroporated as described (Voiculescu et al., 2008).

Tissues from stage XII embryos were dissected using tungsten needles and cultured for 42 hours in collagen gels in medium-199 containing N2 supplement (Streit et al., 1997). Alternate wax sections were processed for in situ hybridization (Etchevers et al., 2001).

Results

BMP inhibition induces neural plate border markers in chick

It was previously shown that BMP inhibition does not induce neural markers (*Sox3, Sox2*) in chick ectoderm (Streit et al., 1998; Streit and Stern, 1999b; Linker and Stern, 2004). However it has not been determined whether this treatment induces neural plate border markers (prospective neural crest/placodes). Electroporation of *Smad6* or *Smad7* into the area opaca epiblast induces *Pax7* (13/14; Fig. 1 A-C), *Dlx5* (9/9; Fig. 1 D-F), *Msx1* (9/10; not shown) and *Slug* (13/14; not shown) but not neural plate (*Sox2*: 0/23; Fig.1 A-F) or mesoderm (*Brachyury*: 0/37; Supplementary Fig. 1 A-L; (Linker and Stern, 2004). It is possible that Smad6 or -7 alone do not inhibit enough BMP-activity for full neural induction. However, even a combination of Smad6 + Smad7 + dominant-negative-BMP-receptor (*dnBMPR*) + Noggin + Chordin + Cerberus, together with FGF and Wnt inhibitors, fails to induce neural markers (*Sox2*: 0/11; Supplementary Fig. 1 J-L). Thus, although BMP inhibition is insufficient for neural induction, it does induce neural plate border markers.

GATA2/3 plays a role in positioning the border

GATA2/3 are targets of BMP signaling (Maeno et al., 1996; Benchabane and Wrana, 2003; Kobielak et al., 2003; Dalgin et al., 2007; Dee et al., 2007) and also induce *BMP4* expression (Sykes et al., 1998). Their expression abuts the anterior/lateral neural plate, partially overlapping with *Sox2* and *Sox3* at late primitive streak stages (Sheng and Stern, 1999). These observations implicate GATA2/3 as candidates to position the neural plate border, perhaps as mediators of BMP activity. To test this, we first confirmed that GATA2 is activated by BMP4 and inhibited by Smad6 in chick epiblast (BMP4: 5/6, Smad6: 7/8, Control: 0/5; Fig. 1 G-L). To test whether GATA2/3 function is required to define the lateral limits of the neural plate or its border, GATA2- and GATA3-morpholinos (MO) were co-electroporated as a line. This causes lateral expansion of *Sox2* expression (6/7; Fig. 1 M-N), but the effect is much less dramatic than misexpression of BMP antagonists near the border of the neural plate (c.f. Fig. 2 A-D). Control-MO (0/7; Fig. 1 Q-R) had no effect and co-electroporation of *GATA2* (lacking the GATA2-MO recognition sequence) rescued the consequences of MO electroporation (8/9; Fig. 1 O-P). These findings are consistent with work in *Xenopus* showing that although inhibition of GATA can mimic some effects of BMP-inhibition, it is not sufficient for neuralization (Sykes et al., 1998). Together, these results implicate GATA2/3 in positioning the neural border, where it may act as a mediator of BMP activity. However, GATA2/3 activity does not completely account for all BMP effects.

Expansion of the neural plate by BMP-inhibition requires cellular continuity of BMP-inhibited cells to the neural plate or its border

Studies using grafts of Chordin- or Noggin-secreting cells have shown that inhibition of BMP affects neural/epidermal choice only at the neural plate border (Streit et al., 1998; Streit and Stern, 1999b; Linker and Stern, 2004). To test whether cell-autonomous BMP antagonists can reproduce this effect, we electroporated *Smad7*, *Smad6* or dominant-negative BMP-receptor (*dnBMPR*) as a line extending outwards from the prospective neural plate. These treatments cause a marked extension in the expression of *Sox2* and *Sox3* into the prospective epidermis and even into the extraembryonic area opaca (*Sox2:* 11/14 [*Smad7*; Fig. 2 A-B], 20/21 [*Smad6*; not shown], 4/5 [*dnBMPR*; not shown], 0/25 [GFP control; not shown]; *Sox3*: 7/7 [*Smad7*; Fig. 2 C-D], 8/8 [*Smad6*; not shown], 0/21 [GFP control; not shown]). Expression of neural plate border markers is also dramatically extended (*Pax7*: 18/18 [Fig. 2 E-G]; *Slug*: 11/12 non shown). Surprisingly, *Pax7* is not restricted to the *Smad*-electroporated cells (Fig. 2 G) but is also seen in neighboring, non-electroporated cells.

This last observation raises the possibility that cells from the host neural plate are stimulated to migrate laterally when BMP is inhibited. To test this, we compared cell movements between the electroporated side and the contralateral side (marked with DiI). No differences were observed between the two sides (Supplementary Movie 1), showing that the expansion of neural plate and border markers by misexpression of cell-autonomous BMP antagonists is due to induction rather than cell recruitment. Together, these results suggest that chick non-neural ectoderm cells can only be induced to express neural markers by BMP-inhibition when these cells form a continuous trail to the neural plate or its border. Without such continuity, only border markers are induced.

Cellular continuity with the neural plate or its border is necessary for neural induction by BMP-inhibition in Xenopus

Does Xenopus ectoderm respond in a similar way? It has been shown that BMP-inhibition is not sufficient to induce neural markers in prospective epidermis (descendants of the A4 blastomeres) and that neural markers are only induced in ventral epidermis by BMP-antagonists when eFGF is also supplied (Linker and Stern, 2004; Delaune et al., 2005). A similar combination (FGF4+Smad6 or Smad7) in chick induces mesodermal markers (Linker and Stern, 2004), raising the possibility that the neural induction by this combination in *Xenopus* is indirect.

First, we confirmed our previous results: inhibition of BMP by injection of *Smad6* (1ng) or *ΔSmad7* (10pg) (Wawersik et al., 2005) does not induce neural markers when injected into the A4 blastomeres (Sox3 [*Smad6* 0/70; *ΔSmad7* 0/237] or Sox2 [*Smad6* 0/60; *ΔSmad7* 0/324] Fig. 3 A-D and Supplementary Fig. 2 A-D). Injection of a combination of *Smad6* (1ng) or *ΔSmad7* (10pg) and *eFGF* (0.16pg) in these blastomeres is now able to induce neural markers (*Sox3* [*Smad6* 108/120; *ΔSmad7* 85/103] *Sox2* [*Smad6* 46/71; *ΔSmad7* 81/102] Fig. 3 E-H, N-O for *Smad6* and Supplementary Fig 2. E-H for *ΔSmad7*).

Next, we analysed whether neural induction by BMP inhibition and FGF activation requires mesoderm. We co-injected *Smad6* (1ng) or *ΔSmad7* (10pg) and *eFGF* (0.16pg) together with the nodal inhibitor *CerS*. To test the effectiveness of CerS, we injected *CerS* in the whole embryo (4 cells at the 4 cell stage, 1.5-2ng). This inhibits the formation of mesoderm (*MyoD* 0/90, *chordin* 0/91, *brachyury* 0/102; not shown) and completely prevents gastrulation, as previously reported (Piccolo et al., 1999). We then tested whether inhibition of Nodal signaling and mesendoderm formation by CerS affects the induction of neural markers by BMP-inhibition+eFGF. Strikingly, co-injection of *CerS* + *eFGF* + *Smad6* or *ΔSmad7* into one A4 blastomere strongly reduces the induction of *Sox3* (*Smad6* from 93% to 20.4%; n=212, Fig. 3 I-J and M or *ΔSmad7* from 82.5% to 6.5%; n=195, Supplementary Fig. 2 I-J) and virtually abolishes induction of *Sox2* (*Smad6* from 62% to 2.7%; n=152, Fig. 3 K-M; *ΔSmad7* from 79.4% to 1.4%; n=174, Supplementary Fig. 2 K-L). Together, these data suggest that in *Xenopus* embryos, as in the chick, the induction of neural markers by eFGF and BMP antagonism is indirect, due to either a prior induction of mesendoderm or to cooperation with Nodal signaling (see also (De Almeida et al., 2008).

To determine whether the activity of eFGF is due to its mesendoderm-inducing ability, we examined whether FGF8a (an isoform without mesoderm inducing activity; (Fletcher et al., 2006) can induce neural markers when injected in combination with BMP inhibitors into ventral epidermis. First, to test the effectiveness of FGF8a, 10-50pg were injected into one cell at the two-cell stage. This did not affect the expression of a mesodermal marker (*Brachyury* 0/60; Fig 4 A), but did expand neural markers (*Sox3* 18/23 not shown, *β-tubulin* 25/28; Fig 4 B), as expected (Fletcher et al., 2006). Next, we tested the effects of injection of *FGF8a* (10-50pg) into the A4 blastomere: neither mesodermal nor neural markers were induced (*Chordin* 0/40, *β-tubulin* 0/17, *Sox2* 0/6, not shown, *Sox3* 0/30; Fig 4 C-D), as was reported

for *eFGF* (Linker and Stern, 2004; Delaune et al., 2005). We then tested if co-injection of *FGF8a* (10-50pg) + *Smad6* (1ng) can induce neural markers in ventral epidermis: neither neural (*Sox2* 0/24 not shown, *Sox3* 0/23; Fig 4 E-F), nor mesodermal markers (Chordin 0/31, not shown) were induced. These results strengthen our previous suggestion that induction of neural markers by FGF activation and BMP antagonism an indirect consequence of mesendoderm induction.

We then analyzed whether BMP-antagonists induce border markers in ventral epidermis in *Xenopus*, as shown above for chick embryos. Indeed, injection of *Smad6* into the A4 blastomere induces the neural border markers *Pax3* (20/26; Fig. 5 A-C), *Slug* (62/73; Fig. 5 D-F), *Hairy2A* (22/33; Fig. 5 G-I) and *Xiro1* (17/19; not shown), but not neural markers (*Sox2*, *Sox3*; Fig. 3 A-D). Thus, as in chick, BMP inhibition in *Xenopus* ventral epidermis induces neural plate border markers.

Finally, we examined if the border of the *Xenopus* neural plate is especially sensitive to BMP-inhibition, as it is in chick. Injection of *Smad6* into the prospective neural plate border (blastomeres A2/3) causes lateral expansion of *Sox3* (43/45; Fig. 5 J-L; white brackets in J and black arrows in L) and *Slug* (38/42; Fig. 5 M-N). These results in chick and *Xenopus* show that although border markers can be induced by BMP-inhibition in lateral/ventral epidermis, neural induction in the same cells requires the BMP-inhibited cells to form a continuous trail to the neural plate and/or its border.

The Xenopus animal cap behaves like a neural plate border and contains prospective border cells

The above results are at odds with the widely reported finding that *Xenopus* animal caps, thought to contain cells destined to contribute to epidermis but not neural tissue, can be neuralized easily by BMP antagonists (Harland, 2000; Muñoz-Sanjuán and Brivanlou, 2002; De Robertis and Kuroda, 2004; Vonica and Brivanlou, 2006). We therefore performed animal cap assays: animal caps were isolated at stage-8 from embryos injected with *Smad6* in the animal pole at the 2-cell-stage. Unlike injections into A4, animal pole injections of *Smad6* induce *Sox3* (Fig. 5O; 38/38). Moreover, co-injection of *Smad6+CerS* does not inhibit *Sox3* induction in animal caps (Fig. 5P; 50/53). This confirms that animal caps can be neuralized by BMP-antagonism and that this is insensitive to Nodal signaling.

The observation that BMP inhibited cells can express neural markers if they form a continuous trail to the neural plate or its border, together with the fact that animal caps are easily neuralized by BMP antagonists, prompted us to test whether animal caps contain prospective neural plate or border cells. To this end, we assessed the contribution of animal cap cells to the neural plate and the neural/epidermal border by fate mapping animal caps. Donor embryos were injected with fluorescein-lysine dextran (FDX) in both cells at the 2 cell stage, and the animal cap excised from these embryos at stage 8. The excised tissue was grafted into an identical region of unlabeled host embryos at the same stage and analyzed at stage-19, examining both fluorescence as a lineage tracer and expression of the neural marker *Sox3* (Fig. 6 A-C). The outlines of all small and all large transplants, at stage 8 and stage 19, were drawn in separate model embryo outlines (see Materials and Methods; Fig. 6 D-E and H-I). In Fig. 6 F and J (stage 8) and G and K (stage 19), the areas that receive a cellular contribution from 60%, 80% and 93% of the transplants are shown in red, orange and yellow, respectively. At stage 19, the region expressing *Sox3* is also shown (grey; Fig. 6 G, K). Surprisingly, 60% of even the smallest caps (Fig. 6 D-G) contribute to the anterior neural plate itself and virtually all caps (>80%) contribute to the anterior neural/epidermal border (prospective placodes; Fig. 6 D-K). These data show that nearly all animal caps dissected at stage 8 contain neural plate and/or neural plate border cells.

Chick epiblast explants behave like the Xenopus animal cap

It has been reported that explants of "lateral" chick epiblast (mainly prospective non-neural ectoderm) can be induced to express neural markers in response to BMP antagonists in culture (Wilson et al., 2000; Wilson et al., 2001). The above results raise the possibility that chick explants are equivalent to *Xenopus* animal caps and are specified as border cells. To assess this we dissected "medial" and "lateral" epiblast (Wilson et al., 2000; Wilson et al., 2001) from stage-XII (Eyal-Giladi and Kochav, 1976) chicken embryos and assessed expression of neural and neural border markers after 42 hours' culture (Fig. 7A). Both medial and lateral explants express neural (*Sox3:* medial 10/11, lateral 8/8; *Sox2:* medial 10/10, lateral 6/6; Fig. 7 B-E) and neural border markers (*Pax7:* medial 9/9, lateral 8/8; *Slug:* medial 8/11, lateral 9/10; *Msx1:* medial 5/9, lateral 8/8; Fig. 7 F-K). These results suggest that under these conditions, epiblast explants from any embryonic region are specified as neural plate and its border, explaining the discrepancy between the results of BMP inhibition *in vivo* (Streit et al., 1998; Streit and Stern, 1999b; Linker and Stern, 2004; De Almeida et al., 2008) and *in vitro* (Wilson et al., 2000; Wilson et al., 2001).

Discussion

BMP-inhibited cells express neural markers only when they form a continuous trail to the neural plate or its border

The default model proposes that BMP inhibition is the only necessary signal for neural induction. Although its simplicity made it very attractive, there has been considerable debate about whether this mechanism is sufficient to explain neural induction (for reviews see (Streit and Stern, 1999a; Stern, 2005, 2006). The current prevailing view is that additional factors are required, and in particular that FGF signaling is important. At least in the chick, BMP inhibitors alone have not been shown to induce any markers (neural or otherwise) in vivo to date. Here we show that BMP inhibition induces border markers in non-neural ectoderm of both chick and *Xenopus* embryos, but neural induction in the same cells only occurs if the BMP-inhibited cells form a continuous trail connecting them to the neural plate and/or its border. These findings suggest that neuralizing factors emanating from the neural plate spread through the ectoderm ("homeogenetic induction", or induction of neural plate by neural plate; (Mangold and Spemann, 1927; Mangold, 1929, 1933; Nieuwkoop et al., 1952; Servetnick and Grainger, 1991), but only between BMP-inhibited cells.

We propose a relay mechanism by which homeogenetic signals can spread from the neural plate only through cells in which BMP signaling is inhibited. Overexpression of BMP inhibitors in cells adjacent to the neural plate or its border allows these cells to respond to homeogenetic neural inducers emanating from the neural plate, resulting in an expansion of the neural territory. In contrast, cells distant from the endogenous neural plate (e.g. the progeny of the A4 blastomere in *Xenopus* or distant epiblast cells in chick, which are competent to make neural tissue in response to an organizer graft or to mesoderm generated by co-injection of eFGF and Samd6) cannot receive homeogenetic inducing signals unless they are connected to it by a continuous trail of BMP-inhibited cells. This provides an explanation for why not all cells injected with BMP antagonist express neural markers (Fig. 5L). Only those cells that are adjacent to the neural plate (black arrows, Fig. 5 L) express neural markers, while cells distant from it (blue arrows, Fig. 5 L) do not.

However, these results also generate a paradox. BMP inhibition in prospective epidermis induces border markers but not neural markers, while inhibition of BMP at the border does induce neural markers. Despite this, increasing the amount of BMP inhibition (in the case of chick even by a combination of Smad6 + Smad7 + dnBMPR + Noggin + Chordin + Cerberus, together with FGF and Wnt inhibitors) outside the border is not sufficient to induce neural

markers. This suggests that the border markers induced by BMP inhibitors alone (*Slug*, *Pax7*, *Dlx5*, *Msx1*) are not indicative of induction of a full-fledged border, and that other factors must also be important. This is consistent with one model of neural crest induction proposing that signals from the underlying mesoderm are required along with BMP-inhibition for neural crest to be specified (Streit and Stern, 1999b; Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005).

What could be the missing signals, present in the neural plate, which can only travel between BMP-inhibited cells? A possible candidate is the Notch pathway, which has been implicated in establishing the border of the neural plate (Kintner, 1992; Cornell and Eisen, 2002; Endo et al., 2002; Glavic et al., 2004) as well as generating boundaries between adjacent domains in many other systems (Bray, 1998; Sanson, 2001; Bray, 2006). Preliminary experiments with Notch inhibitors (DAPT) or NICD overexpression did not produce clear results (unpublished observations), but do not exclude this possibility, which requires further investigation.

The animal cap behaves like the neural plate border and contributes cells to it

The above results prompted us to explore whether animal caps, which are so easily neuralized by misexpression of BMP antagonists, might contain some neural plate and/or border cells. Systematic fate mapping of animal caps of a wide range of sizes revealed that even the smallest caps contribute cells to the anterior neural plate itself in as many as 60% of cases, and nearly all caps contribute to the prospective placodal domain at the border of the anterior neural plate. Furthermore, isolated animal caps express both anterior neural (*Otx2*) and border (*XAG1/XCG1*) markers (Lamb et al., 1993; Knecht et al., 1995; Lamb and Harland, 1995). Although these data are consistent with previous fate maps made at the 32 cell stage (Dale and Slack, 1987; Moody, 1987a, 1987b), our results provide the first demonstration that virtually all animal caps excised at stage 8 contain cells fated to became neural plate border. These findings explain why animal caps can be neuralized so easily by BMP-antagonists.

Thus, animal cap explants contain prospective border cells (as well as prospective neural plate in many cases). This implies that when animal cap assays from BMP-antagonist-injected embryos are used for assessing neural induction, the animal cap preserves cellular continuity between the prospective neural plate/neural plate border and prospective epidermis, through which neural inducing signals can spread (see above). This may also explain why neural marker expression is always restricted to a subset of cells in animal caps excised from BMP-inhibited embryos (e.g.: Fig. 5 O-P). We (Fig. 7) and others (Wilson et al., 2000; Wilson et al., 2001) have made similar observations in explants of chick epiblast.

The animal cap assay was designed by Nieuwkoop to study mesoderm induction (Nieuwkoop, 1969b; Nieuwkoop, 1969a), because the animal pole does not contain prospective mesoderm. It is indisputable that, in addition to providing an understanding of mesodermal induction (e.g.: (Slack et al., 1987; Green et al., 1990; Green and Smith, 1990; Kimelman and Bjornson, 2004), this assay has also identified a number of important functions of BMP signaling (Smith and Harland, 1992; Sasai et al., 1994; Zimmerman et al., 1996). However, our finding that animal caps excised from stage 8 embryos contain prospective neural plate and border cells suggests that this assay is not suitable for studying neural induction because it cannot distinguish between “permissive” (stabilising) and true “instructive” induction (Gurdon, 1987; Streit and Stern, 1999a; Stern, 2001). We propose that experiments targeting the A4 blastomere in *Xenopus* and peripheral misexpression in the chick, provided that the cells are not contiguous to the neural plate, are more rigorous assays for neural inducing signals than animal cap assays or chick epiblast explants.

Interestingly, fate and specification maps of pre-primitive-streak-stage chick embryos reveal that almost the entire epiblast contributes cells to the neural plate and/or its border (Rudnick,

1935, 1938; Hatada and Stern, 1994). One difference between the *Xenopus* animal cap and the chick explant assays is that neural markers are only expressed in the latter. One possible reason for this difference is that chick explants are grown in the presence of complex culture medium that includes N2 supplement (containing a number of factors including insulin, transferrin and others, intended to promote neural differentiation) whereas *Xenopus* animal caps are cultured in simple saline. Together, our results suggest that candidate neural inducing signals revealed by chick epiblast explants or *Xenopus* animal cap assays need to be validated in ectodermal cells distant from the endogenous neural plate and its border.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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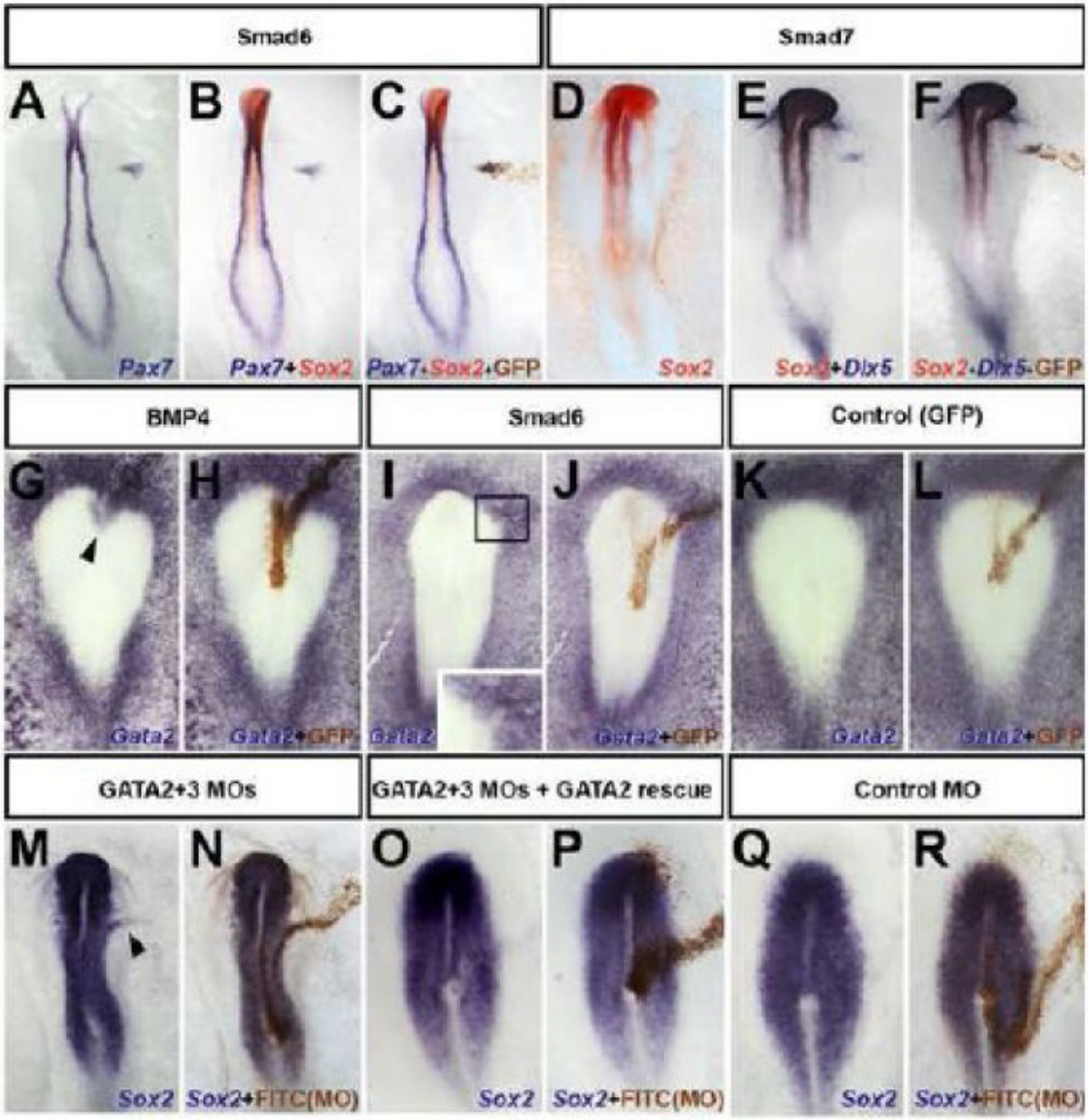

Fig. 1. BMP inhibitors induce neural plate border markers in chick

A-L. Electroporation of Smad6 or Smad7 in prospective epidermis induces *Pax7* (A-C) and *Dlx5* (E-F) in the absence of *Sox2* (B-C and D-F). Electroporation of *BMP4* induces *Gata2* in the neural plate (G-H). Inhibition of BMP by Smad6 inhibits *Gata2* at the neural border (I-J). GFP (control) does not affect *Gata2* (K-L). **M-R**. Gata-2/-3 morpholinos expand *Sox2* into the non-neural territory (M-N) (arrowhead), which is rescued by *Gata2* (O-P), the slight down-regulation of Sox2 in the neural plate is an electroporation artefact; control morpholino has no effect (Q-R). Electroporated cells were stained with anti-GFP antibody (C,F,H,J,L, for the embryos to their left) or with anti-FITC antibody (N,P,R).

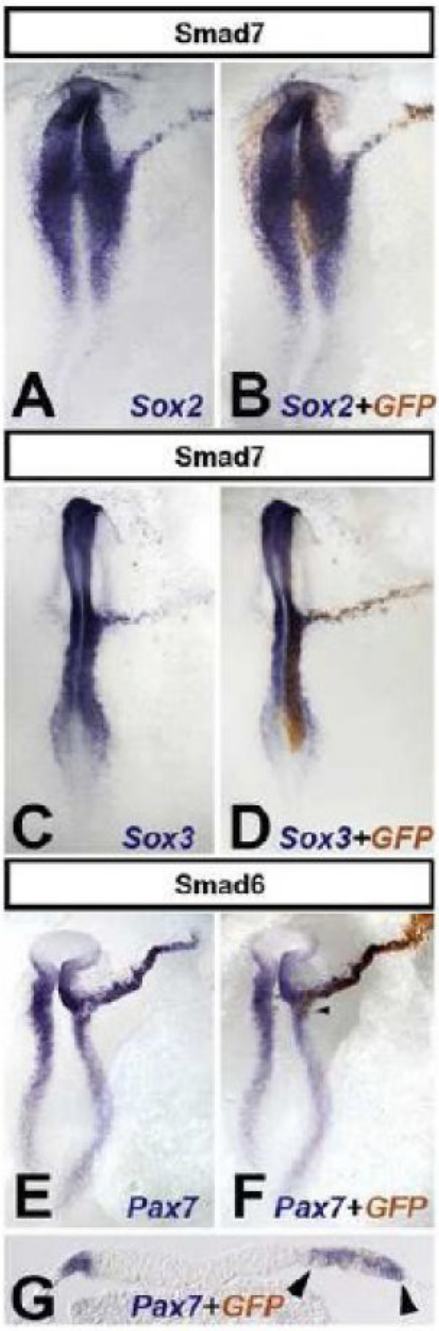

Fig. 2. Only the border of the neural plate is sensitive to BMP in chick

Electroporation of Smad6 or Smad7 as a line extending out from the neural plate induce an expansion in the expression of *Sox2* (A-B), *Sox3* (C-D) and *Pax7* (E-G). G is a section through the embryo in F (arrowhead), showing non-cell-autonomous expansion of *Pax7* (arrowheads). Electroporated cells were stained with anti-GFP antibody (B, D, F and G for the embryos to their left).

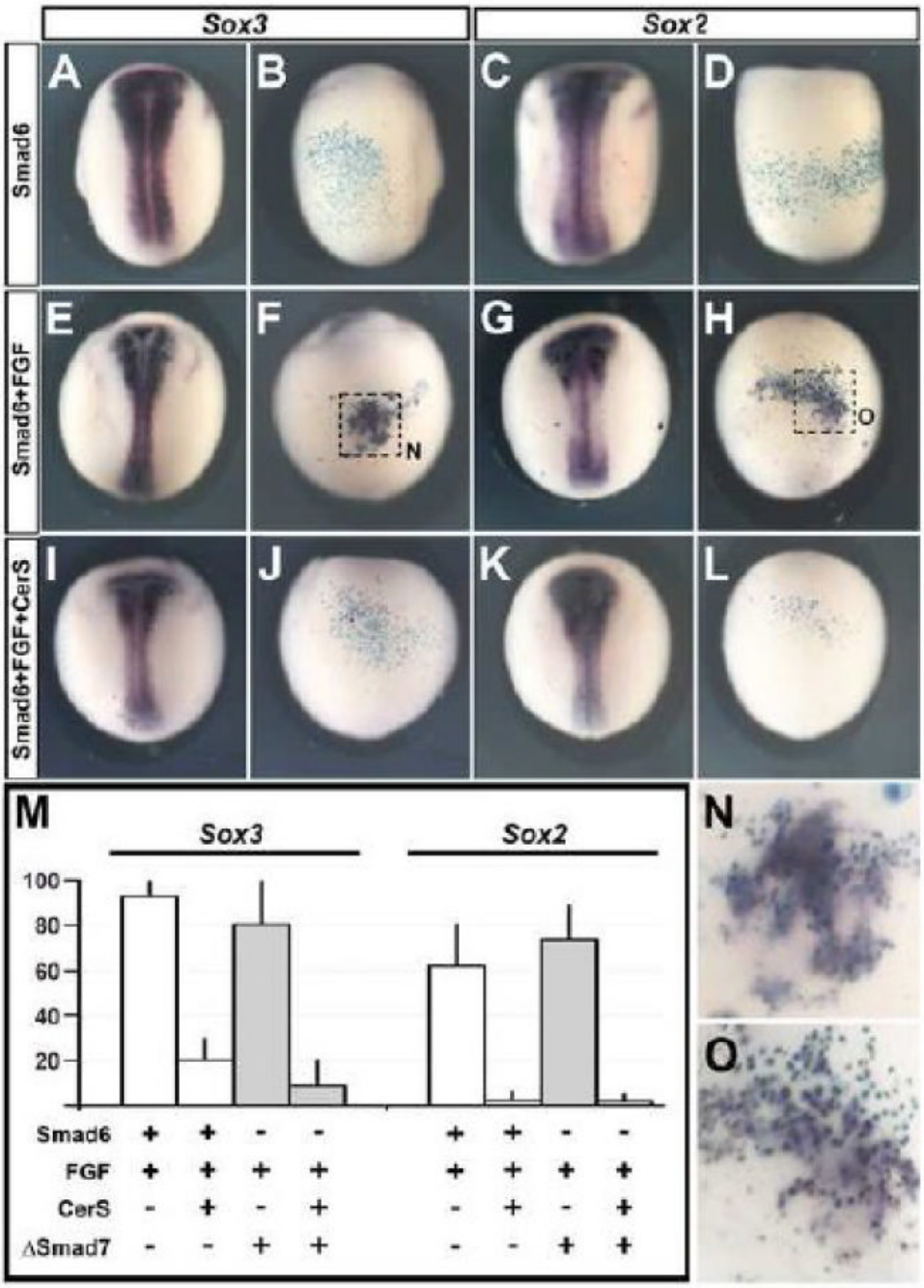

Fig. 3. BMP inhibition together with eFGF activation induces neural marker expression indirectly in *Xenopus*

A-L. Inhibition of BMP by injection of *Smad6* into the A4 blastomere does not induce either *Sox3* (A-B) or *Sox2* (C-D) expression. eFGF together with BMP inhibition into the A4 blastomere induces *Sox3* (E-F, N) and *Sox2* (G-H, O). Neural induction by the former combination is inhibited when Nodal signaling is blocked: injection of *Smad6 + eFGF* together with *CerS* no longer induces *Sox3* (I-J) or *Sox2* (K-L). **M**. Quantification of *Sox3* and *Sox2* expression in the different experiments described above. A, C, E, G, I and K dorsal views. B,D,F,H,J and L ventral views of the embryos to their left. N and O are enlargements of the areas enclosed by a square in F and H, respectively.

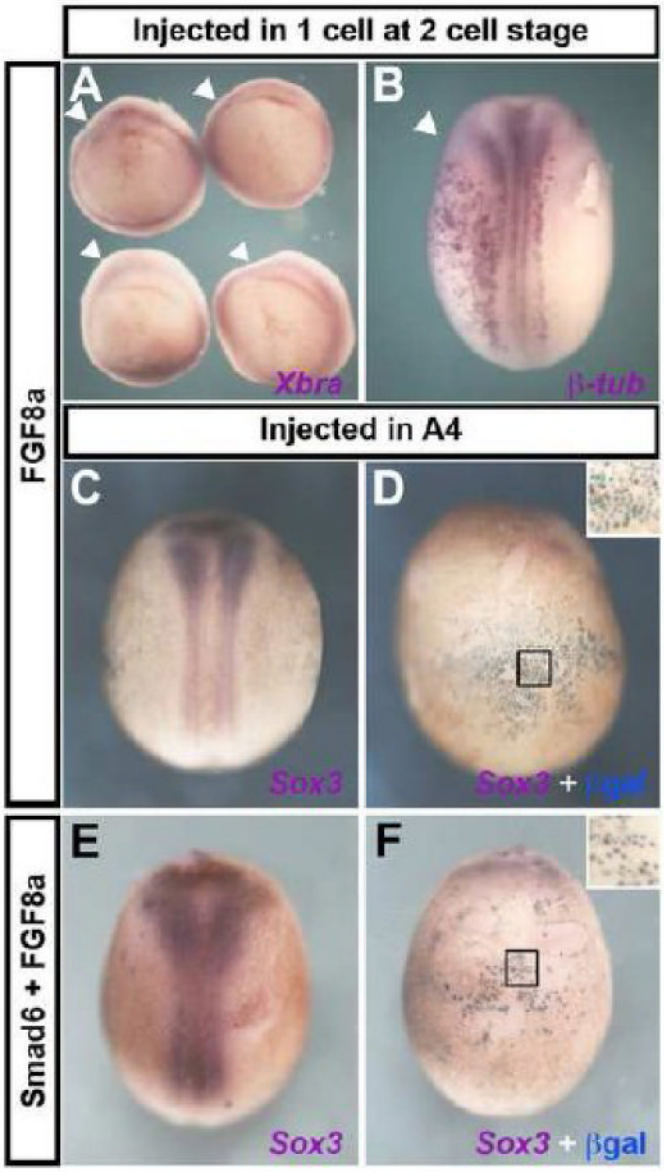

Fig. 4. BMP inhibition together with FGF8a does not induce neural marker expression in *Xenopus*

A-B. Injection of FGF8a into one cell at the two-cell stage does not alter *Brachyury* expression at the gastrula stage (A) but does expand *β-tubulin* expression at the neurula stage (B); arrowheads indicate the injected side. **C-F.** Injection of *FGF8a* into an A4 blastomere, alone (C-D) or in combination with the BMP inhibitor *Smad6* (E-F) does not induce *Sox3* expression in ventral epidermis. A: vegetal view; B, C and E are dorsal views; D and F are ventral views of the embryos to their left. Black squares show the area enlarged in the inset in panels D and F.

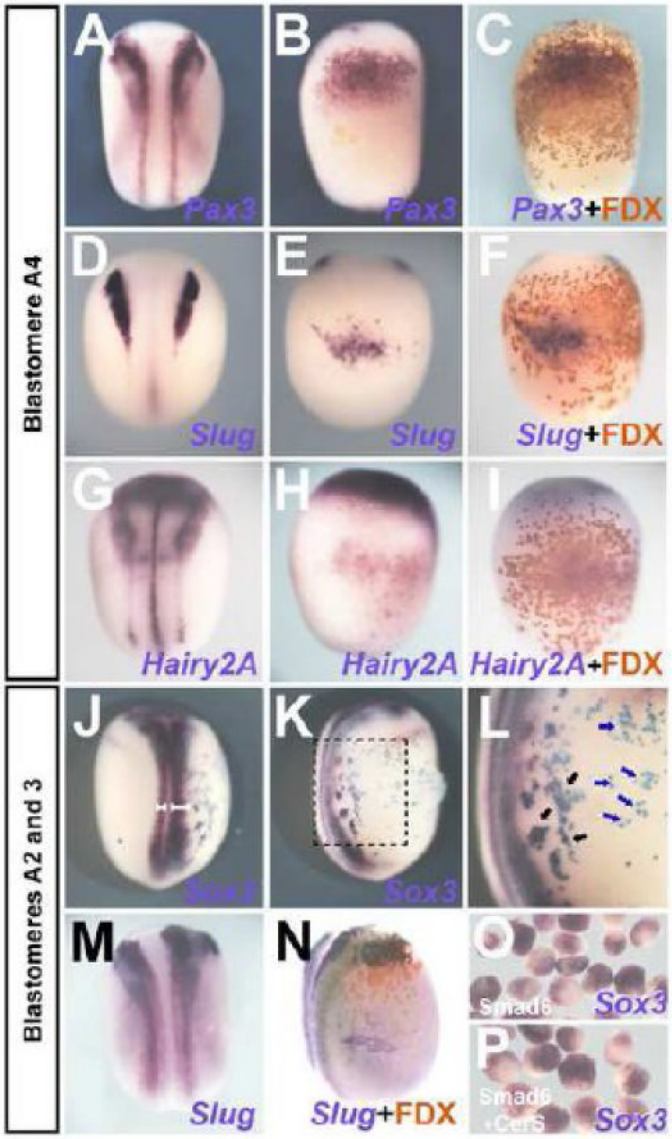

Fig. 5. Only the border of the neural plate is sensitive to BMP inhibition in *Xenopus*

A-I. *Smad6* (1ng) injection into the A4 blastomere induces *Pax3* (A-C), *Slug* (D-F) and *Hairy2A* (G-I). (A, D, and G: dorsal view; B, C, E, F, H and I: ventral view of the embryo to their left). **J-N.** Injection into blastomere A2/3 expands *Sox3* (J-L) and *Slug* (M, N). J, M: dorsal view; K, L and N are lateral views of the embryos to their left. White brackets in J show the extension of the neural plate in the injected and non-injected sides of the embryo. The black square in K indicates the area enlarged in L. Black arrows in L point to injected cells adjacent to the endogenous neural plate, expressing *Sox3*; the blue arrows point to injected cells distant from the endogenous neural plate, which do not express *Sox3*. Injected cells were recognized by FDX or LacZ (C, F, I, K, L and N, for embryos to their left). **O-P**. Animal caps from Smad6-injected embryos at the 2-cell stage express *Sox3* (O), which is not inhibited by *CerS* (P).

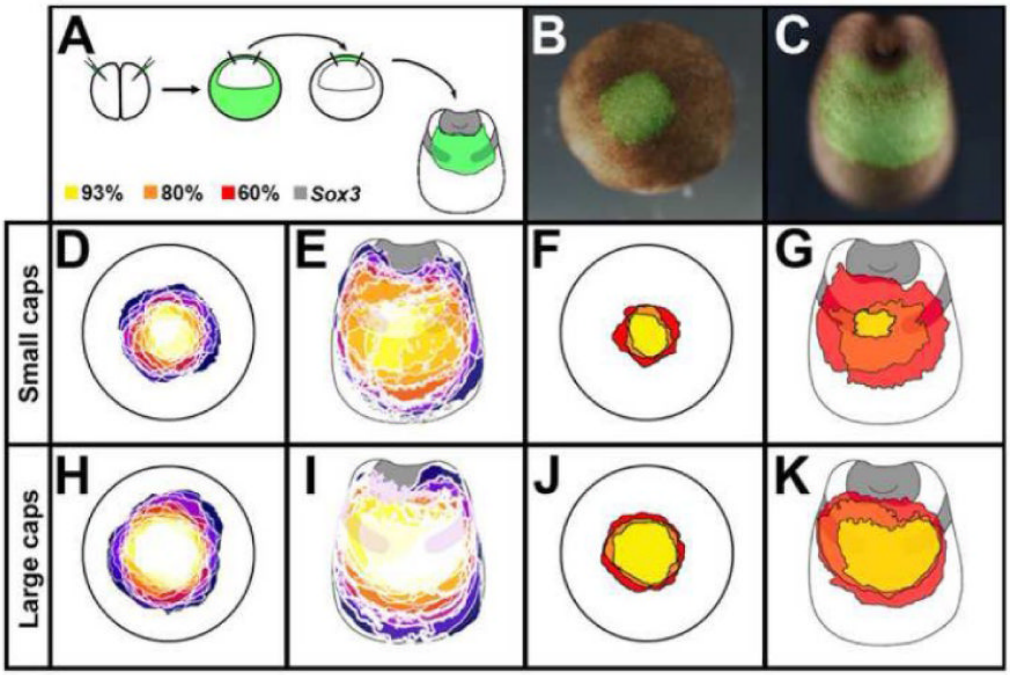

Fig. 6. The *Xenopus* animal cap contains cells fated as anterior neural border

A. Caps from FDX-injected embryos were obtained at stage 8, transplanted to uninjected hosts and analysed for *Sox3* at stage 19. **B.** Example of a transplant at st.8 after 1.5h healing, the same embryo at st.19 (C). **D-K**. Results of all small (D-G; n=14) and large (H-K; n=15) transplants, each in a different colour, at stages 9 (D,H,F,J) and 19 (E,G,I,K; including *Sox3* expression). In D-E and H-I, the regions of overlap are shown in progressively lighter shades, with white indicating a region where all transplanted caps overlap. In F-G and J-K, the areas that receive a cellular contribution from the transplant are in Yellow: 93%; Orange: 80%; Red: 60%.

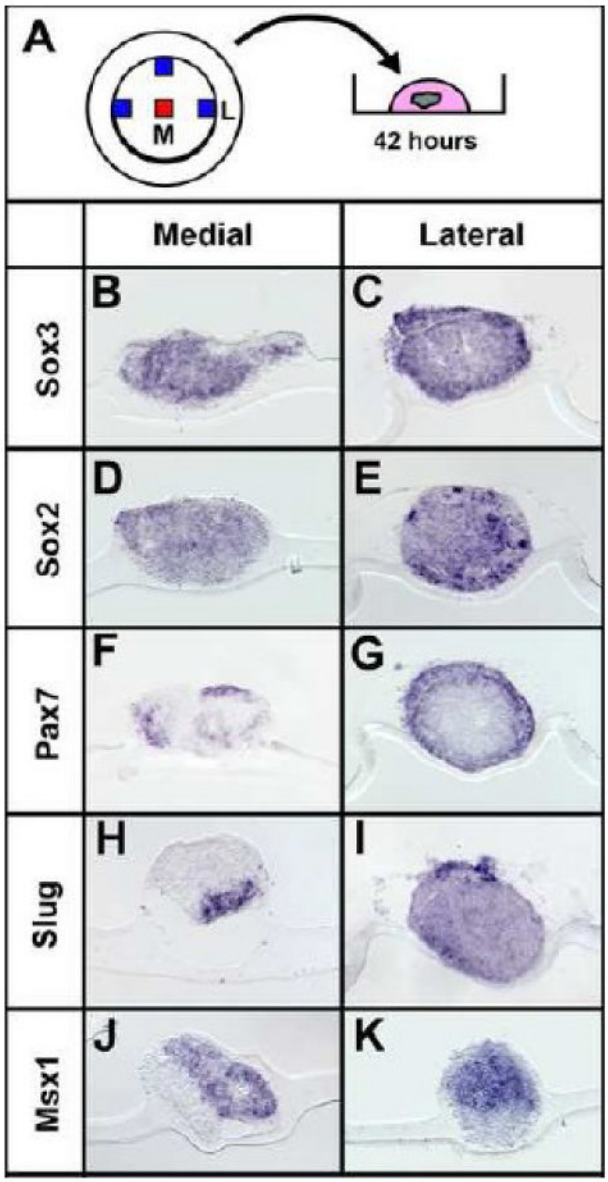

Fig. 7. Chick epiblast explants express neural plate and border markers

A-K. Medial (M) or lateral (L) stage-XII epiblast explants were analysed for neural plate/border markers in alternate sections. All express *Sox3* (B-C), *Sox2* (D-E), *Pax7* (F-G), *Slug* (H-I) and *Msx1* (J-K).