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Neural induction: 10 years on since the 'default model'

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Neural induction is the process by which embryonic cells in the ectoderm make a decision to acquire a neural fate (to form the neural plate) rather than give rise to other structures such as epidermis or mesoderm. An influential model proposed a decade ago, the 'default model', postulated that ectodermal cells will become neurons if they receive no signals at all, but that this is normally inhibited in prospective epidermal cells by the action of bone morphogenetic proteins. Recent results now reveal considerable more complexity and emphasis is shifting from intercellular signalling factors to trying to understand the regulation of expression of key genes within the nucleus.

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Introduction

It is now just over 10 years since data from the laboratories of Eddy De Robertis, Richard Harland, Ali Hemmati-Brivanlou and Doug Melton started to converge to a view that has become very influential: that in the early embryo, ectodermal cells have a 'default' neural fate, which they adopt if they receive no signals at all from neighbouring cells, and that during normal development, bone morphogenetic proteins (BMPs) inhibit this fate and specify epidermis on the ventral side of the embryo (reviewed in [1–4]). While new data strongly suggest that this view may be a little too simplistic, the model continues to hang on.

Current status of the default model

Apparently supporting the model, two important papers from the De Robertis laboratory [5^{••},6^{••}] now show that when three different members of the BMP family (BMPs 2, 4 and 7) are inhibited by injection of morpholino antisense oligonucleotides at the one- or two-cell stage, the embryos have severe trunk defect but still display some dorsoventral polarity, including ventral tissues. However, when a more divergent member of the same family (anti-dorsalising morphogenetic factor, or ADMP)

is simultaneously inhibited, a massive brain develops [6^{••}]. The three BMPs are normally expressed ventrally and are proposed to act as epidermal inducers, but the surprising observation here is that ADMP, which is expressed dorsally (near the neural tissue), paradoxically contributes to specify ventral tissues. Consistent with this, De Robertis and colleagues also show that knock-down of the three BMPs and elimination of the organiser itself by UV treatment or β -catenin depletion has the same effect as eliminating ADMP [5^{••}]. Therefore these studies suggest a certain amount of redundancy in the activities of different members of the BMP family, and partly explain why loss of function of any one of these does not completely neuralise the entire ectoderm.

A similar view emerges concerning the endogenous inhibitors of BMP activity, many of which are expressed in the organiser (the dorsal region of the embryo that is capable of inducing a nervous system when transplanted ventrally). The first such inhibitors to be discovered were Noggin, Chordin and Follistatin (reviewed in [2]). Now, in zebrafish, whose genome has undergone separate gene duplication events during evolution, a new study [7] reveals that a Follistatin-like product (*fstl2*) and *Noggin1* both act redundantly with Chordin to pattern the dorso-ventral axis of the embryo: knockdown of *fstl2* with morpholino oligonucleotides (again, injected at a very early stage of development) is able to remove the dorsal and dorso-lateral territories that remain in Chordin mutants. Interestingly, they are able to rescue the phenotype of *chordin–Noggin1* double-morphants by addition of either *follistatin1* RNA or *fgf8* RNA, apparently supporting the notion that FGF signalling can act as an additional BMP antagonist (see below and [4,8[•],9[•],10]).

A link between epidermal specification at the ventral side and the ventralising activity of BMP-related signals is also revealed by a new study [11] reporting that Ectodermin, a novel RING-type ubiquitin ligase for the TGF β /BMP signal transducer Smad4, also plays a role in neural/epidermal cell fate choices and in dorsoventral patterning of the embryo. Clearly the BMP pathway is important in ventral and epidermal fate specification, accounting for why screens such as this, designed to identify novel players in ventralisation, often uncover components of this pathway.

Previous studies have also implicated FGF signalling in neural induction, although their precise mode of action has remained controversial. It had previously been shown [12] that activation of the MAPK cascade by FGF and related factors like IGF results in the phosphorylation of a

crucial linker region of the BMP effector Smad1, which acts as an inhibitor of the BMP pathway. In two important new studies, the De Robertis and Soriano groups now confirm this activity in *Xenopus* and mouse, respectively [8*,13]. In *Xenopus*, one of the most important experiments cited in support of the default model relates to the behaviour of dissociated ectodermal cells, which acquire a neural fate. Because the effects can be reversed by addition of BMP protein to the medium, this has been interpreted to mean that dissociation results in dilution of the endogenous levels of BMP, which then allows ectoderm cells to acquire their 'default' neural fate. The study from the De Robertis laboratory now shows that, surprisingly, BMP ligands continue to signal during such dissociation, but the dissociation event triggers activation of the Ras/MAPK pathway, which in turn causes phosphorylation of the Smad1 linker region; the authors conclude that it is this mechanism of BMP inhibition that is important in the effect of dissociation on neuralisation, rather than dilution of the extracellular BMP ligand [8*]. However, this study is unable to prove formally that it is linker phosphorylation, rather than some other effect of MAPK activation, that is required for neuralisation in this experiment. In the mouse study [13], a compelling genetic approach is used to separate the roles of Smad1 phosphorylation at this linker from the more 'classical' phosphorylation at the C-terminus, caused by BMP signals. Interestingly, the phenotypes of mutants defective in each of these regions of Smad1 are quite different, with the C-terminus being more important for development while mutations in the linker region allow development to term, without obvious major neural defects.

Two further studies cast more doubt on the simplest version of the default model as a sufficient explanation for neural induction. In *Xenopus*, FGF signalling is suggested to play a role at an earlier stage and independently from its ability to inhibit BMP signalling [9*,14*]. In chick, where the role of BMP signalling in any aspect of neural induction had yet to be demonstrated, it is now shown that BMP signals do inhibit the expression of a 'definitive' neural plate marker, *Sox2*, but not of the earlier marker, *Sox3* [14*]. One very significant difference between these two studies and most of their predecessors is that the assays used for neural induction in these papers focus on cells (descendants of the most ventral animal blastomere at the 32 cell stage, A4) whose normal fate is not to contribute to the neural plate, yet are fully competent to do so when exposed to organiser-derived signals. Most other studies have used animal cap cells (excised from the animal pole), which include many cells fated to contribute at least to neural crest, if not to neural plate itself, and which may therefore have already received some early signals before animal cap excision. Taken together, the view emerging from these studies is that neural induction is not a single step, but rather requires a sequence of signals in a specific order, with

BMP inhibition possibly constituting a relatively late, maintenance event [3].

One view that has floated in the field for some time is that inhibition of BMP transcription may be at least equally important to inhibition of BMP activity [15], although how such a mechanism might influence cell fate is not at all clear. A study in *Xenopus* [10] now also proposes FGF as a regulator of BMP transcription at late blastula stages in addition to its other, better-documented roles. Interestingly, the FGF-mediated repression of BMP transcription occurs in the absence of protein synthesis.

Most of the above studies (especially those using genetic mutants or injection of morpholinos) rely on manipulation of signals at very early stages of development (such as the one- or two-cell stage in *Xenopus*) followed by analysis of the results at much later stages. Since the BMP and FGF pathways are known to be involved in numerous early events, such experiments will reveal the cumulative effects of interference with all of these events, making it difficult to separate neural induction specific roles from other early patterning roles of the same pathways. An interesting recent study tries to address this issue by constructing an ingenious two-component system that allows BMP inhibition to be started at a specific period in *Xenopus* development. The study reports that blocking BMP signals at the gastrula stage (when neural induction was traditionally thought to occur) does not induce neural tissue: rather, BMP inhibition can only result in ectopic neural tissue forming when applied well before the gastrula stage (stage 9) [16**]. However, when late BMP inhibition is combined with early FGF activation, neural induction is greatly enhanced [16**], strongly supporting the idea that an initial FGF signal, probably independent of BMP-blocking activity, is required before BMP inhibition can act as a neural stabilising event [9*,14*].

In addition to FGF, Wnts have also been implicated in neural induction, and their involvement has again been controversial. In chick epiblast explants it was suggested that Wnt inhibition cooperates with FGF to induce neural fates [17], while in *Xenopus* it was proposed that Wnt activation is required for neural induction [18]. A new study is now more consistent with the chick data, showing that blocking canonical (β -catenin-dependent) Wnt signalling by a variety of means results in expansion of the neural plate [19]. The reason for the discrepancy between these sets of results is almost certainly timing. At very early stages, canonical Wnt signalling is required for specification of the dorsal side of the whole embryo, where the organiser will form, and this structure is in turn required for patterning neural and non-neural domains of the later embryo. At later stages, canonical Wnt signalling may have an additional, inhibitory influence on the acquisition of neural fates. However, the stage at which this occurs (if at all) and the sources of

signals responsible for inhibiting Wnt at this later stage remain obscure.

Finally, another study from the De Robertis laboratory [20] focuses on a very early group of cells present on the dorsal side at the gastrula stage of *Xenopus*; these cells already express the BMP antagonists Noggin and Chordin. For this reason the authors refer to these cells as the blastula chordin and noggin expressing centre (BCNE), which they consider to be an early organiser. Apart from the BMP inhibiting activities of the BCNE, the authors propose, as suggested by some of the earlier studies, that at the early blastula stage a β -catenin signal (canonical Wnt) predisposes the future neuroectoderm to subsequent neural induction signals coming from the mesoderm of the organiser. However, the timing of these events and the precise relationships between them in connection with neural induction (as opposed to other early developmental events) remain difficult to disentangle.

In conclusion, there is no question that BMP inhibition (probably at both the transcriptional and post-transcriptional levels) is important for ectodermal cells to acquire a neural plate fate. However, it is also now becoming increasingly clear that neither a neural plate (future CNS) nor mature neurons are default fates for ectodermal cells, and that inhibition of BMP signalling is not sufficient for cells to acquire a neural plate fate.

The elusive Notch

In the fly, the Notch receptor plays several key roles in specifying neuronal fates. It is therefore surprising that so little is known about its possible involvement in similar processes during the early stages of neural development in vertebrates, except perhaps in the choices between glial and neuronal fates and in neural crest identity, both of which occur at relatively later stages of development (see [21] for review). Two new studies begin to address this, both relating not only to the acquisition of generic neural fates but also to a role in the maintenance of self-renewal capacity, a trade-mark of cells with stem cell properties. In mouse and human embryonic stem (ES) cells, Austin Smith's group reveals a very strong effect of Notch signalling upon the differentiation of these cells: unlike treatment of cultures with various growth factors, which at best generates a relatively low proportion of cells that differentiate into neurons, activation of Notch (together with stimulation of FGF signalling) causes a very substantial proportion of the ES cells to exit the cell cycle and differentiate into *Sox1*-expressing neurons, whereas suppression of Notch signalling, either genetically or with γ -secretase-inhibiting drugs, prevents factors that otherwise induce the cells to acquire a neuronal fate from doing so [22••]. These findings strongly implicate Notch signalling as a key component in the acquisition of neural (or at least neuronal) fate and it will be very

interesting to see whether it plays a similar role during early stages of normal development.

In chick embryos during neurulation, the spinal cord elongates as a result of the continuous growth of a special region at the tail end of the somite stage embryo, which has been called the 'stem zone'. This zone contains self-renewing cells that retain an ability to give rise to neuronal and non-neuronal descendants (including cells outside the neural plate). Here, too, Notch plays a key role: FGF signalling, which is required for the maintenance of the stem zone as a self-renewing region, is necessary for expression of *Cash4*, which in turn induces the Notch ligand, *Delta1* [23•]. When Notch signalling is inhibited, cell proliferation in the stem zone is lost, but cells are not pushed along a neural lineage, nor do they become directed out of the stem zone region. The authors propose that, as in the fly, signalling between cells expressing high levels of *Delta1* maintains the neural precursor pool that gives rise to the spinal cord, and that a lateral-inhibition-like event causes individual cells to up-regulate *Delta1* expression and downregulate Notch as FGF activity decreases (which occurs when they leave the stem zone), resulting in their acquisition of a neuronal identity [23•].

Together, these two studies begin to suggest strong parallels between vertebrate and fly nervous systems in that Notch may be required initially for cells to acquire neural identity, while at a later stage it needs to be downregulated in favour of one of its ligands for neuronal differentiation to proceed. It seems that this see-saw continues at later stages still, since it has been shown that in cultured mammalian neurospheres there is an initial step during which Notch promotes glial fates at the expense of neurons, whereas glial fates are further refined at later stages by Notch promoting astrocyte differentiation and inhibiting both neurons and oligodendrocytes [24].

Heads or tails?

From very early on, when Spemann and Mangold discovered the organiser and the phenomenon of neural induction in 1924 [25], neural induction has been intimately connected with head-tail patterning. The original study discovered that grafts of the organiser generate complete ectopic neural axes, extending from the brain to the tip of the tail. However, some investigators subsequently suggested that induction of the brain and of the trunk and tail are mediated by signals from different ('head', 'trunk' and 'tail') organisers, although this is still the subject of heated debate (for review see [26]). Three new studies [27•–29•] now add fuel to the fire, suggesting that neural induction in the most anterior regions is mediated by BMP inhibition alone, while neural induction of the trunk and tail are mediated by FGF signalling alone (emanating from a ventral signalling centre), with BMP acting mainly to impart caudal fates to this

prospective spinal cord. But again these studies do not separate the inducing events in time, and it remains likely that the manipulations described, which are performed at very early stages of development, affect the pattern of the whole embryo, and the decision between neural and epidermal fates only as a secondary effect of alterations in gross embryo patterning. One very important question that remains to be addressed clearly is the stage is the stage at which rostral-caudal identity is imparted to the CNS. The assumption in many of these studies in fish and frog is that this occurs at least as early as the neural/non-neural decision, but other studies in amniotes suggest that although the most rostral regions may indeed start to be defined early, the CNS does not acquire a discernible regional pattern until much later in development, and then in a progressive way (for review see [30]).

The view from the nucleus

The neural induction field has traditionally concentrated on identifying signalling factors secreted by the organiser that are able to induce neural fates in other cells. While ultimately we need to understand such signals and when and how they act, a full understanding of the process will only be gleaned when we can also uncover the mechanisms responsible for directly activating neural-specific genes and repressing those required for specifying other cell identities. Studies such as these have only just begun. Perhaps surprisingly, it is the chick, rather than *Xenopus*, which is currently in the lead, largely because of huge strides made by Hisato Kondoh's group in Japan. They have concentrated on one important gene, *Sox2*, the first general neural marker whose spatio-temporal expression pattern correlates with the commitment of cells to a neural plate fate and their acquisition of neural plate character. First, a compelling molecular dissection of very complex regulatory regions both upstream and downstream of the reading frame of this gene revealed no less than 23 separate enhancers, each responsible for directing expression to a very specific sub-set of structures [31]. Among these enhancers, two turned out to be particularly relevant to the neural induction process: N1 and N2. The former directs expression to the caudal neural plate (hindbrain and spinal cord), while the latter is responsible for *Sox2* expression in the fore- and midbrain. Each of these is itself complex, containing binding sites for numerous transcription factors. In the case of N1 (which in the chick is first activated relatively late, after gastrulation), activity can be recapitulated by a minimal 56 base pair core element (N-1c), which contains binding sites for components of the FGF and Wnt signalling pathways but not for components of the BMP pathway [32*].

In another study by the same group, the regulation of the neural-specific Nestin gene was examined, and a requirement for SoxB1 sub-class (to which Sox2 itself belongs) and class III POU-domain transcription factors (such as Brn2) demonstrated [33]. Then the group turned its

attention to N-cadherin, which is co-expressed with SoxB1 class genes in the neural plate and primordia of the cranial sensory organs. Here again several enhancers were identified, responsible for different aspects of its expression, and a crucial role for SoxB1-subclass proteins was uncovered [34].

New transcription factors involved in the regulation of these target genes are also starting to be identified and their roles understood. *Churchill* encodes a zinc finger transcription factor shown to regulate the choice, near the embryo's midline, between neural and mesodermal fates (by stopping the continued ingression of cells at the end of gastrulation) [35]. *Churchill* is a transcriptional activator that exerts its actions through its target, Smad-interacting protein 1 (Sip1) [35]. These studies had been conducted largely in the chick, and Makoto Asashima's laboratory have now shown that Sip1 is essential for neural plate specification in *Xenopus* independently of a separate requirement for SoxD [36], while Dale Frank's group reveal, also in *Xenopus*, that another POU domain gene (the Oct3/4 homologue POU91) is an essential regulator of *Churchill* expression and that loss of POU91 function can be rescued by expression of either *Churchill* or Sip1 [37*]. Finally in the mouse, two different Sip1-related genes (Sip1 itself and δ -EF1) are shown to be expressed in complementary patterns and to interact genetically to specify nervous system [38].

Apart from transcription factors and their roles in regulating specific elements to direct expression of critical target genes, a new area of investigation is starting to emerge: larger-scale changes in chromatin structure and the factors responsible for these modifications. At the moment of writing, this has only been applied to the study of the acquisition of neural fate by various cells in culture rather than in the context of the normal embryo, but it is only a matter of time before this is recognised more widely as a key regulatory mechanism in neural induction and other key developmental events. In a pioneering study, Amanda Fisher's group concentrated on the regulation of *Mash1*, a key locus involved in the acquisition of neural fates by ES cells *in vitro*, discovering that the timing of replication of this locus during the cell cycle shifts from late to early during S-phase after 'neural induction' of these cells, and that histone acetylation and methylation play a key role in the regulation of its expression. In addition, the location of chromatin containing the *Mash1* locus moves from a peripheral location to a more central location in the nucleus when the cells are stimulated to differentiate along the neuronal lineage [39*].

Conclusions

Classical experiments (starting from Spemann's original finding that a graft of the organiser induces a complete nervous system in ectopic regions of the host embryo) have often been interpreted as indicating that neural

induction is a single event, occurring at a discrete time in development and involving not just the generation of a neural plate but of one that already displays a considerable amount of organisation. This view was even enhanced by the influential 'default model', whose most extreme form implies that BMP inhibition is sufficient to explain the activity of the organiser. We have come a long way in this decade since the first description of the model. Findings in the last few years have emphasised the complexity of the process of early neural plate development and that this involves sequential, interacting steps. It seems as if we are only now starting to be able to ask the right questions that will lead to a real understanding of the mechanisms that govern this important set of embryonic cell behaviours.

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