

REVIEWS

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Neural Induction Takes a Transcriptional Twist

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ABSTRACT Over the past decade, several molecules have been identified that influence neural cell fate in vertebrate embryos during gastrulation. The first neural inducers studied were proteins produced by dorsal mesoderm (the Spemann organizer); most of these proteins act by directly binding to and antagonizing the function of bone morphogenetic proteins (BMPs). Recent experiments have suggested that other secreted signals, such as Wnt and FGF, may neuralize ectoderm before organizer function by a different mechanism. Neural effector genes that mediate the response of ectoderm to secreted neuralizing signals have also been discovered. Interestingly, most of these newly identified neuralizing pathways continue the theme of BMP antagonism, but rather than antagonizing BMP protein function, they may neuralize tissue by suppressing *Bmp* expression. Down-regulation of *Bmp* expression in the prospective neural plate during gastrulation seems to be a shared feature of neural induction in vertebrate embryos. However, the signals used to accomplish this task seem to vary among vertebrates. Here, we will discuss the role of the recently identified secreted signals and neural effector genes in vertebrate neurogenesis. © 2001 Wiley-Liss, Inc.

Key words: bone morphogenetic protein (BMP); embryo; gastrulation; neural effector genes; neural induction; *Xenopus*

INTRODUCTION

The vertebrate neural plate arises from the embryonic ectoderm during gastrulation. Study of the signals involved in vertebrate neural induction began with Spemann and Mangold's seminal experiment (Spemann and Mangold, 1924) in which the amphibian dorsal blastopore lip or Spemann organizer was shown to induce a secondary nervous system after transplantation to the ventral side of a host embryo. Subsequent experiments have demonstrated the existence of neural-inducing information within a similar domain (the shield of zebrafish and primitive node of chick and mouse) in other vertebrate embryos (Waddington, 1930; Beddington, 1994; Shih and Fraser, 1996). At the molecular level, our understanding of neural induction by the organizer has improved dramatically over the past decade. Several excellent reviews concerning the role of the organizer in neural induction have been published recently (Sasai, 1998; Chitnis, 1999; Streit

and Stern, 1999c; Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000; Robertis and Arechaga, 2001); thus, we will only briefly summarize this topic below. This review will instead focus on newly identified players that act both upstream and downstream of organizer signaling during vertebrate neural plate formation.

BMPs are a subgroup of the TGF-beta family of secreted growth factors, which have been shown to play a role in establishing epidermal versus neural cell fate. The first direct neuralizing agent to be described was a truncated type II activin receptor originally designed to test the role of the TGF-beta family member activin in *Xenopus* mesoderm induction. Surprisingly, while embryos expressing this dominant-negative molecule were blocked in mesoderm formation, neural tissue was generated as a result of this perturbation (Hemmati-Brivanlou and Melton, 1994). Additional work by several groups demonstrated that *Xenopus* ectoderm could be neuralized by cell dissociation (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989), suggesting that intact ectodermal tissue might normally contain a secreted inhibitor of neural cell fate that was washed away by the manipulation. Taken together, this evidence suggested a TGF-beta family member as the soluble neural cell fate suppressor. Although activin did not seem to fulfill this role, BMP4 demonstrated potent epidermal-inducing activity (Wilson and Hemmati-Brivanlou, 1995) and was expressed in early *Xenopus* embryos at the correct place and time to be involved in suppression of neural cell fate. Further experiments demonstrated that the dominant-negative type II activin receptor originally used to induce neural tissue also blocked signaling through the BMP pathway.

During this same time period, several structurally diverse secreted molecules were also identified that could induce neural cell fate directly (without induction of mesoderm with neural-inducing capacity). These molecules, Noggin, Chordin, Follistatin, Xnr3, and Cerberus, were all expressed in the organizer region during late blastula and gastrula stages, when neural-inducing signals were transmitted to adjacent ectoderm.

J. Bainter and A. Boos made an equal contribution to this work.

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Rather than triggering a signal transduction cascade, all of these molecules were found to induce neural tissue by interfering with BMP signaling (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995; Bouwmeester et al., 1996; Hansen et al., 1997). This interference occurred either by direct binding to BMP ligands to prevent interaction with their receptors, or by binding to BMP receptors and competing with BMPs for receptor binding sites (*Xnr3*). Since these discoveries, many additional means of disrupting the BMP signal transduction cascade have also been shown to result in neural tissue formation (Onichtchouk et al., 1999; Zhu et al., 1999; Massague and Chen, 2000).

For several years, BMP protein antagonism by the organizer has been viewed as the central and initiating event in neural induction. Recently, however, a role in neural induction has also been proposed for the Wnt and FGF signaling pathways. These pathways play an essential role in other aspects of early axial patterning. The activities of the Wnt and FGF pathways in neural induction may precede organizer signaling and are almost certainly not localized to organizer mesoderm. In addition, molecules have recently been identified that mediate neuralization within ectoderm immediately downstream of neural induction signals. These neural effector genes may link neural induction to later processes of neuronal differentiation. An interesting theme has emerged from these diverse studies: although BMP antagonism still seems central to neural induction, the new neural-inducing signals and several of the neural effector genes can act on the BMP pathway at the transcriptional rather than posttranslational level. We will discuss the recent data pertaining to this topic and re-evaluate several features of neural induction: the time at which neural tissue is induced, the requirements for the organizer in this process, and the possible involvement of nonorganizer-dependent factors and pathways.

NEURAL INDUCTION: BEYOND THE ORGANIZER?

Until recently, neural induction had been thought to occur mainly during the cell movements of gastrulation and had been attributed to a fully formed organizer acting on overlying ectoderm (Holtfreter and Hamburger, 1955; Otte and Moon, 1992; Harland and Gerhart, 1997). Experiments in *Xenopus* demonstrated that neural specification occurs only by mid-gastrulation (Blitz and Cho, 1995) when ectoderm is competent to respond to neural-inducing signals (Grainger and Gurdon, 1989; Servetnick and Grainger, 1991a). However, it now seems that induction of at least anterior neuroectoderm may occur much earlier, possibly even before gastrulation. In *Xenopus*, expression of some genes localizes to presumptive neuroectoderm by the onset of gastrulation, demonstrating that ectoderm is already divided into distinct neural and non-neural territories (reviewed in Sasai, 1998; Chitnis, 1999; Gamse and Sive, 2000). In zebrafish embryos, dorsal

presumptive neuroectoderm explanted at early gastrula stages is already specified as neural tissue (Grinblat et al., 1998). Recent work in chick embryos has shown that neural specification occurs still earlier, before gastrulation. A very early neural marker, *ERN1*, is expressed in presumptive neural tissue by pre-gastrula stages (Streit et al., 2000) and explants of presumptive ectoderm are specified as neural tissue well before gastrulation (stages VII and VIII) (Wilson et al., 2000). Thus, the process of neural induction may begin before or by the onset of gastrulation in many vertebrates.

Organizer expression of BMP antagonists occurs by the late blastula stages, early enough to initiate neural induction, even on the revised timetable proposed above. However, in several vertebrates, it now seems that organizer signaling may be neither sufficient nor required for neural induction in vivo. In mouse embryos, neural development occurs in the absence of the activities of Noggin, Chordin, or Follistatin and double mutants for *noggin* and *chordin* form some neural tissue, although anterior neural tissue (forebrain) is lost (Matzuk et al., 1995; McMahon et al., 1998; Bachiller et al., 2000). Furthermore, mouse embryos mutant for *HNF3-beta* lack a recognizable node and most node-specific gene expression, including *Noggin* and *Chordin*, yet still form neural tissue (Klingensmith et al., 1999). In zebrafish, embryos mutant for *chordin* retain a nervous system, as do embryos in which the embryonic shield has been removed surgically (Shih and Fraser, 1996; Driever et al., 1997). The organizer and all derivatives are also lost in several zebrafish mutants, including double mutants for *squint* (*sqt*) and *cyclops* (*cyc*), TGF-beta molecules of the Nodal class, and mutants for *one-eyed pinhead* (*oep*), a protein required for Nodal function (Feldman et al., 1998; Zhang et al., 1998; Gritsman et al., 1999). All of these embryos retain well-patterned nervous systems.

In avian embryos, perhaps the strongest case can be made that BMP antagonists produced by the organizer are neither necessary nor sufficient for neural induction. Although Henson's node has the ability to induce neural tissue, the BMP antagonists Noggin and Chordin cannot mimic that activity: addition of BMP antagonists to competent ectoderm outside of the presumptive neural plate does not neuralize this ectoderm unless it has received prior signals from the organizer (Streit et al., 1998; Streit and Stern, 1999b). The timing of expression of three BMP antagonists that have been identified in chick (Noggin, Chordin, and Follistatin) does not correlate with the ability of the organizer to induce neural tissue in vivo (Storey et al., 1992; Levin, 1998; Streit et al., 1998). In addition, very early explants from prestreak stage embryos become specified as neural tissue in vitro, although they never express the secreted BMP inhibitors Noggin, Chordin, Follistatin, or Caronte (Wilson et al., 2000). All of these data indicate that generation of neural cells in chick embryos is unlikely to require the production of BMP antagonists by the organizer.

Although the data above suggest alternative modes of neural induction independent of a fully formed organizer (recognizable by specific morphologic or molecular criteria), several caveats must be considered. The organizer signals *noggin* and *chordin* do seem to be essential for forebrain development, as demonstrated by the loss of forebrain in *Noggin-/Chordin*-mutant mice (Bachiller et al., 2000). However, because the organizer expresses a large number of known and potentially additional unknown BMP antagonists, even loss of function of several of these proteins might not be expected to cause loss of all neural tissue. Furthermore, when the organizer is removed surgically, BMP antagonists acting before the surgery might be sufficient for neural tissue formation. Low-level expression of antagonists outside of the organizer, which might be difficult to detect by conventional methods, could be sufficient to initiate neural induction in this context. Additionally, in chick, fish, and mouse, surgical ablation of the organizer at early time points can lead to partial or complete organizer regeneration from neighboring cells (reviewed in Joubin and Stern, 2001).

THE USUAL (AND UNUSUAL) SUSPECTS

Although some caveats apply, mounting experimental evidence suggests that the events that initiate neural induction may occur quite early in development. They may be associated as much with the early signaling pathways that contribute to embryonic axis formation as with the organizer. As mentioned above, recent data have implicated the FGF and Wnt signaling pathways in initiating neural induction. In addition, several neural effector genes have been identified recently; these genes are expressed within presumptive neuroectoderm during early gastrulation and can mediate neuralization downstream of secreted signals. Neuralization of ectoderm correlates closely with down-regulation of *Bmp* expression in many vertebrate embryos: zebrafish (*Bmp2b* and *Bmp4*), *Xenopus* (*Bmp4*), and chick (*Bmp4* and *Bmp7*) (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Nikaido et al., 1997; Streit et al., 1998; Dick et al., 2000; Wilson et al., 2000). Interestingly, many of these newly identified neuralizing factors seem to carry on the theme of BMP pathway antagonism, but do so at the transcriptional rather than the posttranslational level.

FGF SIGNALING IN NEURAL INDUCTION

FGFs have been implicated in neural induction in the chick embryo (Alvarez et al., 1998; Storey et al., 1998). FGF ligands (FGF3 and FGF4) and their receptors (FGFR1b and FGFR2b) are expressed in gastrula stage embryos (Mahmood et al., 1995; Shamim and Mason, 1999; Walshe and Mason, 2000; Wilson et al., 2000). FGF seems to act very early in chick neural induction, as addition of FGF8 to explants induces the early neural marker *ERNI* at pregastrula stages (Streit et al., 2000). Furthermore, active FGF signaling seems to be required for neural induction in the chick.

FGF signaling inhibitors (SU5402 and a dominant-negative FGF receptor) block expression of neural markers *in vivo* and also block induction of an ectopic neural plate by a grafted organizer (Streit et al., 2000). Inhibition of FGF signaling both blocks neuralization and down-regulates *Fgf3* expression, effects that are rescued by FGF3 addition to the explants (Wilson et al., 2000). All of these data suggest that FGF signaling is essential for neural plate formation in the chick.

The role of FGF signaling in neural induction has also been studied extensively in *Xenopus*, but results have been less suggestive of an essential function in neural induction. FGF signaling seems to be active at the right place and time to contribute to early neural induction in *Xenopus*: the receptor tyrosine kinase pathway downstream of FGF is activated in ectoderm at late blastula stages, although it shows no distinction between presumptive neural versus non-neural tissue (LaBonne and Whitman, 1997; Uzgare et al., 1998; Christen and Slack, 1999; Curran and Grainger, 2000). FGFs can directly induce neural markers in *Xenopus* gastrula ectoderm under some experimental circumstances (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). However, neural induction by FGFs is dependent on sensitization of tissue, for example by culturing in media that lowers cell adhesion and partially attenuates BMP signaling (Lamb and Harland, 1995). Various studies have examined the *in vivo* requirement for FGF signaling in neural induction. The bulk of data suggests that neural tissue forms even in the presence of dominant inhibitory molecules that interfere with FGF signaling (Kroll and Amaya, 1996; McGrew et al., 1997; Barnett et al., 1998; Bang et al., 1999; Holowacz and Sokol, 1999; Ribisi et al., 2000). However, in a few cases neuralization of explants has been blocked by expression of dominant-negative FGF receptors (Launay et al., 1996; Sasai et al., 1996; Barnett et al., 1998). Taken together, these data suggest that low levels of FGF signaling may sensitize the ectoderm to respond to other neural-inducing cues *in vivo*. This role has been attributed to FGF signaling during mesoderm induction (Isaacs, 1997). Such a role may also be played by FGFs in the chick, as addition of FGF to competent ectoderm induces neural markers but neural marker expression is only transient without subsequent maintenance mechanisms (Storey et al., 1998; Streit and Stern, 1999a). It is possible that the dorsal ectoderm might receive a combination of sensitizing signals before gastrulation with neural induction triggered or enhanced by the additive effects of several of these signals.

How might FGF signaling influence the formation of neural tissue? Recent work in avian embryos suggests a link to down-regulation of *Bmp* expression. Expression of *Bmp4* and *Bmp7* is normally suppressed in prospective neuroectoderm at the onset of gastrulation (Streit et al., 1998; Wilson et al., 2000). When FGF signaling is inhibited, this down-regulation does not occur and no neural tissue is formed (Wilson et al.,

2000). Does this suppression of *Bmp* expression account for FGFs neuralizing activity? In tissue blocked for FGF signaling, BMP pathway antagonists can rescue neural cell fate (Wilson et al., 2001). A dose dependence of rescue is seen, suggesting that two pathways downstream of FGF act to neuralize tissue. One pathway may down-regulate *Bmp* expression, whereas the second may be independent of *Bmp* expression levels (Wilson et al., 2001). We do not yet know which downstream components act in these pathways or how they are integrated during neurogenesis. It also remains to be determined whether FGF signaling impacts neural induction in *Xenopus* by suppressing *Bmp* expression.

WNT SIGNALING IN NEURAL INDUCTION

Although a role for Wnt signaling in early dorsal-ventral patterning is well established, Wnt signaling has only recently been shown to contribute to neural induction (Baker et al., 1999; Wilson et al., 2001). The Wnt signaling cascade includes the secreted Wnt ligands, Frizzled receptors, and intracellular proteins such as Dishevelled and glycogen synthase kinase 3 (GSK3). After activation of the Wnt signaling pathway, GSK3 inhibition by Dishevelled allows stabilization and nuclear localization of beta-catenin, which complexes with the TCF DNA-binding protein to activate transcriptional targets, including *Xnr3* and *siamois*. Shortly after fertilization in *Xenopus*, nuclear localization of beta-catenin occurs in dorsal cells, creating a dorsovegetal signaling center, which organizes the dorsal-ventral embryonic axis (reviewed in Moon and Kimelman, 1998).

Neural tissue is induced directly in *Xenopus* ectodermal explants by various components of the Wnt signaling pathway, including several Wnt ligands, the receptor *Xfrz8*, *Xenopus* Dishevelled, a dominant-negative form of GSK3, and a constitutively active form of beta-catenin (Baker et al., 1999). Misexpression of Wnts during pregastrula but not gastrula stages is sufficient to neuralize tissue, suggesting that Wnts act at pregastrula stages (Baker et al., 1999). Indeed, Wnt signaling may be essential for neural induction in vivo: embryos overexpressing beta-catenin in ectoderm have expanded neural tissue, whereas a dominant-negative form of TCF blocks neural tissue formation in and around the TCF-expressing cells (Baker et al., 1999). Although it is not yet certain how Wnt signaling neuralizes *Xenopus* ectoderm, this process may be mediated by regulation of *Bmp* expression. In *Xenopus*, *Bmp4* is initially expressed throughout ectoderm but is down-regulated in prospective neuroectoderm at early gastrula (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). Although Noggin cannot down-regulate *Bmp4* expression during gastrulation, Wnt signaling can perform this function (Baker et al., 1999). Activation of BMP4 signaling in Wnt-injected ectoderm blocks both neuralization and Wnt-mediated suppression of *Bmp4* expression, suggesting that the ability of

Wnts to regulate *Bmp* expression could account for their neuralizing activity (Baker et al., 1999).

How might the Wnt signaling pathway down-regulate *Bmp* expression to neuralize ectoderm in *Xenopus*? Although TCF seems to be essential for this process, the downstream target genes *Xnr3* and *siamois* do not (Baker et al., 1999). In some contexts, TCF functions as a transcriptional corepressor, suggesting that it could directly repress *Bmp* expression. However, upon Wnt pathway activation, TCF generally serves as a transcriptional coactivator. Thus, TCF is likely to use a repressor as an intermediary for this function. Recently, the homeodomain protein Xiro1, a transcriptional repressor, has been proposed to suppress *Bmp* expression downstream of Wnt signaling (see below) (Gomez-Skarmeta et al., 2001). This connection could account for the ability of Wnt signaling to neuralize tissue.

In zebrafish, Wnt signaling seems to play a more central role in neural patterning than in neural induction. Mutations in the Wnt signaling pathway include *headless* (*Tcf3*) and *bozozok*, a homeodomain protein downstream of Wnt signaling (Driever et al., 1996; Solnica-Krezel et al., 1996; Kim et al., 2000). Embryos carrying mutations in either of these genes have defects in anterior-posterior neural patterning, but neural tissue is still induced (Fekany-Lee et al., 2000; Kim et al., 2000). *bozozok* mutants do have a reduction of total neural tissue, suggesting a possible involvement of Wnt signaling in neural induction (Fekany-Lee et al., 2000). As in *Xenopus*, the zebrafish Wnt pathway also directly regulates BMP ligands at the transcriptional level. *Bozozok* can function as a transcriptional repressor (reviewed in Solnica-Krezel and Driever, 2001). The *bmp2b* (*swirl*) gene is likely to be a direct transcriptional target of *Bozozok*: ectopic expression of *Bozozok* suppresses *bmp2b* expression in pregastrula embryos, in *bozozok* mutant embryos the expression domains of *bmp2b* and *bmp4* are enlarged, and *Bozozok* may directly bind the *bmp2b* promoter (Koo and Ho, 1999; Fekany-Lee et al., 2000; Solnica-Krezel and Driever, 2001). Thus, the minor defects in neural induction seen in *bozozok* mutants are most likely due to an increase in BMP2b activity and expression. Although the Wnt pathway is not required for neural induction, its ability to modulate *Bmp* expression suggests that Wnt signaling may be one of several redundant pathways contributing to neural induction.

In chick embryos, Wnt signaling seems to play a critical role in neural induction, but interestingly, as an antagonist of neural induction rather than an inducer of neural tissue. The pattern of nuclear beta-catenin localization differs substantially from that found in *Xenopus* and zebrafish: by pregastrula stages, nuclear localization of beta-catenin and expression of *wnt3A* and *wnt8C* are restricted to lateral epiblast tissue, which acquires an epidermal fate (Roeser et al., 1999; Wilson et al., 2001). Furthermore, Wnt3A or Wnt8 treatment of presumptive neural tissue blocks

neural development (Wilson et al., 2001). Wnt signaling also seems to be essential for epidermal development, as interference with Wnt signaling (by using a soluble fragment of the mouse Frizzled protein) converts presumptive epidermal tissue to neural cell types (Wilson et al., 2001).

Based on these data, the use of Wnt signaling in neural induction in chick seems to differ greatly from that described above for *Xenopus*. This difference may actually represent an alternate regulation of the FGF and/or BMP pathways, which contribute to neural induction in both organisms. In chick, the ability of Wnts to epidermalize presumptive neuroectoderm (medial epiblast) seems to be entirely due to antagonism of FGF signaling. Wnt ligands block the ability of FGF to induce neural differentiation and mimic the FGF signaling antagonist SU5402, preventing down-regulation of *Bmp4* expression in presumptive neuroectoderm (Wilson et al., 2001). However, this FGF connection to Wnt activity may not be true for presumptive epidermis (lateral epiblast). Studies of these signaling pathways are still in the early stages. We will only appreciate the similarities and differences between various vertebrates when we understand how these pathways interact to contribute to neurogenesis.

CONNECTING THE DOTS: EFFECTOR GENES THAT MEDIATE THE FORMATION OF NEURAL TISSUE

Immediately downstream of neural induction, the molecular circuitry that establishes neural cell fate in vertebrates remains poorly understood. However, several effector molecules that promote neural fate within dorsal ectodermal cells have been recently identified. These neural effectors include members of the Iroquois, Sox, and Zic families of transcription factors, the zinc-finger transcription factors XSIP1/ZEB2 and Kheper, and a novel coiled-coil protein, Geminin. RNAs corresponding to these proteins become restricted to future neuroectoderm by early to mid-gastrulation. Misexpression of these genes is sufficient to neuralize competent non-neural ectoderm. The neural effector genes are, therefore, likely to connect neural induction with later neuronal cell fate determination and differentiation processes. Here, we have summarized data regarding the role of particular genes in early neurogenesis, including temporal and spatial expression patterns, as well as sufficiency and/or necessity for neurogenesis in various vertebrates.

Xiro1 and Xiro2

The Xiro1 and Xiro2 homeodomain proteins are *Xenopus* homologs of the *Drosophila Iroquois* genes *caupolican*, *araucan*, and *mirror* (Gomez-Skarmeta et al., 1996, 1998, 2001; Leyns et al., 1996; McNeill et al., 1997) These proteins contain a highly conserved homeodomain and a C-terminal IRO box. *Xiro1* and *Xiro2* are initially expressed at early gastrulation throughout the presumptive neural plate (stage 10) (Gomez-

Skarmeta et al., 1998) and, thus, are expressed sufficiently early in development to act as neural effector genes. Functional studies in *Xenopus* also suggest an essential role for these genes in neural cell fate determination. When misexpressed in *Xenopus*, *Xiro1* and *Xiro2* cause ectopic neurogenesis and expansion of the neural plate (Gomez-Skarmeta et al., 1998, 2001). The mechanism of neuralization by *Iroquois* genes has been most extensively characterized for Xiro1. Neuralization is suppressed by a VP16 activation domain fusion to Xiro1 that behaves as a dominant-negative molecule; these data demonstrate that Xiro1 acts as a transcriptional repressor and is essential for early neural development. Xiro1 may control neural cell fate by regulating *Bmp* expression: Xiro1 and BMP4 can suppress each other's expression during gastrulation and *Bmp4* is ectopically expressed in embryos injected with dominant-negative Xiro1 (Gomez-Skarmeta et al., 2001). In addition, induction of neural tissue by a dominant-negative BMP receptor is blocked by coexpression of dominant-negative Xiro1, demonstrating an interaction between these pathways. Finally, *in vitro* studies have shown that Xiro1 can bind directly to the *Bmp4* promoter (Gomez-Skarmeta et al., 2001). Therefore, substantial evidence indicates that Xiro1 participates in repressing *Bmp4* expression during early gastrulation to contribute to neuralization of the ectoderm. As *Drosophila* studies originally identified these genes as regulators of domains of proneural gene expression (Gomez-Skarmeta et al., 1996; Leyns et al., 1996; McNeill et al., 1997), this regulation of *Bmp* expression may represent an alternate or additional mechanism for *Iroquois* gene function. Many of the functional similarities between vertebrate and invertebrate *Iroquois* genes still remain to be elucidated.

Gomez-Skarmeta et al. (2001) have also demonstrated a link between the Wnt signaling pathway and Xiro1 in *Xenopus*. Activation of the Wnt pathway in ectodermal explants stimulates *Xiro1* expression. *Xiro1* expression and neural tissue formation is reduced in early gastrula embryos injected with dominant repressors of the Wnt pathway (GSK3 or dominant-negative TCF-3). Furthermore, a dominant-negative form of Xiro1 blocks the ability of beta-catenin to induce neural tissue in embryonic explants. These data suggest that *Xiro1* expression may be activated by Wnt signaling *in vivo* and may be an essential mediator of *Bmp* transcriptional suppression downstream of the Wnt pathway to promote neural tissue formation.

Although expression and functional data in *Xenopus* strongly suggest a role for Xiro1 and Xiro2 in the earliest stages of neural cell fate specification, in several other vertebrates (chick, zebrafish, and mouse), expression of *Iroquois* genes is more consistent with a role in neural patterning than in the earliest neural cell specification events. Three chick, six mouse, and one zebrafish homolog have been identified (Bosse et al., 1997, 2000; Bellefroid et al., 1998; Goriely et al., 1999; Tan et al., 1999; Peters et al., 2000). The only zebrafish

Iroquois gene characterized thus far, *Ziro3*, is first expressed in neural tissue at the end of gastrulation (10 hr postfertilization) (Tan et al., 1999). In chick embryos, *c-Irx2* is first detected at late gastrula to early neurula stages in the neural plate (Goriely et al., 1999). However, zebrafish orthologs of *Iroquois 1* or *Iroquois 2* and a chick ortholog of *Iroquois 1* have not yet been isolated; these could localize to neural tissue during early gastrulation as in *Xenopus*. In the mouse, both *Irx1* and *Irx2* have been isolated and show only post-gastrula expression. *Irx3* shows the earliest expression in mouse, with expression initiating at the late gastrula stage (Bosse et al., 1997). Thus, expression and functional data suggest a role for *Iroquois* genes in neural cell specification in *Xenopus*, but this has not yet been established for other vertebrates.

SoxD

The Sox family encompasses a variety of proteins with high conservation in their HMG-domains (<50% amino acid similarity) (reviewed in Wegner, 1999; Sasai, 2001a,b). Among the Sox genes, *Xenopus* SoxD best fits the criteria for a neural effector gene. *SoxD* is first expressed in the entire ectoderm at late blastula and restricted to neuroectoderm by mid-gastrula. Overexpression of SoxD causes the formation of ectopic neural tissue of anterior character. SoxD also seems to be essential for the formation or patterning of anterior neural tissue, as a dominant-negative molecule blocks neuralization of ectoderm in animal caps and suppresses anterior neural tissue formation in embryos (Mizuseki et al., 1998b). The mechanism by which SoxD neuralizes ectoderm is not yet known. Sox protein specificity may be defined by a target or a cell-/tissue-specific partner with Sox proteins acting as either transcriptional activators or repressors in different contexts (reviewed in Kamachi et al., 2000). Identification of neural specific cofactors and targets is needed to determine how SoxD neuralizes ectoderm and whether there is any connection to regulation of *Bmp* expression or function. Although *Xenopus* studies reveal SoxD as a strong neuralizer, it is not yet clear whether this role is conserved because homologs of SoxD have not yet been identified in other vertebrates. (Wegner, 1999).

Zic1/Opl and Zic3

The *Zic* gene family consists of zinc finger transcription factors that are the vertebrate homologs of *Drosophila odd-paired* (Benedyk et al., 1994). These genes contain five tandemly repeated C₂H₂-type zinc finger motifs, and were originally identified as proteins expressed in adult mouse cerebella (Aruga et al., 1994). Two members of the *Zic* gene family, *Zic1* (also called *Opl*) and *Zic3*, are neural effector gene candidates based on their activities and expression in *Xenopus*. In *Xenopus*, *Zic1/Opl* and *Zic3* are expressed throughout the prospective neural tissue by early gastrulation, consistent with a role in the earliest stages of neural

cell specification (Nakata et al., 1997, 1998; Kuo et al., 1998; Mizuseki et al., 1998a). In several studies, misexpression of *Zic1/Opl* or *Zic3* was sufficient to directly neuralize ectoderm and to expand the neural plate (Nakata et al., 1997, 1998; Mizuseki et al., 1998a). However, one study found *Zic1/Opl* to be insufficient to induce neural tissue but capable of sensitizing ectoderm to neuralization by low doses of Noggin (Kuo et al., 1998). Future studies are needed to establish how the *Zic* genes induce neural cell fate. *Zic1/Opl* and *Zic3* are proposed to act as transcriptional activators (Kuo et al., 1998; Koyabu et al., 2001; Mizugishi et al., 2001). Because these genes are likely to be involved in early neural fate determination, it will be important to identify their direct transcriptional targets and determine whether a downstream connection to control of *Bmp* expression exists.

Zic1 and *Zic3* homologs have also been characterized in mouse and zebrafish. In zebrafish, *Zic1/Opl* also begins to be expressed in neural tissue during gastrulation. However, its expression is limited to a region of the anterior neural plate suggesting a role in mediating induction or patterning of anterior tissue rather than a general role in early neuralization (Grinblat et al., 1998; Rohr et al., 1999). In mouse, *Zic1* and *Zic3* seem to be expressed in neuroectoderm at some point during gastrulation, but data are limited (Nagai et al., 1997). Loss of function studies in the mouse reveal neural patterning functions for *Zic1* and *Zic3*; however, embryos lacking *Zic1* or *Zic3* function did not have deficits in neural induction (Aruga et al., 1998; Klootwijk et al., 2000; Nagai et al., 2000). If the *Zic* proteins have partially redundant functions, it may be necessary to remove the function of multiple genes to reveal a deficiency in neural induction. Another *Zic*-family gene *Opr* was also recently identified in mouse and shows pregastrula expression in the embryonic ectoderm, consistent with a possible role in early neuralization, but functional characterization has not yet been performed (Furushima et al., 2000). Thus, although in *Xenopus* the expression and activities of *Zic1* and *Zic3* are consistent with a neural effector gene function, further data in other vertebrates are needed.

XSIP1/ZEB2 and Kheper

XSIP1/ZEB2 and Kheper are recently identified members of the ZFH (zinc-finger and homeodomain protein)/DeltaEF1 transcription factor family (Eisaki et al., 2000; Muraoka et al., 2000; van Grunsven et al., 2000). In *Xenopus*, *XSIP1/ZEB2* is expressed in the prospective neural territory at early gastrulation. Misexpression expands the neuroectoderm or induces ectopic neurogenesis (Eisaki et al., 2000; van Grunsven et al., 2000). XSIP1 has been shown to interact with Smad1, an intracellular mediator of BMP signaling (van Grunsven et al., 2000). Some members of the DeltaEF1 family behave as transcriptional repressors by interacting with the corepressor CtBP (Postigo and Dean, 1999). Interaction of XSIP1 with Smad1 and/or

CtBP could allow it to repress *Bmp* expression to neuralize tissue. However, this possibility has not yet been tested.

Similarly in zebrafish, *Kheper* is expressed in early gastrula embryos in prospective neural tissue and overexpression induces ectopic neurogenesis (Muraoka et al., 2000). There is direct evidence that *Kheper* behaves as a transcriptional repressor: fusion with the VP16 activation domain produces a dominant-negative protein that suppresses neural tissue formation when misexpressed in embryos (Muraoka et al., 2000). Although characterization of these genes is still in the early stages, they meet the criteria for neural effector genes. Further studies will be needed to determine whether either of these genes neuralize tissue by regulating *Bmp* expression.

Geminin

Geminin is a novel coiled-coil protein with characterized vertebrate homologs in *Xenopus*, mouse, and human (Kroll et al., 1998; McGarry and Kirschner, 1998). Geminin has two separable functional domains, one that can neuralize ectoderm and one involved in inhibiting DNA re-replication (Kroll et al., 1998; McGarry and Kirschner, 1998). In *Xenopus*, *Geminin* transcripts show pregastrula expression throughout the ectoderm, becoming restricted to the presumptive neural territory at the onset of gastrulation. Gain-of-function studies in *Xenopus* show Geminin to be sufficient to neuralize ectoderm and suppress epidermal development. Evidence that Geminin function may be required for some aspect of early neurogenesis is derived from a putative dominant-negative truncation of Geminin: this protein suppresses neural and induces epidermal markers in frog embryos, effects that are rescued by full-length Geminin (Kroll et al., 1998). Although these observations are promising, confirmation with other methods is required given that the dominant-negative Geminin truncation contains a domain that affects cell cycle function and a coiled-coil domain that might lead to unknown nonphysiological effects. Geminin's ability to suppress *Bmp4* expression during gastrulation may account for its neuralizing activity. In support of this hypothesis, neuralization by *Geminin* mRNA is blocked by coinjection of *Bmp4* mRNA (Kroll et al., 1998). In the mouse, *Geminin* is expressed from early cleavages onward; early expression seems to be ubiquitous, but later expression occurs at high levels in neural tissue (Kroll et al., 1998). Functional data in other vertebrates, in particular loss of function studies, are needed to determine whether Geminin plays a conserved role in vertebrate neurogenesis.

Other Genes Involved in Early Neurogenesis

Several other genes have also been implicated in controlling neurogenesis, but expression or functional data is more consistent with a role in sensitizing ectoderm toward a neural fate, or in patterning or maintenance

of neuroectoderm. For example, although the HMG-domain protein Sox2 is expressed in presumptive neural tissue by early gastrulation in *Xenopus*, it is insufficient to induce neural markers in ectodermal explants. Rather, Sox2 can enhance neural responsiveness of ectoderm to FGF (Mizuseki et al., 1998a). Furthermore, a dominant-negative Sox2 protein does not affect initiation of neurogenesis at early gastrulation but does suppress neural marker expression in late gastrulae (Kishi et al., 2000). This finding suggests that Sox2 is required for the maintenance but not the initial induction of neural tissue and would place Sox2 functionally downstream of Sox2 (Wegner, 1999). In mouse and chick, *Sox2* shows a similar localization to presumptive neural tissue, suggesting a conserved role in vertebrate neurogenesis (Collignon et al., 1996; Rex et al., 1997; Wood and Episkopou, 1999). It has not been possible to determine whether Sox2 plays a role in initiation or maintenance of neural tissue in the mouse, because homozygous knockout embryos die at the time of implantation (Pevny et al., 1998). A conditional knockout is needed to address this question.

XBF-1 and *XBF-2* encode winged-helix transcription factors that are expressed first at late gastrulation in the anterior neural plate of *Xenopus* (Papalopulu and Kintner, 1996; Mariani and Harland, 1998). The timing of expression of these genes suggests a role in neural patterning or differentiation rather than in initiating neural specification (Papalopulu and Kintner, 1996). In *Xenopus* embryos, misexpression of *XBF-1* or *XBF-2* induces neural markers (Bourguignon et al., 1998; Mariani and Harland, 1998; Hardcastle and Papalopulu, 2000). In explant assays, *XBF-2* can behave as a transcriptional repressor to down-regulate *Bmp4* expression, which may account for its ability to induce neural markers in explant assays (Mariani and Harland, 1998). Thus, although *XBF-1* and *XBF-2* share some characteristics with the neural effector genes, their temporal expression is suggestive of a later role.

Neural Effector Genes and Control of BMP Expression

The neural effectors were defined in *Xenopus* as molecules that may bridge the gap between secreted neural-inducing signals from the organizer and later neurogenesis. They are expressed in the future neuroectoderm by early to mid-gastrulation and have the ability to neuralize competent ectoderm. Direct transcriptional targets of most of these genes have not been identified but could include other neural effector genes or downstream neuronal determination genes. Additionally, several neural effectors have been shown recently to suppress *Bmp* expression (*Xiro1*, *geminin*), whereas others are transcriptional repressors with unknown targets (*XSIP1* and *Kheper*). These data suggest that suppression of *Bmp* expression may represent a shared activity of several of the neural effector genes.

Identification and functional characterization of the neural effector genes has been performed primarily in

Xenopus. Homologs of these genes have been identified in mouse, chick, and zebrafish. Some of these homologs may play a conserved role as neural effectors in many vertebrates, whereas others seem to be expressed at the wrong time or place to participate in early neurogenesis. The apparent lack of functional conservation of these genes suggests that additional, unidentified neural effector genes are likely to exist. Some molecular mechanisms of early neurogenesis, such as down-regulation of *Bmp* expression, may be shared among vertebrates, whereas others such as the role of Wnt signaling or of specific neural effector genes may have diverged. Further work is needed to elucidate the pathways that regulate formation of the neural plate in each vertebrate.

PERSPECTIVES

Although studies of the pathways leading from neural induction to neural cell fate determination in vertebrates are still in the early stages, recent work has converged upon an interesting theme. In most instances, regulation of BMP signaling still seems to be central to the epidermal-neural cell fate decision. However, this regulation is not limited to extracellular antagonism of BMP proteins, but also includes various modes of intracellular antagonism, such as transcriptional control of *Bmp* expression.

Several lines of evidence suggest that transcriptional regulation of *Bmps* is involved in neurogenesis. In all vertebrates that have been analyzed, down-regulation of *Bmp* expression occurs within the future neural plate during early development and this correlates with the acquisition of neural cell fate. Suppression of *Bmp* expression can be carried out by the Wnt and FGF signaling pathways as well as by several of the neural effector genes (*Geminin*, *Xiro1*); in the case of *Xiro1*, evidence suggests that this down-regulation could be mediated by direct binding to the *Bmp* promoter. Some other genes with neural effector capacity (*SIP1/ZEB2* and *Kheper*) are also apparent transcriptional repressors, which could act by targeting *Bmp* expression. The mode of action of the *Sox* and *Zic* genes is more ambiguous, as different family members or cofactor complexes can give activator or repressor activity and direct targets have not yet been defined. A *Bmp* regulatory connection may emerge when the transcriptional targets and cofactors of these molecules are identified.

Is suppression of *Bmp* expression by the Wnt and FGF signaling pathways and the neural effector genes essential to their ability to neuralize ectoderm? Neuralization by *Geminin* and *Xiro1* can be counteracted by BMP4 injection. These results support the hypothesis that *Xiro1* and *Geminin* may down-regulate *Bmp* expression to neuralize ectoderm. FGF signaling in the chick appears to use both a BMP antagonistic pathway and a pathway independent of *Bmp* expression or activity. Coexpression of BMP antagonists in embryos blocked for FGF signaling sometimes rescues a neural

fate. These results suggest that down-regulation of *Bmp* expression may be necessary for the abilities of Wnt, FGF, and some of the neural effector genes to mediate neural cell fate determination.

As is the case for BMP protein antagonists produced by the organizer, several factors with apparently redundant or similar functions may cooperate to suppress *Bmp* expression. It is not yet known how many of these genes or pathways interact to establish neural cell fate or which may operate as independent parallel pathways. We suggest that neural cell fate in vivo is likely to be induced and maintained by pathways that act additively. Figure 1 shows a model, based on data from *Xenopus*, demonstrating how the overlapping activities of these pathways could spatially delineate the neuroectoderm during early development. Molecules that interfere with BMP signaling at both the transcriptional and posttranslational levels may reinforce attenuation of the BMP signal transduction pathway sufficiently to allow cells to develop as neural tissue within the proper spatial domain. The ability of a molecule to induce neurogenesis in explant assays or when misexpressed at high levels in embryos does not necessarily represent its in vivo activity. Many of these molecules may actually be insufficient for neuralization in vivo, but may instead nudge ectoderm closer to the threshold level of BMP signal attenuation necessary for neural tissue formation.

Signals contributing additively to neuralization may account for observations of organizer-less embryos that generate neural tissue and for differences between vertebrates in neural induction mechanisms. For example, in *HNF3-beta* mutant mouse embryos that apparently lack an organizer and its derivatives, a diffusely distributed neural-inducing signal is still present, which most likely represents Wnt or FGF signaling or an as yet unidentified neuralizing signal (Klingensmith et al., 1999). Furthermore, *Xenopus* and chick embryos appear to vary with respect to the use of the FGF and Wnt signaling pathways in neural induction. FGF signaling does not seem to be as critical for generating neural tissue in *Xenopus* as in chick. The role of the Wnt pathway also varies: Wnt ligands act as positive regulators of neurogenesis in *Xenopus* and as negative regulators through the FGF signaling pathway in chick. If each vertebrate has several contributing mechanisms to modulate BMP activity, it may not be necessary to conserve the function of any single molecule or pathway to accomplish this purpose.

Many aspects of the pathways underlying early neurogenesis remain relatively undefined at the molecular level. For the neural effector genes, direct downstream targets have not yet been identified and the relationships between these genes that may contribute to neural fate determination remain unknown. It is also unclear whether these genes are directly connected to the FGF and Wnt signaling pathways. In the case of FGF signaling in avian embryos, both BMP signaling-dependent and -independent modes of neuralization have

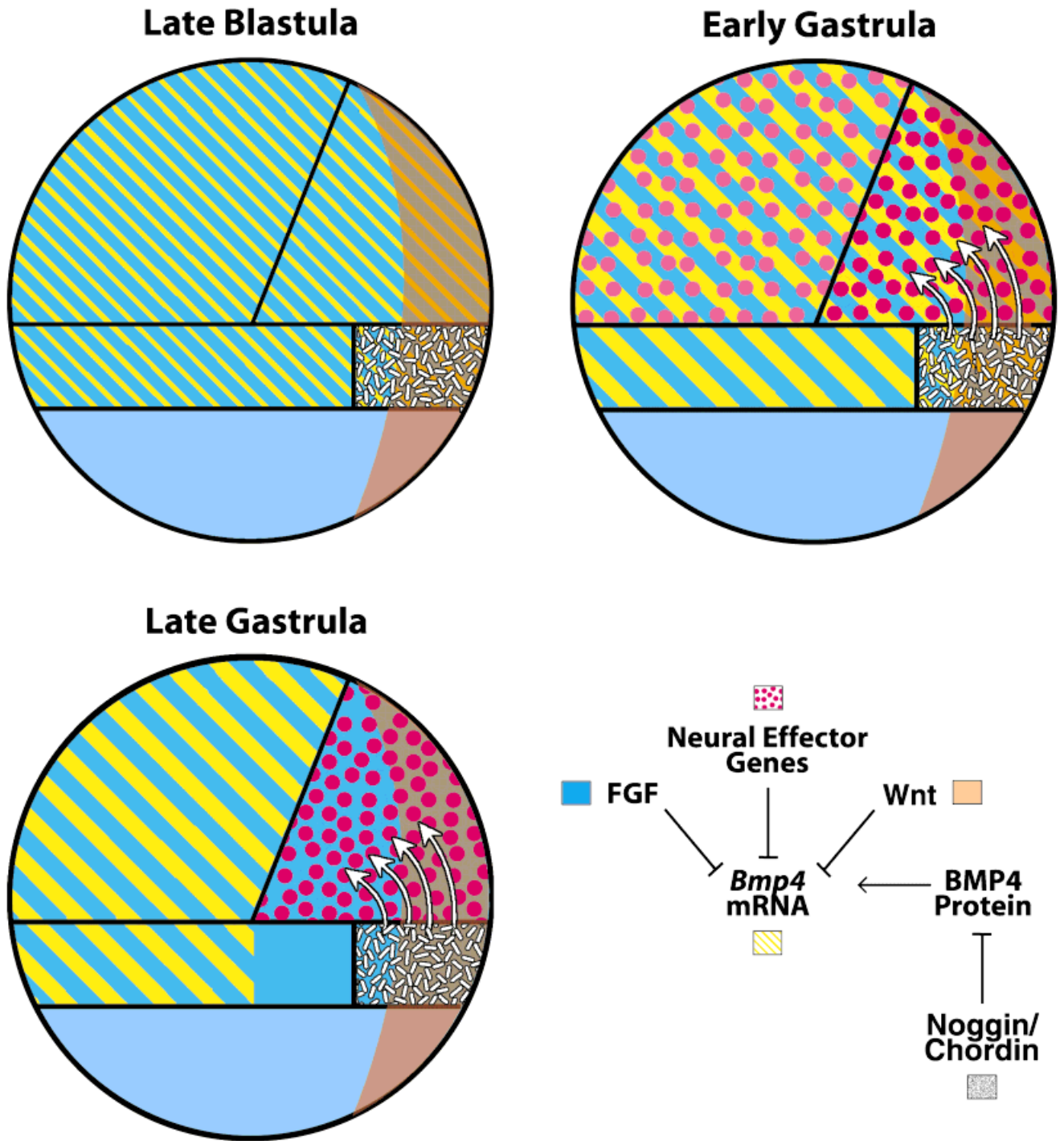


Fig. 1. Additive pathways may regulate Bmp expression to neuralize ectoderm. In the schematized *Xenopus* late blastula embryo, FGF signaling (blue) is ubiquitous with potentially higher levels in the animal and marginal zones (LaBonne and Whitman, 1997; Uzgare et al., 1998; Christen and Slack, 1999; Curran and Grainger, 2000). Low levels of *Bmp4* mRNA (yellow) are also found throughout the animal and marginal zones (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Nikaido et al., 1997; Streit et al., 1998; Dick et al., 2000; Wilson et al., 2000). Active Wnt pathway signaling (nuclear localized beta-catenin)(orange) occurs along the entire future dorsal side of the embryo (reviewed in Moon and Kimelman, 1998). BMP protein antagonists begin to be expressed in the organizer (white speckles)(Sasai, 1998; Chitnis, 1999; Streit and Stern, 1999c; Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000; Robertis and Arechaga, 2001). By the early gastrula stage, *Bmp4* mRNA levels have increased throughout the animal and marginal

zones. The Wnt and FGF signaling pathways are still active as are BMP protein antagonists produced by the organizer. Neural effector genes begin to be expressed; some are localized to the presumptive neural territory (dark pink) whereas others are expressed throughout the animal hemisphere (light and dark pink)(Sasai, 1998; Chitnis, 1999; Streit and Stern, 1999c; Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000; Robertis and Arechaga, 2001). By the late gastrula stages, *Bmp4* mRNA has been cleared from the prospective neuroectoderm and dorsal marginal zone. Neural effector gene expression is localized to the prospective neural territory or subdomains within this territory. Lower right panel: the FGF and Wnt pathways and some of the neural effector genes can negatively regulate *Bmp* expression to neuralize tissue. As BMP activity can maintain *Bmp* expression, BMP protein antagonists could also indirectly affect *Bmp* expression levels (Hammerschmidt et al., 1996; Piccolo et al., 1997; Nguyen et al., 1998).

been shown to occur but little is known about the molecular networks that mediate neuralization downstream of either of these pathways. The mechanism by which the FGF signaling pathway is negatively regulated by Wnt signaling in avian neural induction is also undefined. Although BMP antagonism can positively regulate expression of many of the neural effector genes, possibly by alleviating negative BMP pathway regulation (Sasai, 1998; Chitnis, 1999), the direct connections between these molecules in vivo remain unknown.

There also seem to be additional forms of neuralization that have no apparent connection to regulation of BMPs. Neuralization in the chick can occur without apparent BMP regulation at either the transcriptional or posttranslational level. In *Xenopus* embryos, neural plate tissue and paraxial mesoderm (somite) can neuralize competent ectoderm (Jones and Woodland, 1989; Hemmati-Brivanlou et al., 1990; Servetnick and Grainger, 1991b; Barnett et al., 1998). The molecular basis of these activities is unknown. Additionally, recent experiments in zebrafish have demonstrated that embryos lacking both BMP antagonists produced by the organizer and a functional Wnt signaling pathway retain neural tissue (Sirotkin et al., 2000). Thus, we are still identifying modes of neuralization in the embryo that lack an obvious molecular explanation.

In summary, antagonism of BMP signaling seems to be a shared feature of vertebrate neurogenesis. Although early studies focused on the control of BMP protein activity, recent data suggest that transcriptional control of *Bmp* expression may be another mechanism by which neuralization occurs. In vivo, multiple, possibly additive, molecular mechanisms may contribute to neural cell fate determination. Yet, we are only now beginning to assemble the first pieces to this puzzle. Although we have come a long way toward understanding the molecular networks underlying neurogenesis in the past century, many connections remain to be made.

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