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Active signals, gradient formation and regional specificity in neural induction

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Abstract

The question of how the vertebrate embryo gives rise to a nervous system is of paramount interest in developmental biology. Neural induction constitutes the earliest step in this process and is tightly connected with development of the embryonic body axes. In the *Xenopus* embryo, perpendicular gradients of BMP and Wnt signals pattern the dorsoventral and anteroposterior body axes. Both pathways need to be inhibited to allow anterior neural induction to occur. FGF8 and IGF are active neural inducers that together with BMP and Wnt signals are integrated at the level of Smad phosphorylation. Hedgehog (Hh) also contributes to anterior neural induction. Suppressor-of-fused plays an important role in intertwining the Hh and Wnt pathways. Distinct mechanisms are discussed that establish morphogen gradients and integrate retinoic acid and FGF signals during posterior development. These findings not only improve our understanding of regional specification in neural induction, but have profound implications for mammalian stem cell research and regenerative medicine.

The organizer and regional specification of the CNS

Few experiments in biology have been as influential as the transplantation experiment reported by Hans Spemann and Hilde Mangold in 1924. Grafting of the dorsal blastopore lip from a salamander gastrula into the ventral side of a host gastrula induced a twinned embryo with a brain and spinal cord (Spemann and Mangold, 1924). How can the Spemann-Mangold organizer (as the dorsal lip and homologous cell clusters in other vertebrates are now called) induce surrounding ectoderm to become central nervous system (CNS) and confer axial polarity to the developing embryo? The activation-transformation model of Peter Nieuwkoop postulates that the initially induced neural tissue is of anterior (forebrain) character, and that later signals from the chordamesoderm transform parts of it to more posterior midbrain, hindbrain, and spinal cord tissue (Nieuwkoop, 1952). As discussed below, anterior neural tissue is considered to be a default state that requires inhibition of bone morphogenetic protein (BMP) and Wnt signalling. Wnt, retinoic acid (RA) and fibroblast growth factor (FGF) signals are distributed in a gradual manner along the rostrocaudal neuraxis and exert posteriorizing activity.

In this review, we focus on active signals that contribute to anterior neural induction and ask of how they are integrated in the *Xenopus* embryo. We further discuss the mechanisms that establish a linear RA gradient and long-range FGF signalling in embryonic patterning. Finally we review how RA, FGF, and Wnt signals interact during posterior development in different vertebrates. An important message is that active signals not only play an important role in neural induction but are re-used and engage in complex feedback interactions during regionalization of the CNS.

Soluble BMP and Wnt antagonists promote anterior neural induction

Studies primarily in *Xenopus* have shown that two main signalling gradients exist in the early embryo, activated by the BMP and Wnt proteins (Eivers et al., 2009; Niehrs, 2010). These two gradients are perpendicular to each other and allocate positional information along the dorsoventral (DV) and anteroposterior (AP) body axes. The main BMPs in the *Xenopus* embryo are the ventrally expressed BMP4 and BMP7 and the dorsally expressed BMP2 and ADMP. Depletion of all four BMPs by antisense morpholino oligonucleotides converts the entire ectoderm into CNS, mostly brain tissue (Reversade and De Robertis, 2005). At the onset of gastrulation, the organizer secretes the soluble BMP antagonists Noggin, Chordin, and Follistatin (Weinstein and Hemmati-Brivanlou, 1997; Harland, 2000; De Robertis, 2009). Removal of all three BMP inhibitors in *X. tropicalis* results in severe loss of neural and expansion of ventral tissue (Khokha et al., 2005), stressing the essential function of BMP antagonism in the induction of neural fate.

In frogs, sperm entry causes Wnt-mediated stabilization of beta-catenin in the dorsal blastomeres, leading to initial DV asymmetry (Harland, 2000). At the blastula stage, dorsal Wnt/beta-catenin signals induce neural fate in the dorsal ectoderm through blocking *BMP4* transcription (Baker et al., 1999) and promoting expression of secreted BMP antagonists (Wessely et al., 2001). However, Wnt/beta-catenin signals have also been reported to stimulate epidermis at the expense of neural fate (Heeg-Truesdell and LaBonne, 2006). Temporal analysis with an inducible Tcf construct showed that canonical Wnt signalling can inhibit neural plate formation at the onset of gastrulation (Min et al., 2011). Thus, Wnt signals appear to shift from an early pro-neural to a late anti-neural function. The organizer secretes the Wnt antagonists

Frzb1, Cerberus, and Dkk1, which during gastrulation translocate to the anterior pole of the embryo and establish a Wnt/beta-catenin gradient that determines the AP polarity of the neural plate (Eivers et al., 2009; Niehrs, 2010). Thus, anterior neural tissue is a default state that depends on the dual inhibition of BMP and Wnt signals.

Perpendicular activity gradients of BMP and Wnt signals are not restricted to *Xenopus*. Chordin and BMP have universal functions in bilateria for patterning the DV axis during gastrulation (De Robertis, 2009). Key roles for anterior Wnt inhibition by Dkk and posterior Wnt signals have been validated in most animals (Niehrs, 2010).

FGF and IGF signals are active neural inducers

Studies in the chick have challenged the idea that neural induction relies exclusively on the extracellular suppression of BMP signalling and suggested that instructive signals like FGF8 participate in the induction of neural tissue. FGF is now a well-established neural inducer in planarians, ascidians, fish, *Xenopus* and the chick (Stern, 2005; Böttcher and Niehrs, 2005).

In *Xenopus*, it was first shown that IGFs (insulin-like growth factors) are important for head and neural induction (Pera et al., 2001; 2003; Richard-Parpaillon et al., 2002). Misexpression of IGF and IGFBP5 (IGF binding protein-5) leads to the induction of ectopic head-like structures containing eye and brain tissue (Fig. 1A,A'). IGF signals promote anterior neural induction through combined inhibition of BMP and Wnt signals. The inhibition of the Wnt pathway occurs upstream or at the level of beta-catenin.

Thus, multiple signals promote neural induction, raising the question of whether a common molecular explanation may exist that integrates these different pathways?

The BMP signal transducer Smad1/5/8 integrates multiple neural inducers

Recent studies performed by the De Robertis laboratory in the frog gave evidence for a unifying mechanism by which FGF8, IGF and Wnt converge with BMP signals at the level of Smad1/5/8, resulting in differential phosphorylation of this transcription

factor (Eivers et al., 2009). The BMP antagonists Noggin and Chordin block phosphorylation of Smad1 at its C-terminal end and allow the ectoderm to acquire a neural fate (Harland, 2000; De Robertis, 2009), whereas high ventral BMP levels through BMP receptor kinase activation increase the intensity of Smad1 signals and instruct the ectoderm to become epidermis (Weinstein and Hemmati-Brivanlou, 1997). Activation of the IGF or FGF receptor triggers MAPK (mitogen-activated protein kinase) to phosphorylate Smad1 in the central linker region, thus inhibiting Smad1 transcriptional activity and promoting neural induction (Pera et al., 2003). MAPK phosphorylation in turn primes GSK3 (Glycogen synthase kinase-3)-mediated linker phosphorylation, which triggers ubiquitination and proteasome-mediated degradation of Smad1, terminating the BMP signal (Fuentealba et al., 2007). Wnt signals inhibit GSK3, thereby stabilizing Smad1 in a beta-catenin-dependent manner and prolonging the duration of BMP signalling. The mechanisms by which Smad1/5/8 serves as a common platform for BMP, MAPK and GSK3 signals not only operates in neural induction and embryonic patterning, but is evolutionarily conserved and important for organogenesis (Eivers et al., 2009).

Hedgehog signals participate in anterior neural induction

The finding that Hedgehog (Hh) signals can induce anterior neural markers in cultured embryonic explants (Lai et al., 1995) received more recent support by the demonstration that misexpression of Hh ligands or their transcriptional activator Gli1 causes enlargement of the neural plate and promotes anterior development in whole embryos (Min et al., 2011). Importantly, inhibition of Hh signalling by overexpression of a dominant-negative Patched1 receptor or a carboxyterminally truncated Gli3 protein significantly expands epidermal at the expense of neural tissue, suggesting that Hh signals and activation of the Gli family of zinc-finger transcription factors play an important role in neural induction (Min et al., 2011). It remains to be shown, whether Hh is a conserved neural inducer in other vertebrates. An interesting question is of whether Hh and Wnt signalling interconnect and whether a common regulatory mechanism may exist that integrates the two pathways in neural induction and patterning?

Sufu is a dual regulator of Hh/Gli and Wnt/beta-catenin signals during neural induction and anteroposterior patterning

Suppressor-of-fused (Sufu) is a recognized Hh antagonist that binds to and inhibits Gli proteins in *Drosophila* and mammals (Cheng and Yue, 2008). Sufu can also bind to and inhibit beta-catenin in cultured cells (Meng et al., 2001), and evidence for a functional relevance of this interaction has recently been given in the frog (Min et al., 2011). Sufu promotes epidermal at the expense of neural fate through inhibiting Hh/Gli and early Wnt/beta-catenin signalling. Intriguingly, both depletion and overexpression of Sufu causes anteriorization of the neural plate, suggesting a biphasic model in which lowered Sufu concentration de-represses anterior Hh/Gli signalling, and high Sufu protein levels inhibit posterior Wnt/beta-catenin signalling. Sufu also has a critical role in the crosstalk of both signalling pathways, in which Hh inhibits Wnt signalling, and vice versa, Wnt stimulates Hh signalling (Min et al., 2011).

A biosynthetic enzyme code establishes a retinoic acid gradient in the organizer and the neural plate

The vitamin A-derived RA is a small lipophilic molecule that signals via nuclear receptors and patterns multiple tissues including the CNS in vertebrate embryos (Rhinn and Dollé, 2012). Its concentration is tightly controlled by developmentally regulated enzymes, in which Rdh10 (Retinol dehydrogenase-10) and Raldh2 (Retinal Dehydrogenase-2) sequentially mediate the two-step biosynthesis from Vitamin A via retinal to RA, whereas the hydroxylase Cyp26A1 triggers RA degradation (Fig. 2A). In *Xenopus*, exogenous RA can stimulate *Cyp26A1* (Holleman et al., 1998) and inhibit *Raldh2* transcription (Chen et al., 2001). The finding that endogenous RA blocks *Rdh10* gene expression in the frog embryo suggests feedback regulation already at the first step of RA biosynthesis (Strate et al., 2009), which has been confirmed in the zebrafish (Feng et al., 2010) and mouse (Sandell et al., 2012).

During gastrulation, the expression of Rdh10 and Raldh2 overlap in the dorsal mesoderm, while Cyp26A1 shows non-overlapping expression in the adjacent dorsal ectoderm and ventral mesoderm (Holleman et al., 1998; Chen et al., 2001; Strate et

al., 2009) in agreement with active RA signalling in the Spemann-Mangold organizer (Chen et al., 1994) (Fig. 2B). Morpholino oligonucleotide-mediated depletion of *Rdh10* or *Raldh2* causes a ventralized phenotype (Strate et al., 2009), suggesting an important role of RA signals in dorsalizing the embryo. Interestingly, overexpression of *Rdh10* or RA treatment upregulated *Chordin* and downregulated *ADMP* expression, while knockdown of *Rdh10* or *Raldh2* had the opposite effects (Strate et al., 2009). ADMP (Anti-dorsalizing morphogenetic protein) is secreted from the organizer and activates BMP receptor/Smad1 signalling (Reversade and De Robertis, 2005). The opposite transcriptional regulation of the BMP antagonist *Chordin* and the BMP agonist ADMP by RA (Strate et al., 2009) adds the *Rdh10*-*Raldh2*-RA axis as new modulator of the *Chordin*-BMP pathway for DV patterning during gastrulation.

In early neurula embryos, the *Rdh10* and *Raldh2* genes exhibit nested gene expression patterns in the presomitic mesoderm, with *Rdh10* localized more anteriorly than *Raldh2* expressing cells (Chen et al., 2001; Strate et al., 2009) (Fig. 2C,D). These domains are flanked by non-overlapping *Cyp26A1* expression in the anterior and posterior parts of the neural plate (Hollemann et al., 1998). The nested gene expression and sequential action of *Rdh10* and *Raldh2* causes a posterior flow of retinal, which is converted into a RA gradient in the anterior trunk mesoderm with a peak at the level of the hindbrain-spinal cord boundary. Subsequent diffusion of RA creates two gradients across the hindbrain and spinal cord, which acquire their final shape through CYP26A1-mediated degradation at the anterior and posterior ends of the neural plate. Support for a two-tailed gradient of endogenous RA came recently from a live-imaging study using genetically engineered RA probes in zebrafish embryos, which showed highest RA concentrations anteriorly within the *RALDH2*-expressing region (Schimozono et al., 2013). It is of interest that RA-responsive Hox genes, such as *HoxD1*, show a sharp anterior border of expression and posteriorly declining transcript levels (Fig. 2D), underscoring the gradual RA distribution generated by *Rdh10* and *Raldh2*. The combinatorial gene expression of two enzymes that act back-to-back to produce a signal is referred to as „biosynthetic enzyme code“ (Strate et al., 2009) and constitutes a novel mechanism for forming a morphogen gradient. An essential role of *Rdh10* in RA biosynthesis during anteroposterior hindbrain patterning in *Xenopus* (Strate et al., 2009) has been validated in the mouse (Rhinn et al., 2011).

Long-range FGF signalling by the secreted serine protease HtrA1 in mesoderm induction and posteriorization

In addition to their early function in neural induction, FGFs are involved in mesoderm induction and posteriorization of the neural plate in vertebrate embryos (Böttcher and Niehrs, 2005). Proteoglycans tether FGF ligands to the cell surface and facilitate their interaction with FGF receptors through their heparan or dermatan sulfate chains (Matsuo and Kimura-Yoshida, 2013; Thelin et al., 2013). In *Xenopus*, the secreted serine protease HtrA1 stimulates long-range FGF signalling during mesoderm induction and posterior development (Hou et al., 2007). Microinjection of HtrA1 mRNA leads to induction of tail-like structures, including secondary spinal cord, notochord and somite tissue, that is characteristic of spino-caudal development (Fig. 1B,B'). The secreted serine protease HtrA1 triggers the cleavage of Biglycan, Glypican-4, and Syndecan-4, thereby releasing FGF/proteoglycan messages that activate FGF receptors at distance (Fig. 1C). A critical function of secreted serine proteases in proteoglycan cleavage and spreading of FGF signals has recently been shown in extraembryonic ectoderm development in the mouse (Shimokawa et al., 2011). FGFs stimulate *HtrA1* transcription in *Xenopus* (Hou et al., 2007) and the chick (Ferrer-Vaquer et al., 2008), suggesting positive feedback regulation. It is obvious that the proteolytic activity of HtrA1 needs to be tightly regulated to protect the integrity of the extracellular matrix and fine-tune growth factor signalling in the embryo. In *Xenopus*, a developmentally regulated protease inhibitor of the serpin family regulates FGF signalling by binding and inhibiting HtrA1 in mesoderm induction and AP neural patterning (H.A., D. Iliev, H.M.T. Grahn, N.G., and E.M.P, unpublished data).

Interaction of FGF, WNT and retinoic acid signalling during posterior development

Some of the signals that are implicated in tail induction in *Xenopus* also regulate body axis extension in higher vertebrates (Wilson et al., 2009) (Fig. 2F). In chick and mouse embryos, Fgf8 and Wnt signals maintain a pool of axial stem cells in the

caudal epiblast that gives rise to the spinal cord and somites. FGF signals protect the tail end from RA signalling by attenuating *Raldh2* expression in the paraxial mesoderm and promoting *Cyp26a* expression in the mouse caudal epiblast. In the chick, Fgf8 also stimulates expression of *Wnt8*, which maintains *Raldh2* expression. RA in turn promotes neuronal differentiation and somitogenesis by inhibiting *Fgf8* and *Wnt8* expression. The arrest of body axis elongation appears to be linked to the differentiation process and involves downregulation of FGFs and Wnts (Wilson et al., 2009). A mutual antagonism of FGF and RA signals is also employed in *Xenopus* somitogenesis (Moreno and Kintner, 2004), and Fgf8 maintains *Raldh2* expression in the frog and zebrafish body axes (Shiotsugu et al., 2004; Shimozone et al., 2013), supporting the conservation of signalling mechanisms that regulate spino-caudal development.

Conclusion

Since Spemann and Mangold's discovery of neural induction 90 years ago, the question of how the early CNS is forming has attracted many generations of scientists and continues to do. Through studies initially performed in *Xenopus*, we now know that the Spemann-Mangold organizer forms as a result of Wnt/beta-catenin signals that accumulate on the dorsal side of the early blastula embryo. Neural tissue is commonly viewed as default state, which arises through inhibition of ventral BMP signalling at the gastrula stage (Fig. 2E). Chordin and the active signals FGF, IGF, and retinoic acid (RA) are generated in the organizer and mediate neural induction by repressing the BMP/Smad1 pathway. Hedgehog (Hh)/Gli1 signals participate in neural induction through an unknown mechanism.

The neural tissue induced by Dkk, IGF and Hh is of anterior (forebrain) character due to inhibition of Wnt/beta-catenin signalling (Fig. 2F). The intracellular protein Sufu is essential for both the repression of Wnt signalling by Hh and the reciprocal activation of Hh signalling by Wnt. A two-tailed RA gradient forms in the trunk of the neurula embryo to specify the hindbrain and anterior spinal cord. Wnt and FGF signals gradually increase towards the caudal end of the embryo and specify the posterior spinal cord. The new molecular data add questions to Nieuwkoop's activation-transformation model, because some of the signals (Wnt, RA, FGF) turn out to

mediate both induction of anterior neural tissue (activation) and its posteriorization (transformation). Moreover, Wnt, RA, and FGF signals interact with each other in mutually interactive feedback loops during extension of the body axis, suggesting more complex interactions during regional specification of the CNS.

How morphogen gradients form is a long-standing question in developmental biology. The biosynthetic enzyme code for the upbuilding of RA gradients and the establishment of long-range FGF signalling by a developmentally regulated protease provide new avenues to our understanding of how growth factors are produced and delivered in right concentrations to their responsive targets. Feedback control in RA and FGF signalling further contributes to robustness of patterns. As stem cell research is moving from petridishes to 3D-organ culture, knowledge gained from experimental models will be most valuable for regenerative medicine. A nice example is the anterior neural inducer IGF that plays a decisive role not only in the frog embryo but for the conversion of human embryonic stem cells into retinal neuroepithelium (Lamba et al., 2006) and hence may be useful for the treatment of retinal degenerations. Overactivation of Wnt and Hedgehog signalling is a frequent cause of cancer. As mutations in Sufu predispose to medulloblastoma (Taylor et al., 2002), a better understanding of how the tumor suppressor protein Sufu interacts with two prominent oncogenic pathways is highly relevant to develop targeted therapies for this most prevalent malignant brain tumor in children. Thus, the study of neural induction and patterning is not only relevant to understand the first events in CNS formation, but helps to develop therapies in neurodegenerative diseases and cancer.

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Figure legends:

Fig. 1. Head and tail induction in *Xenopus* embryos. Embryos were microinjected with the indicated synthetic mRNA into the animal pole of one blastomere at the 4-cell stage. Tadpole embryos are shown as whole-mount (A, B) and transversal sections upon staining with hematoxylin-eosin (A', B').

(A, A') Ventral injection of a combination of *IGF2* and *IGFBP5* (400 pg each) causes ectopic head structures. Note secondary cement gland, eye, olfactory placode, and brain structures.

(B, B'). *HtrA1* (80 pg) leads to loss of head (*) and formation of supernumerous tail structures (arrowhead). Note secondary spinal cord, notochord and somite structures.

(C) Model of how the secreted serine protease HtrA1 promotes long-range FGF signaling in the embryo.

br, brain; cg, cement gland; ey, eye; nc, notochord; op, olfactory placode; PG, proteoglycan; sc, spinal cord; so, somite. Adapted from Pera et al., 2001 (A, A') and Hou et al., 2007 (B, B', C), with permission.

Fig. 2. Establishment of the retinoic acid gradient and interaction of signals in the early *Xenopus* embryo.

(A) Feedback interaction of RA and enzymes that are involved in its biosynthesis (*Rdh10*, *Raldh2*) and degradation (*Cyp26A1*). Thin arrows and bars indicate transcriptional regulation.

(B) Vegetal view of gastrula embryo. The overlapping expression of *Rdh10* and *Raldh2* in the Spemann-Mangold organizer is flanked by *Cyp26A1* expression in the dorsal ectoderm and ventrolateral blastopore lip. RA transcriptionally induces *Chordin* and reduces *ADMP* expression in the organizer.

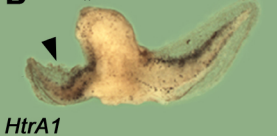
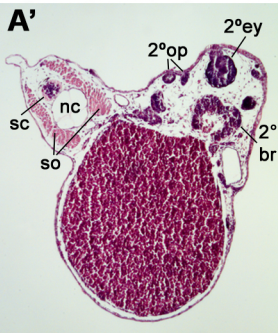
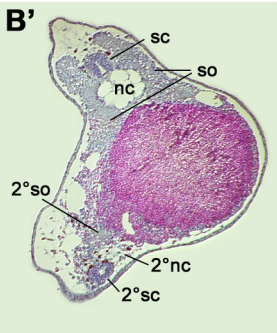
(C) Dorsal view of early neurula embryo. Nested gene expression of *Rdh10* and *Raldh2* in the trunk mesoderm is surrounded by *Cyp26A1* expression in the anterior

and posterior ectoderm, giving rise to a 2-tailed RA gradient that activates 3' Hox genes.

(D) Dorsal view of early neurula embryos after whole mount *in situ* hybridization with antisense RNA probes against *Rdh10*, *Raldh2*, *Cyp26A1*, and *HoxD1*.

(E,F) Summary of the interaction of signals and activity gradients in early gastrula (E, vegetal view) and early neurula embryos (F, dorsal view). Active signals are depicted in bold, secreted antagonists in regular letters. Bars show inhibitory interactions of signals in *Xenopus*. Stripped arrows indicate stimulatory interactions in the chick (see Wilson et al., 2009).

ADMP, anti-dorsalizing morphogenetic protein; BMP, bone morphogenetic protein; Chd, Chordin; Cyp26A1, Cytochrome 450 hydroxylase 26A1; Dkk, Dickkopf-1; fb, forebrain; FGF, fibroblast growth factor; Hh, Hedgehog; mb, midbrain; hb, hindbrain; RA, retinoic acid; Raldh2, Retinal-Dehydrogenase-2; Rdh10, Retinol-Dehydrogenase-10; SMO, Spemann-Mangold organizer; Sufu, Suppressor-of-fused. Panel (D) adapted from Strate et al., 2009, with permission.

A**B****A'****B'****C**