Global and local mechanisms of forebrain and midbrain patterning
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During the past years, major advances have been made in understanding the sequential events involved in neural plate patterning. Positional information is already conferred to cells of the neural plate at the time of its induction in the ectoderm. The interplay between the BMP- and the Fgf- signaling pathways leads to the induction of neural cell fates. Thus, neural induction and neural plate patterning are overlapping processes. Later, at the end of gastrulation, positional cell identities within the neural plate are refined and maintained by the action of several neural plate organizers. By locally emitting signaling molecules, they influence the fate of the developing nervous system with high regional specificity. Recent advances have been made both in understanding the mechanisms that dictate the relative position of these organizers and in how signaling molecules spread from them with high spatial and temporal resolution.

Introduction
The complex shape and highly partitioned functional organization of the adult vertebrate brain makes it a challenging task to understand how this structure forms during embryonic development. This review focuses on the early patterning events during development of the forming brain, including the events that lead to the establishment of the neural plate and its later regional subdivision that are under the control of local neural plate organizers. We describe how these organizers are positioned within the early neural plate and how they mediate activity in the neighboring tissues through secreted signaling molecules. Because the propagation of these signals determines the range of organizer activity, we also discuss recent advances in the analysis of these factors at a cellular level of resolution.

Neural induction and posteriorization during gastrulation
The nervous system arises from the neural plate, which is induced during gastrulation. At the end of gastrulation the neural plate comprises the primordia of the fore-, mid- and hind-brain and spinal cord. Cells acquire neural identity through the interplay of extracellular antagonists of the bone morphogenetic proteins (BMPs), that is, chordin, noggin and follistatin, in addition to antagonists of Wnt, that is, cerberus, dickkopf, sFrp and Tle ([1]; reviewed in [2–4]). Initially all of the induced neural plate expresses the sox2 gene, which is later restricted to the fore- and mid-brain primordia, suggesting that the induced neural tissue initially has anterior identity (reviewed in [2]). So then, how is posterior identity determined in the early neural plate? Work during the past 20 years has suggested the presence of posteriorizing factors including Fgfs, Wnt, Nodals and retinoic acid, which are present only in the posterior region of the embryo and lead to the formation of neural tissue with posterior character (reviewed in [2]). The current model is that neural induction and posteriorization are two closely linked events in time and space that generate neural tissue with anterior and posterior character. Recent work has shown that posterior neural tissue is not only generated by a ‘transformation’ of anterior neural tissue but is also directly induced by an Fgf signal [5**]. The sox3 gene in the zebrafish is expressed in two distinct domains: one in the anterior and one in the posterior-most neuroectoderm, enabling the simultaneous analysis of anterior and posterior cell fates [7]. Experimental inhibition of BMP antagonists or BMP gain-of-function experiments lead to the loss of the anterior sox3 domain (presumptive forebrain), whereas the posterior neuroectoderm is specified correctly, which suggests that BMP signaling has a differential effect on anterior and posterior neuroectoderm. By contrast, inhibition of Fgf signaling leads to a loss of sox3 expression in the posterior neuroectoderm and an expansion of non-neural ectoderm, an effect that cannot be compensated for by blocking BMP signaling. First, this shows that Fgfs are capable of inducing neural tissue. Second, because anterior expression of sox3 is not affected or only slightly expanded upon Fgf inhibition, this demonstrates that Fgfs are required for the induction of posterior fates in the neural plate. Taken together, this suggests that only a combination of BMP gain-of-function and Fgf blocking can lead to the complete loss of neural plate, or its conversion to non-neuronal ectoderm [5**]. Similarly, data obtained in Xenopus and in chicken embryos show that BMP inhibition alone is not sufficient for neural induction, which also involves Fgf signaling ([8,9*]; reviewed in [4]). It has been suggested that Fgfs are...
required for the extracellular BMP inhibitor chordin expression [10], but Fgf5 might induce neural specification indirectly by blocking Bmp expression [10,11,12] or BMP signaling [13]. Fgfs can also act independently of BMP signaling [6]. The fact that other factors might contribute to neural induction cannot be excluded ([14]; reviewed in [4]).

**Local signaling centers subdivide the neural plate at the end of gastrulation**

At the end of gastrulation, several local signaling centers, in the following referred to as neural plate organizers, are established to maintain and further refine positional cell identities along the antero-posterior (AP) axis of the neural plate. These organizers have been described at several positions along the AP axis of the neural plate and produce signals that influence cellular fate, histogenic organization and growth of adjacent tissue in a position-specific manner. Patterning of the forebrain primordium is controlled by a small group of cells at the anterior tip of the neural plate called row1, anterior neural ridge (ANR) or anterior neural boundary (ANB) [1,15], and by the zona limitans intrathalamica (ZLI) at the boundary between prosomeres 2 and 3 [16,17] (Figure 1a). The midbrain–hindbrain boundary (MHB) organizer induces and maintains positional cell identities in the mid- and hind-brain (reviewed in [18–20]), and rhombomere 4 (rh4) controls patterning of the adjacent rh 3–5 [21,22] in addition to forming the ear placode [23]. The tail organizer controls patterning in the posterior tip of the embryo [24] (Figure 1a).

Two properties crucially determine the local activity of these neural plate organizers: first, their relative position within the field of the neural plate, and second, the range and activity of their inductive signals that mediate position-specific patterning-responses at the target site.

**Positioning local organizers in the neural plate**

All neural plate organizers are established at very precise positions within the early neural plate. Progress has been made in understanding how their position is determined by global patterning of the neural plate. Initial work suggested that the MHB might be positioned by the axial mesendoderm, through vertical signals [25–28]. Nevertheless, in mouse and zebrafish embryos lacking a notochord a well-formed neural axis develops and the MHB organizer is positioned correctly [29–31]. Consistent with this finding, recent work shows that the position of the MHB organizer is a direct consequence of ‘global’ posteriorization of the neural plate. The position of the MHB is prefigured by the interface between anterior otx2 expression in the future fore- and mid-brain and posterior gbx1 expression in the future hindbrain (reviewed in [18,19]).

![Figure 1](current-opinion-in-neurobiology-2006-16-5-12.png)

Local signaling centers and molecules mediating their activity. Schematic drawing of an embryo at the end of gastrulation (dorsal view, anterior to the top). (a) Distribution of local neural plate organizers along the anterior–posterior axis. Different signaling molecules mediating the activity of the neural plate organizers are involved: Fgf3 and/or Fgf8, (b) Shh and (c) Wnts.
Experiments in zebrafish demonstrated that Wnt- but not Fgf- or Nodal-signaling needs to be active to position correctly the otx2–gbx1 interface at the end of gastrulation. The signal is encoded by wnt8 emanating from the margin of the embryo. Inhibition of Wnt signaling in the anterior neural plate leads to ectopic, posterior expression of the midbrain marker otx2 in the territory of the hindbrain primordium. Thus, it appears that the MHB is positioned in response to a Wnt8 gradient, which possibly spreads from the posterior end of the neural plate and suppresses fore- and mid-brain fates [32] (Figure 2).

Similar to the interface between the otx2 and the gbx1 expression domains at the MHB, the prospective ZLI is established between an anterior Six3 expression domain and a posterior Irx3 expression domain. Explant-culture experiments in chicken determined that the position of this interface is also controlled by Wnt signaling. Wnt induces expression of Irx3 and represses Six3 [33]. As for the MHB, the ZLI forms at a precise threshold of Wnt activity in the gastrulating embryo. The mechanisms that determine the position of the other neural plate organizers are currently not known.

Signaling molecules as effectors of organizer activity
The patterning range of neural plate organizers clearly depends on how the signaling molecules, which act as the effectors of organizer activity, spread, and how signals are translated into position-specific responses at target sites. Interestingly, one factor expressed in many neural plate organizers is Fgf8 (Figure 1b). Fgf8 is expressed in the ANB and influences gene expression in the telencephalon [15]. Indeed, Fgf8b beads can restore the expression of the forebrain marker BF1 in explants in which the ANB has been ablated. Conversely, inhibitors of Fgf function reduce BF1 expression in neural plate explants [34]. However, Fgf8 is expressed too late during development to be the primary inducer, and indeed development of the telencephalon occurs in absence of Fgf8 in zebrafish and mice [35,36]. Fgf3 is co-expressed with Fgf8 in the ANB [37,38]. Simultaneous inhibition of Fgf3 and Fgf8 in the zebrafish embryo mimics ANB ablation, which suggests combinatorial or redundant signaling of these Fgfs during forebrain patterning [39]. Later, during somitogenesis stages, the Fgf8-expressing ANB cells converge towards the dorsal midline of the head, where they form the telencephalic primordium. Results from the zebrafish suggest that at this stage a combined Fgf signal, including Fgf8, from telencephalic primordium is bilaterally received by the evaginating optic vesicles and thereby determines axial patterning of the prospective neural retina [40]. Thus, in different spatio-temporal contexts Fgfs elicit specific organizer-related induction and patterning steps.

Furthermore, antagonists of Wnt activity mimic the telencephalon-inducing property of the ANB. A secreted Frizzled-related Wnt antagonist, Tle, which is expressed in ANB cells, can non-autonomously promote telencephalic gene expression in a concentration-dependent...
manner [41] (Figure 1c). In summary, induction of the forebrain and further subdivision into telencephalon, eye and diencephalon is controlled by Fgf signals accompanied by graded modulation of Wnt signaling in the anterior neural plate.

Fgfs also regulate patterning events in the midbrain and hindbrain. A complex genetic network that includes pax2, engrailed (eng), pou2, fgf8 and wnt1 performs several aspects of MHB formation: initiation of the MHB program, maintenance of gene expression, morphogenesis and lineage restriction (reviewed in [18,19]; [42,43]). Notably, Fgf8 expressed at the MHB regulates patterning and later aspects of development of the adjacent territories (Figure 1b). This function for Fgf8 in the mid- and hindbrain has been demonstrated in the zebrafish mutant acerebellar (fgf8<sup>−/−</sup>) [44] and in mice [45]. In addition, Fgf8 beads can induce ectopic expression of midbrain markers, suggesting that Fgf8 is the critical molecule that mediates MHB organizer activity [46]. Fgf8, together with eng2/3, is also necessary to maintain the position between the diencephalon and the mesencephalon (DMB). There, Fgf8 is both necessary and sufficient to repress pax6, a key regulator for forebrain development, and to shift the DMB anteriorly. The source of the Fgf8 signal is probably the MHB. Because the MHB is located a significant distance from the cells of the DMB (15 cells at tailbud stage), this suggests a possible long-range effect of Fgf8 signaling [47]. Taken together, Fgf signaling from the MHB is required for correct midbrain and forebrain patterning. As previously mentioned, Wnt molecules are also part of the MHB organizer activity. However, in contrast to Fgf8, Wnt1 is unable to mimic the activity of the organizer when misexpressed (reviewed in [18,19]). In zebrafish, wnt1, wnt10b and wnt3a are partially redundant in their capacity to regulate gene expression at the MHB, and they are required to maintain threshold levels of pax2.1 and fgf8 [48,49] (Figure 1c). Thus, the MHB patterns adjacent midbrain and hindbrain structures through the activity of Wnts and Fgfs.

As mentioned above, Fgf signaling is a common feature of the activity of all mentioned organizers, except for the ZLI. The ZLI is a boundary-cell population that develops between the ventral and the dorsal thalamus. Recently, it has been shown in chick and zebrafish that the ZLI functions as a local organizer through the production of sonic hedgehog (Shh), a member of the hedgehog family of secreted proteins. The ZLI regulates the acquisition of cellular identity of the adjacent diencephalic region (prethalamic and thalamus) [47,50,51]. Wnt8b, a signaling molecule of the Wnt family, is expressed dorsally and in the ZLI itself (reviewed in [52]) but the effect of Shh is not mediated through Wnt expression because Shh overexpression has no effect on Wnt. Instead, Wnt might function earlier to regulate regionalization of the diencephalon along the AP axis and define prospective thalamic and prethalamic areas [17**].

Two organizers have been identified in the posterior region of the developing embryo. In zebrafish, rhombomere 4 (rh4) is the first hindbrain segment to be formed [22] and is distinct from the others because it expresses Fgf8 and Fgf3. Studies in mouse and chicken suggest that this signaling activity of rh4 is conserved across vertebrates and is also mediated through Fgf signaling [21,22,53]. Rh4 is locally required for development of the adjacent rhombomeres, rh3 and particularly rh5 and 6 [21,22]. An organizer required for tail development has recently been identified in zebrafish [24]. Loss-of-function experiments revealed that the BMP, Nodal and Wnt8 signaling pathways are required for tail development.

**Molecular and cellular control of inductive signal propagation**

Advances are being made in the analysis of how signaling molecules spread dynamically in the early vertebrate embryo on a cellular and subcellular level. This is of prime importance, because only the identification of the cellular compartments involved in signaling from embryonic organizers will yield a full understanding of the inductive mechanisms involved. Many of the signaling molecules discussed are believed to be bona fide morphogens. Morphogens first, form concentration gradients across the cellular field to be patterned and second, elicit patterning responses, such as the induction of gene expression, in a concentration dependent manner (reviewed in [54,55]). In particular, in vivo expression and analysis of fluorescently tagged morphogens has been instrumental in the visualization of spreading inductive molecules in the multicellular system of the developing embryo.

**Fibroblast growth factor**

In a recent study, spreading of Fgf8 protein labeled in vitro with Cy3 chromophore from a local source was monitored to determine how Fgf8 spreads through tissue of the gastrulating zebrafish embryo. The labeled Fgf8 protein accumulated in Rab5-positive early endosomes and in lysosomes at a distance away from the source. Reducing the rate of endocytosis led to an extracellular accumulation of Fgf8 and resulted in broader domains of Fgf-target gene expression, suggesting increased spreading of Fgf8. Thus, the signaling range of extracellular Fgf8 is controlled through endocytosis and subsequent degradation (Figure 3a–e) [56**]. This mechanism is opposite to the one proposed for spreading of the transforming growth factor-β (TGF-β) molecule decapentaplegic (Dpp) in Drosophila. Dpp spreading is enhanced when endocytosis is increased [55]. The endogenous graded activity of Fgf has not been directly visualized around the ANB or the MHB organizers. However, additional evidence that Fgf does indeed function as a...
morphogen comes from experiments with *Xenopus*, in which cells of the animal cap ectoderm were cultured in the presence of various concentrations of bFGF (Fgf2). Low doses of bFGF induced ectodermal cells of the gastrula to express anterior neural markers, and increasing doses of bFGF induced progressively more posterior markers. Thus, bFGF induces target-gene expression in a concentration dependent manner, inferring it to be a morphogen [57]. This is consistent with the finding that application of Fgf8-beads or ectopic, clonal Fgf8 expression activates the transcription of the *erm*, *pea3* and *sprouty4* genes in a nested fashion, also suggesting a concentration-dependent response of morphogen signaling [37,38,56**].

Fgf8 also functions during inductive events at post-gastrulation stages, often in an epithelial context in which an alternative cellular mechanism of Fgf spreading has been proposed.

In the *Drosophila* wing disc, Fgf propagation might employ an alternative mode of distribution. Indeed, tracheoblasts form filopodia that have several properties in common with cytonemes [58]. The formation of these filopodia is Fgf-dependent, and their presence suggests a possible mechanism for Fgf signal propagation from the source into the surrounding tissue. However, both in *Drosophila* and in vertebrate systems Fgf activity and specificity can be modulated by heparan-sulfate proteoglycans (HSPGs) and in turn by enzymes that synthesize and degrade HSPGs [59]. Because different HSPGs can be membrane-associated or truly extracellular, it is unclear whether this suggests propagation of the Fgf signal in the extracellular matrix or Fgf propagation in association with membranous compartments [58].

**WNT**

Graded activity of Wnt signaling in the neural plate during gastrulation stages is required for proper positioning of the MHB organizer and the ZLI. To date, no direct visualization of Wnt-signal distribution has been described in vertebrates. Several studies have indirectly shown graded Wnt activity in the early neural plate: chick neural plate explants express different regional markers in response to different concentrations of Wnt-conditioned medium [60], and the nuclear localization of β-catenin, a transcriptional activator of the canonical Wnt signaling pathway, is high in posterior and low in anterior areas of the neural plate [61].
Furthermore, clonal analysis of wnt8 expressing cells shows that gbx1 is activated in the host tissue one or two cells distant from the transplanted cells, and that otx2 is repressed four or five cells distant from the transplanted cells, suggesting that otx2 is sensitive to lower doses of Wnt8 in comparison with gbx1 [32**]. These cell transplantation experiments show that Wnt8 signaling acts non-cell autonomously and directly, suggesting a morphogen-mode of signaling for Wnt8 [32**]. Wnt molecules also mediate the activity of neural plate organizers. Evidence from studies in mice, chicks and zebrafish supports the idea that within the forebrain the prospective diencephalon is a source of Wnt/β-catenin signals that promote posterior diencephalic identity and suppress telencephalic fates [41]; reviewed in [62]. Wnt8b is produced in the posterior diencephalon and might spread anteriorly in the neural plate, and is required in the midbrain and diencephalon. Wnt8b is also required to establish the posterior boundary of the eye field, which is located only a few cell rows away from the anterior boundary of the wnt8b domain, and has been suggested to function as a short-range signal there [63*].

Taken together, studies on Wnt signaling in vertebrates are consistent with the ability of Wnt molecules to form gradients and to activate target genes in a concentration-dependent manner, similar to their function in the Drosophila wing imaginal discs [64,65]. Studies on wingless (Wg), the Drosophila wnt-homolog, in the embryonic epidermis and the wing disc led to several models addressing the cellular mechanisms of spread of signaling molecules across a tissue: in addition to extracellular signal propagation, planar transcytosis via dynamin-mediated endocytosis is discussed (reviewed in [55,66,67]). Other mechanisms of Wg protein transport have been described, namely cellular processes called cytokinemes [68] and argosomes [69,70**]. In recent years, various studies have revealed significant roles for cell surface molecules such as receptors and HSPGs in the distribution of morphogens [66,67,71–75]. However, the exact mechanism of Wg transport, and the molecules regulating this process, are currently still under intensive debate.

All the described mechanisms for Wg propagation in Drosophila tissue could be analogous to those of Wnt-propagation in vertebrates. To date, only a few studies have addressed the question of how a Wnt gradient is established in vertebrates. Using zebrafish, visualization of GFP-tagged Wnt8 protein around a clone of cells overexpressing the tagged protein showed that the it mostly associates in patches on the cell surface or extracellular matrix [32**]. Wnt gradients are sharpened by inhibitors that attenuate Wnt signaling: secreted Frizzled related (sFRRP), cerberus, dickkopf and Tle are considered to antagonize Wnt activity by sequestering secreted Wnt ligand. The ANB and the prechordal plate mesoderm can function as source of these antagonists, which might spread in the neural plate and generate a Wnt-antagonizing counter gradient.

**Conclusions**

to date, the primary focus of work on neural plate patterning was to identify the molecular players, such as transcriptions factors or signaling molecules. To define how and when they act was crucial for the understanding of how neural plate organizers are established and how they function. It is now evident that complicated regulatory loops between these factors are necessary to maintain the organizing activities. However, it is also emerging that cellular mechanisms operating in and around the target cells greatly influence the range of organizer-derived signals. The activity of an organizer is mostly mediated through secreted molecules that have to travel short or long distances in the neural plate, which determines their activity range on the cellular level. These types of studies are now advancing rapidly and it will be a challenge to determine how far these cellular mechanisms can modulate signaling events during forebrain and midbrain patterning. We’re in for an increasing merge of cell biology and development in this exciting field.

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**References and recommended reading**
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


The authors provide gain- and loss-of-function evidence in zebrafish showing that only anterior neuroectoderm is induced by inhibition of Bmp signalling, whereas posterior neuroectoderm is induced by Fgf signalling. They further show that at its endogenous site of neural induction, Fgf signalling induces neural specification independently of Bmp signalling.
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